MELAMINE EXCRETION PATHWAYS IN LACTATING DAIRY COWS

by
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Promotor: Prof CW Cruywagen

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ii

ABSTRACT

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Degree: **PhD (Agric)**

In this study, five trials were conducted to examine *in vitro* and *in vivo* degradation, excretion and absorption parameters of melamine (MEL) in dairy cows that have not been studied before or where limited information is available. The first two trials were *in vitro* studies conducted to determine the extent of MEL degradation in rumen liquor and the effects of MEL on ruminal ammonia (NH_3) and volatile fatty acid (VFA) concentrations. For both trials, rumen liquor was collected from ruminally cannulated lactating Holstein cows. For the first and second trial, rumen liquor was collected from three and two cows, respectively. For both trials, Erlenmeyer flasks contained 1 g substrate and 100 mL incubation medium consisting of 20 mL rumen liquor and 80 mL reduced buffer solution. In the first trial, each flask contained 100 mg of MEL, resulting in an initial MEL concentration of 1000 mg/L. The flasks were incubated at 39° C for 0 (Control), 6, 24 or 48 hours under strictly anaerobic conditions. In all the trials, MEL concentrations were determined by LC/MSMS. MEL degradation was low after 6 and 24 h of incubation (3.2 and 5.5%, respectively) and increased to 13.6% after 48 h of incubation. In the second trial where VFA and NH_3 concentrations were determined, the flasks contained either 0 (Control), 0.2 (T1) or 0.4 mg (T2) of MEL. The flasks were incubated for 6, 24 or 48 h. Treatment had no effect on individual or total VFA concentrations or NH_3 concentrations at 6 and 48 h. At 24 h, T2 resulted in an inexplicable higher NH_3 concentration. This study showed that the addition of melamine would not result increased rumen NH_3 concentrations *in vitro*. Melamine would also not affect the production of different VFA's. Therefore, it was concluded that the rumen micro-organisms present in rumen liquor would be unable to utilize MEL as a source of nitrogen and that the microbial production of VFA's remains unaffected by the presence of MEL. In the third trial, MEL excretion in lactating cows was determined. Five cows were randomly allocated to treatments according to a 5 x 5 Latin square design. Cows received the treatment diets for 7 d followed by 8 d of MEL withdrawal during each of the five periods. The experimental treatments were formulated to provide a daily MEL intake of 0 (M0), 500 (M1),

1000 (M2), 5000 (M3) or 10000 mg (M4) via 15 kg of dairy concentrate pellets. Calculations based on the work of Newton & Utley (1978) suggested that a melamine intake of 0.16 g/kg of live weight would not result in detrimental health effects of ruminant animals. Therefore, a 600 kg lactating dairy cow should not be at risk when consuming 100 g of melamine. In this trial, the highest melamine treatment (M4 = 10 g/d) included a 10-fold safety factor from the suggested safe amount from the work of Newton & Utley (1978) and should not pose a health risk to the cows. Treatments had no effect on DMI, milk yield or milk composition. MEL was detected in the milk 8 h after initial MEL ingestion, increased rapidly and peaked on d 3 and was undetectable after 8 d. Treatments had no effect on MEL excretion efficiencies which ranged from 1.5 to 2.1%. The mean apparent digestibility of MEL was 78%. Mean faecal and urinary MEL excretions were 22 and 54 % of ingested MEL, respectively. Higher milk, urine and faecal MEL concentrations were observed with higher levels of dietary MEL. It was concluded that MEL appeared in the milk soon after first ingestion and a withdrawal period of 8 d was required for all milk, faecal and urine samples to reach undetectable levels of MEL. Urine and faeces were the primary routes for MEL excretion. The fourth trial was conducted to determine MEL absorption by the mammary gland in lactating dairy cows through arterio-venous (A-V) difference. Five cows received 10 g of MEL/d for three consecutive days. Day 3 of the trial was selected for commencement of blood sampling as previous studies (Cruywagen *et al*., 2009; Shen *et al*., 2010; Sun *et al*., 2011) reported the milk melamine concentration to reach a peak on d 3 of continuous melamine consumption by dairy cows. Early on d 3, catheters were inserted into the caudal superficial epigastric vein (milk vein) and caudal auricular artery. The blood sampling period commenced after residual milk removal from the udder following oxytocin administration. Blood from both locations were collected hourly for 9 hours. Following the final blood collection, oxytocin was administered again, catheters were carefully removed and cows were milked immediately thereafter. All blood samples were centrifuged and the decanted plasma was analysed for MEL, as well as for amino acid contents to calculate mammary blood flow. The positive MEL flux (calculated from A-V difference) confirmed net absorption of MEL into the mammary gland with an efficiency of absorption of 0.29%. Melamine excretion into milk was 5.63 mg/h. The mean plasma and milk MEL concentrations were 5.2 and 3.9 mg/kg, respectively. Melamine excretion efficiency to milk, expressed as percentage of the ingested amount, was 1.47%. It was concluded that melamine ingested by cows will result in net MEL absorption by the mammary gland, but that the absorption efficiency is low. The final trial of the study aimed to determine the effects that fermentation processes during the

manufacturing of cheese, yoghurt and kefir would have on their MEL content if these products were made from MEL contaminated milk. Another objective was to determine if MEL in cheese would be degraded during the curing process. Cheese, yoghurt and kefir were made from milk with a MEL content of 6.77 mg/kg. The cheese was then cured for 2 wk at 6° C. The MEL contents of the yoghurt and kefir were 6.76 and 6.78 mg/kg, respectively, indicating that the different fermentation processes used in yoghurt and kefir production had no effect on their MEL content and that MEL was not degraded during the short fermentation periods. The percentage of milk MEL partitioned to whey and cheese were 97.4 and 6.5 %, respectively. It was concluded that the different fermentation processes involved during the manufacturing of yoghurt and kefir from MEL tainted milk did not decrease the MEL concentration. The milk MEL was predominantly partitioned to whey, with little MEL transferred to cheese. It was also concluded that MEL was not degraded in cheese during a 2-wk curing period. It was finally concluded that dietary MEL is readily absorbed by dairy cows and mainly excreted via the urine. The mammary gland has a low affinity for MEL absorption and approximately 2% of ingested MEL is excreted in the milk. When cheese is made from MEL tainted milk, the majority of MEL will concentrate in the whey fraction and only 6.5% will be present in the cheese.

UITTREKSEL

Melamien uitskeidings roetes in lakterende melkkoeie

deur

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Graad: PhD (Agric)

Vyf proewe is gedoen om *in vitro-* en *in vivo*-degradering, uitskeiding en absorpsie parameters van melamien (MEL) na te gaan waaroor daar min of geen inligting bekend was nie. Die eerste twee proewe was *in vitro*-studies, uitgevoer om die mate van MEL degradeerbaarheid in rumenvloeistof na te gaan, asook die invloed van MEL op rumen-NH_3 en vlugtige vetsuur (VVS)-konsentrasies. Vir beide proewe is rumenvloeistof van lakterende, rumengekannuleerde Holsteinkoeie verkry. Vir die eerste en tweede *in vitro*-studies, was rumenvloeistof verkry vanaf drie en twee koeie, onderskeidelik. In albei proewe is 1 g substraat in Erlen-meyerflessies afgeweeg en 100 mL inkubasiemedium bygevoeg wat uit 20 mL rumenvloeistof en 80 mL van 'n buffermedium bestaan het. In die eerste proef is 100 mg MEL by die substraat gevoeg, sodat die aanvanklike MEL konsentrasie in die flessies 1000 mg/L was. Die flessies is by 39° C geïnkubeer vir 0 (Kontrole), 6, 24 of 48 ure, onder streng anaerobiese kondisies. Met die beïndiging van die inkubasieperiode is 100 mL van 'n 0.2 M perchloorsuuroplossing bygevoeg om enige melamien wat nie gedegradeer was nie, op te los. In al die proewe is melamienbepalings by wyse van LC/MSMS gedoen. Melamiendegradering was laag na 6 en 24 h inkubasie (3.2 en 5.5%, respektiewelik) en teen 48 h inkubasie het dit toegeneem tot 13.6%. In die tweede proef het die flessies 0 (Kontrole), 0.2 (T1) of 0.4 mg (T2) melamien bevat. Behandeling het geen invloed op individuele of totale VVS-konsentrasies by enige van die inkubasietye gehad nie en ook nie op NH_3-konsentrasies by 6 en 48 h nie. Om een of ander onverklaarbare rede het die T2-behandeling gelei tot hoër NH_3-konsentrasies by 24 h. Die gevolgtrekking is gemaak dat die byvoeging van MEL geen effek op rumen NH_3-konsentrasies het nie en dat die mikroorganismes in die rumen nie daartoe in staat sal wees om MEL as 'n stikstof-bron sal kan benut nie. In die derde proef is die uitskeiding van MEL in melkkoeie ondersoek. Vyf lakterende Holsteinkoeie is ewekansig aan vyf behandelings toegeken in 'n 5 x 5 Latynsevierkantontwerp. Gedurende elke periode het koeie die behandelings vir 7 d ontvang, gevolg deur 'n 8 d MEL-onttrekkingsperiode. Die eksperimentele diëte is geformuleer om 'n daaglikse MEL-inname van 0 (M0), 500 (M1), 1000 (M2), 5000 (M3) of 10000 mg (M4) per koei/dag te verseker, toegedien via 15 kg/d van 'n suiwelkonsentraat in pilvorm. Berekeninge gebasseer op die werk van Newton & Utley (1978) stel voor dat 'n MEL inname van 0.16 g/kg lewende massa, geen negatiewe effek op herkouers se

gesondheid sal hê nie. Dus, 'n koei wat 600 kg weeg, sal geen skade lei deur die inname van 100 g MEL nie. In hierdie proef was die hoogste MEL behandeling (M4 = 10g/d) tien keer laer as die voorgestelde veiligheidsvlak van Newton & Utley (1978). Behandeling het geen invloed op DMI, melkopbrengs of melksamestelling gehad nie. Melamien is so gou as 8 h na eerste inname in die melk waargeneem, waarna die konsentrasie vinnig toegeneem het en 'n piek na 3 d bereik het. Behandeling het geen invloed op die uitskeidingsdoeltreffendheid van melamien in melk gehad nie en waardes het gewissel van 1.5 tot 2.1%. Die gemiddelde skynbare verteerbaarheid van MEL was 78%. Die gemiddelde mis- en uriene-MEL-konsentrasies was 22 en 54%, onderskeidelik. Hoër melk-, mis- en uriene-MEL-konsentrasies is waargeneem namate die MEL-inhoud van die diëte gestyg het. Die gevolgtrekking is gemaak dat MEL spoedig na eerste inname in die melk verskyn en dat 'n onttrekkingsperiode van 8 d benodig word voordat melk-, mis- en uriene-MEL onwaarneembare vlakke bereik. Uriene en mis is die primêre uitskeidingsroetes van ingenome MEL. Die vierde proef is onderneem om MEL-absorpsie in die melkklier met behulp van arterio-veneuse (A-V) verskille te ondersoek. Vyf koeie het elk 10 g MEL/d vir drie agtereen-volgende dae ontvang. Dag 3 van die proef is gekies vir bloedkolleksies aangesien vorige studies (Cruywagen *et al*., 2009; Shen *et al*., 2010; Sun *et al*., 2011) gewys het dat melk MEL op dag 3 van MEL inname, piek konsentrasies beryk. Vroeg gedurende die oggend van d 3 is kateters in die kaudale oppervlakkige epigastriese aar (melkaar) en die kaudale aurikulêre slagaar geplaas. Die bloedtrekkingsperiode het 'n aanvang geneem direk nadat die koeie volledig uitgemelk is na toediening van oksitosien om te verseker dat soveel as moontlik residuele melk verwyder word. Monsters van veneuse-, sowel as arteriële bloed, is 9-uurliks geneem. Na die finale bloedtrekking is oksitosien weer toegedien, die kateters is versigtig verwyder en die koeie is direk daarna weer gemelk. Al die bloedmonsters is gesentrifugeer en plasmamonsters is ontleed vir MEL, asook vir aminosuursamestelling ten einde bloedtoevoer na die uier te bereken. Die positiewe fluks (bereken van A-V verskil) het bevestig dat netto MEL absorpsie in die melkklier plaasvind, met 'n doeltreffendheid van 0.29%. Melamienuitskeiding in die melk was teen 'n tempo van 5.63 mg/h. Die gemiddelde plasma- en melk-MEL konsentrasies was 5.2 en 3.9 mg/kg, onderskeidelik. Die uitskeidingsdoeltreffendheid van MEL na melk, uitgedruk as persentasie van ingenome MEL, was 1.47%. Die gevolgtrekking is gemaak dat MEL wat deur koeie ingeneem word, tot netto MEL-absorpsie in die melkklier sal lei, maar dat die absorpsiedoeltreffendheid baie laag is. In die finale proef is daar gepoog om die invloed van fermentasieprosesse gedurende die vervaardiging van kaas, joghurt en kefir op die produkte se melamieninhoud na te gaan indien die produkte van melamienbevattende melk gemaak sou word. 'n Tweede doel van hierdie proef was om te bepaal of MEL in kaas gedegradeer kan word tydens rypwording. Kaas, joghurt en kefir is gemaak van melk wat 'n MEL-inhoud van 6.77 mg/kg

gehad het. Die kaas is vervolgens vir twee weke by 6° C rypgemaak. Die MEL-inhoud van die joghurt en kefir was 6.76 en 6.78 mg/kg, onderskeidelik, wat daarop dui dat die onderskeie fermentasieprosesse wat tydens die bereiding van joghurt en kefir plaasvind, geen invloed op hul MEL-inhoud gehad het nie en dat MEL nie gedurende hierdie kort fermentasieperiodes gedegradeer is nie. Die persentasie MEL na wei en kaas versprei was 97.4 en 6.5%, onderskeidelik. Die gevolgtrekking is gemaak dat die verskillende fermentasieprosesse betrokke tydens die vervaardiging van joghurt en kefir wat van melamienbesmette melk gemaak word, nie die MEL-konsentrasie verlaag het nie. Tydens die vervaardiging van kaas, word die MEL hoofsaaklik na die weikomponent versprei en baie min na kaas. Melamien word ook nie in kaas afgebreek gedurende 'n verouderingsproses van twee weke nie. Die finale gevolgtrekkings is gemaak dat MEL maklik deur melkkoeie geabsorbeer word en dat die hoof uitskeidingsroete via urine is. Die uier het 'n lae affiniteit vir MEL absorpsie en ongeveer 2% van ingenome MEL is in die melk uitgeskei. Wanneer kaas van MEL besmette melk gemaak word, sal die meerderheid van die MEL in die weifraksie konsentreer, met slegs 6.5% teenwoordig in die kaas.

ACKNOWLEDGEMENTS

NOTES

The language and style used in this dissertation are in accordance with the requirements of the *South African Journal of Animal Science*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has been unavoidable.

TABLE OF CONTENTS

LIST OF FIGURES:

LIST OF TABLES:

CHAPTER 1

General introduction

Hard (2002) estimated the global population to exceed 7.5 billion by the year 2020. The Population Reference Bureau reported the world population has reached the 7 billion mark on 31 October 2011 with the current world population standing at 7.099 billion. Future population growth in under-developed countries is expected to increase the most. From a South African perspective, the country's population growth has experienced significant increases since 1970 (Figure 1.1). Therefore, in order to supply the population's demand for protein sources, the production of animal protein products is required to increase accordingly. As society is becoming more health conscious and concerned about animal welfare, the global meat consumption trends are fluctuating and decreasing due to social trends. However, milk consumption trends are not likely to decrease or fluctuate compared to meat consumption trends, as milk and milk products are subjected to fewer religious and social restrictions (McDonald *et al*., 2002). Figure 1.2 presents the annual per capita (kg) meat (red and white), egg and milk consumption trends for the South African population. Figures 1.1 and 1.2 are adapted from Agricultural Statistics South Africa (2011).
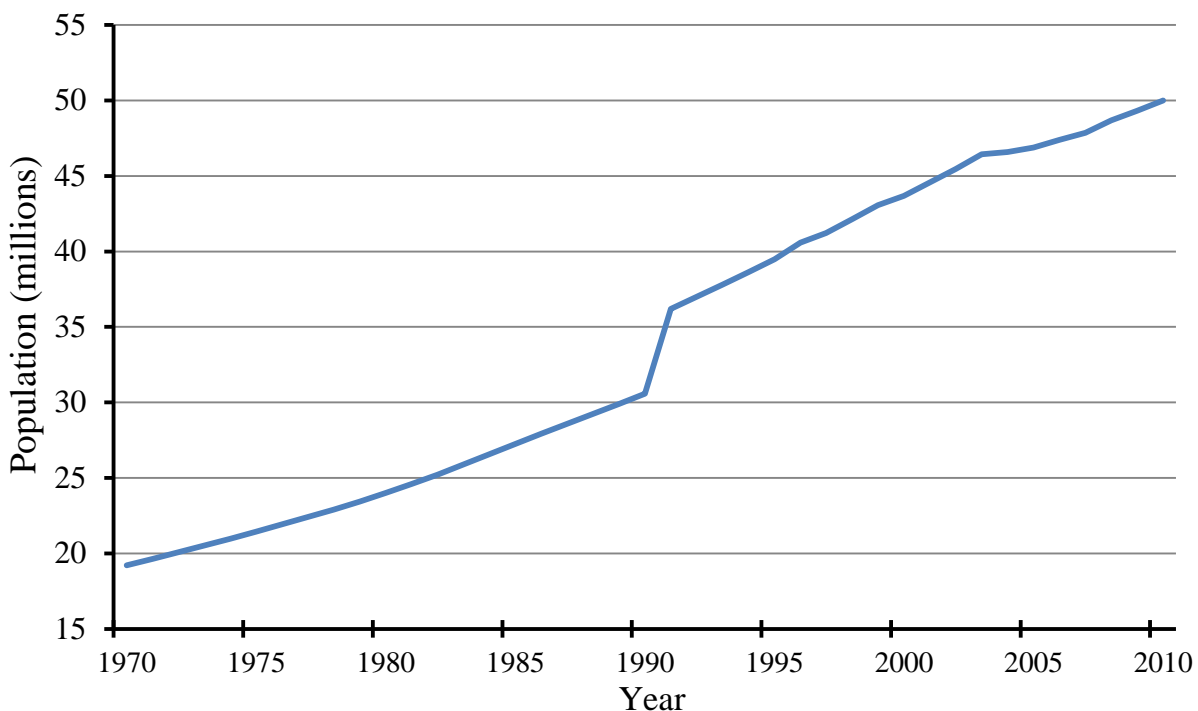
Figure 1.1 South African population growth over the last four decades.

1
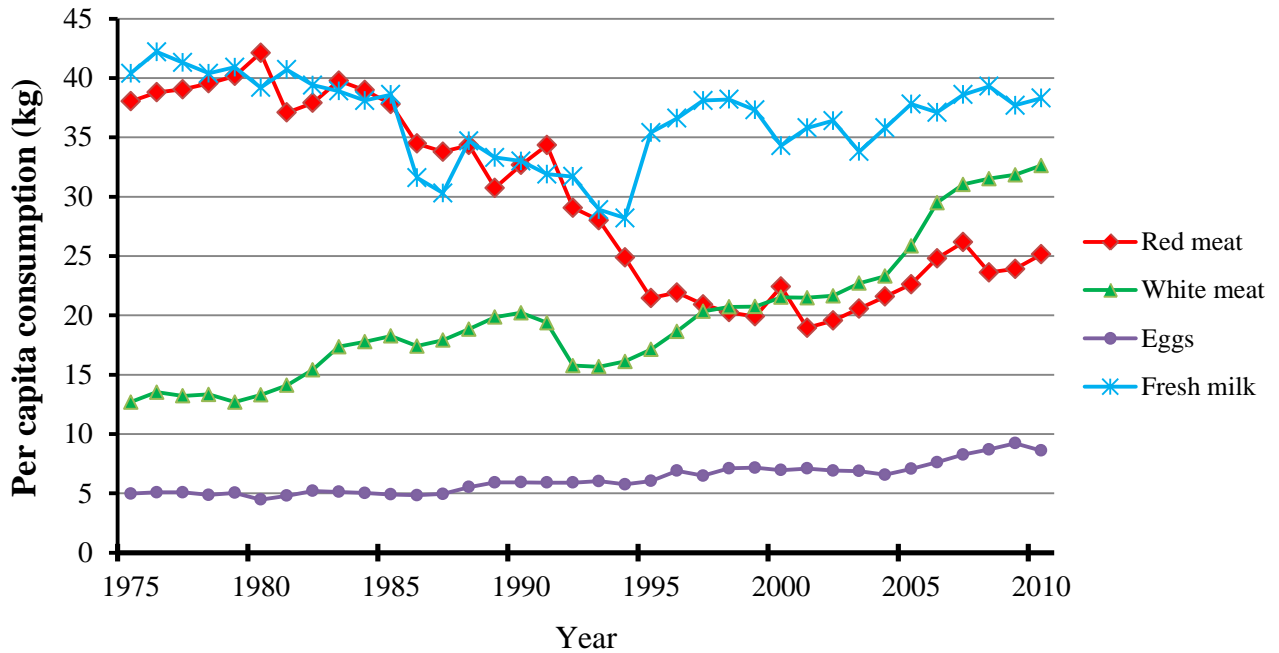
Figure 1.2 Annual per capita animal product consumption (kg) in South Africa.

As a result of the increasing population trends, the animal industry is under pressure to keep up with society's demand for nutritious, safe and high quality protein sources of animal origin (including meat, milk and eggs). In turn, this has resulted in animal production systems becoming more intensified. With the increased intensive animal production systems, managerial practises employed should be as such to ensure optimal animal welfare and nutrition in order to ensure optimal animal productivity and therefore maintaining profitability to the farmer/producer. Apart from the high demand, producers of such products are competing with each other for market share to maintain profitability. It is clear that the producer's greatest expenditure is obtaining high quality feeds. Therefore, the challenges faced by producers to ensure and maintain profitability include feeding the animals high quality feeds at the lowest prices while producing high quality products which are sold at competitive prices to ensure a significant market share. Due to limitations in available land, the expansion of infrastructure to increase animal numbers are sometimes not possible when attempts are made to increase profit levels. Therefore, the only way to increase a business' profit margin is to reduce feed costs. It is well known that high quality feeds are more expensive than low quality feeds, especially protein feedstuffs. However, with intensive production systems, feed quality cannot be jeopardized in order to reduce feed costs. Thus, in order to reduce feed costs, producers have to compare prices of competitive animal feed manufacturers to ensure they obtain the best quality feeds at the lowest prices.

The increased intensification of animal production systems have also resulted in increased numbers of feed companies and feed manufacturers. In an attempt to sell feeds to producers/farmers at competitive prices, feed companies are sourcing from local and international feed manufacturers. This has led to the globalization of the feed supply chain. Tse & Tan (2012) reported the increased risks associated with feed quality control as the supply chains extend via outsourcing and stretched by globalization. The increased risks associated with feed quality can be ascribed due to the fact that quality regulation is primarily local in a global market (Brown & Brown, 2010). This is especially true when feed ingredients are sourced from countries known to have lax quality control regulations. The predominant quality risk associated with feeds includes the adulteration of feed ingredients.

Adulteration of feedstuffs includes substituting, diluting, or modifying a feed ingredient, resulting in a physical and/or chemical alteration of the original feed ingredient (Moore *et al*., 2012). The adulteration of feed ingredients used in the animal industry is a concerning reality. The primary reason for adulteration is purely for economic gain which predisposes expensive feed ingredients to adulteration (Moore *et al*., 2012). This is especially true for protein feed ingredients used in the animal feed industry. A contributing complication factor in protein ingredient adulteration is the fact that the crude protein content of such ingredients is routinely determined from their nitrogen content (N x 6.25; AOAC, 2002). Therefore, the addition of a non-protein nitrogen source to a protein ingredient would increase the nitrogen content while deceiving the potential buyer by false information on the crude protein content of that ingredient. This was exactly the case when various protein sources for animal feeds originating from China were adulterated with melamine waste and exported to various countries.

The commercial scale manufacturing of industrial melamine in China results in vast amounts of melamine waste. Melamine waste consists predominantly of melamine (~70%) and its analogues, including cyanuric acid (Kirk-Othmer, 1978). China produces 46% of the world's melamine (IHS Chemical, 2010). In 2006, China's melamine production exceeded its demands and resulted in a severe surplus of melamine (Wang, 2006). During this time, the increased urea price (which serves as the feedstock for melamine production) and the global melamine price remaining stable, the profit margin for melamine production decreased. As the demand for high protein feed ingredients increased, some feed manufacturing companies in China attempted to increase their profits by incorporating melamine waste to protein

3

feedstuffs in order to artificially increase the crude protein content and selling their products at reduced prices. Clearly, the high apparent crude protein content of the protein ingredients offered at such competitive prices, gained much attention from the international market.

China exported various melamine adulterated protein ingredients to various countries including rice proteins, wheat gluten, soy products and maize gluten (EFSA, 2010). The incorporation of the adulterated wheat and maize gluten in various countries into pet foods resulted in a large number of mortalities amongst dogs and cats worldwide. The cause of these deaths was reported to have resulted due to kidney failure related to the physical obstructions of melamine-cyanurate crystals (Brown *et al*., 2007; Puschner *et al*., 2007). Due to the incorporation of melamine waste into the wheat and maize gluten, the animals have consumed melamine and cyanuric acid simultaneously. The predominant elimination pathway for both melamine and cyanuric acid is via the kidneys in urine (Mast *et al*., 1983; WHO, 2008). Subsequently, this resulted in the formation of kidney stones as melamine and cyanuric acid binds on a 1:1 basis to form melamine-cyanurate crystals (Perdigão *et al*., 2006), which consequently precipitates in the kidneys as their size prevents excretion via urine.

The American population expressed their health related concerns following reports that pigs were provided with the pet food that was recalled. Consumer concerns increased especially after China announced kidney-related illnesses and deaths in infants consuming melamine adulterated infant formula during 2008 (WHO, 2008). Mankind is extremely vulnerable to contaminated foods and therefore health authorities have a great responsibility in controlling and limiting such health risks (Yang & Batlle, 2008). Hence, health regulatory authorities (including the World Health Organization (WHO), United States Food and Drug Association (US FDA) and European Food Safety Authority (EFSA) rapidly responded to the public's health concerns by implementing and reviewing regulations with regards to the presence of melamine in foods and feeds. Through risk assessment estimates and considering all contributing factors resulting in possible melamine exposure to humans, the maximum melamine concentration allowed in infant foods and other food and feed sources without posing risks to human and animal health were reported as 0.1 and 2.5 mg/kg, respectively (WHO, 2008; EFSA, 2010). The recommended Tolerable Daily Intake (TDI) of melamine for humans were reported as 0.5, 0.2 and 0.63 mg/kg body weight by EFSA (2010), the WHO (2009) and FDA (2007), respectively. As infants are more susceptible to melamine exposure,

the FDA (2009) adjusted the TDI of 0.63 mg/kg for infants by incorporating a tenfold safety factor. Hence, a recommended TDI for infants of 0.063 mg/kg body weight was suggested by the FDA (2009).

Various studies have confirmed the transmission of melamine to various animal products used for human consumption. Melamine excretion into milk (Cruywagen *et al*., 2009) and transmission to cheese (Battaglia *et al*., 2010), sheep meat (Cruywagen *et al*., 2011; Lv *et al*., 2010), poultry meat (Lü *et al*., 2009; Sirilaophaisan *et al*., 2010; Brand *et al*., 2012) and eggs (Bai *et al*., 2010; Valat *et al*., 2011; Gallo *et al*., 2012) have been reported. Following the enforcement of maximum allowable levels of melamine concentrations for food and feeds, various studies investigating melamine transmission to animal products focussed on determining whether the animal products would have resulted in melamine concentrations exceeding the limit of 2.5 mg/kg. Even though the majority of studies reported animal products from animals exposed to melamine to contain lower melamine levels than the recommended level, it should not be regarded as a justification for administering melamine adulterated feeds to animals. Cruywagen *et al*. (2009) made an important remark when they illustrated milk powder to exceed the melamine safe limit by approximately 2-fold when produced from milk with a melamine concentration of 0.4 mg/kg which would be regarded as "safe".

It is important to note that apart from the set maximum allowed melamine levels in feeds and foods, as well as the recommended TDI's, these values are calculated from animal studies and there is no literature available to prove the safety of these values. In addition, the rate of toxicity remains yet to be determined. As milk and milk products are important sources of protein to mankind, very little is known about melamine metabolism and excretion in dairy cows.

Therefore the current study investigated the following objectives to determine:
1. Whether melamine can be degraded *in vitro* in rumen liquor;
2. The effect of melamine on *in vitro* ammonia and volatile fatty acid concentrations of rumen liquor;
3. Melamine excretion via milk, urine and faeces lactating dairy cows;
4. Melamine absorption in the udder of lactating dairy cows;
5. Melamine transmission from melamine tainted milk to cheese, whey, yogurt and kefir and also whether melamine degradation occurs during the conditioning of cheese.

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6

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CHAPTER 2

Literature Review

Melamine: What we know thus far

2.1 Introduction

Melamine ($C_3H_6N_6$) or 1,3,5-triazine-2,4,6-triamine is an industrial chemical that has the appearance of fine, white, powder crystals. On a molecular weight basis, pure melamine contains 667 g/kg nitrogen (Merck, 2001). Melamine was first synthesized by a German chemist, Justus von Liebig, in 1834, by converting calcium cyanamide to dicyandiamide and heating it above its melting temperature to produce melamine. Since then, melamine is produced from urea. The manufacturing of melamine is commonly integrated into urea production systems (which use ammonia as feedstock) due to the fact that urea serves as feedstock for the production of melamine. Melamine resin is produced by combining melamine with formaldehyde, resulting in a highly durable plastic used in the manufacturing of laminates, plastic ware, glues and flame retardants.

The initial use of melamine was for industrial purposes, until the 1950's and 1960's, when it was proposed to use triazines as a source of nitrogen fertilizer (Hauck & Stephenson, 1964) due to its high nitrogen content. However, the application of melamine as fertilizer proved to be inefficient due to the fact that the nitrogen from melamine is released very slowly. This was confirmed by Scholl *et al*. (1937), when they reported that as little as 1% of melamine nitrogen was converted to nitrate when applied to plants as a source of nitrogen fertilizer. In addition, it was concluded that melamine should not be used as fertilizer for grass as it results in a low growth response (Wehner & Martin, 1989) due to the slow release of nitrogen into the soil (Mosdell *et al*., 1987).

It was also during the early 1950's to 1960's when researchers gained interest in studying the use of urea as a non-protein nitrogen (NPN) source for ruminants. This interest was stimulated following the reports made by Loosli *et al*. (1949) that sheep and goats fed purified diets containing urea as sole source of dietary nitrogen, could result in the synthesis of all essential amino acids by the rumen microbial population. These results were later confirmed by Duncan *et al*. (1953), Virtanen (1966) and Oltjen (1969). Researchers then shifted their focus to various other NPN sources in an attempt to improve the utilization of

NPN sources in ruminants. Other NPN sources (apart from urea) under investigation included biuret, triuret, cyanuric acid (Clark *et al*., 1965) and melamine. In 1958, Colbey and Mesler patented the use of melamine as a non-protein nitrogen source for cattle.

In 1966, MacKenzie investigated the possible use of melamine as a source of NPN for sheep fed roughage based diets deficient in protein. MacKenzie's rationale for selecting melamine was based on the fact that the structure of melamine was similar to that of cyanuric acid which has been reported to be a safe and effective source of NPN for sheep (Clark *et al*., 1965). In addition, the nitrogen content of cyanuric acid is lower when compared to melamine (32% *vs*. 66%). This may result in a more efficient utilization of nitrogen when using melamine as a source of NPN. However, MacKenzie reported the use of melamine as a NPN source to be unreliable due to observed reductions in apparent nitrogen digestibility, reduced feed intake and the inexplicable deaths of five sheep that were fed 10 g melamine per day.

Following these results, Clark (1966) investigated the possible toxic effects of melamine. It was concluded that sheep fed 10 g melamine per day succumbed due to uraemia as a result of crystalluria. It was concluded from this trial that melamine cannot be considered as a safe source of NPN for ruminants.

Newton & Utley (1978) also investigated the effectiveness of melamine as a NPN source for ruminants. Three mature steers fitted with permanent fistulae were used in this study. Even though they managed to prove an increase in rumen ammonia concentration via an *in vitro* trial, they reported that the rate of melamine hydrolysis in the rumen was insufficient to promote maximum ruminal protein synthesis and confirmed Clark's (1966) recommendation that melamine is not an acceptable NPN source for ruminants.

Since these studies, and based on the overall conclusions that melamine is an ineffective NPN source for ruminants, further investigations on the use of melamine in animal studies ceased. It was not until early 2006, when melamine made headlines in the food and feed industries, that melamine received renewed attention from the scientific world. This was due to the international havoc caused by the 2007 pet food recall and human health concerns associated with the 2008 Chinese milk scandal. Since the first report of melamine adulterated pet food, vast amounts of research pertaining to melamine toxicity and transmission, were done in various fields of interests, including human health risks and animal studies.

This review aims to provide a summary on melamine adulteration in feeds and foods with reference to the 2007 pet food recall and the 2008 Chinese milk scandal. However, the main focus of this review is to provide an update on the recent knowledge pertaining to melamine in the feed and food chain by discussing melamine transfer from animal feeds to animal products used for human consumption. Therefore, the potential human risks associated with consuming melamine tainted animal products will also be discussed.

2.2 Melamine as a protein adulterant

The adulteration of raw ingredients and final products has become a great concern in both the feed and food industries. This could be attributed to the fact that the supply chain of feed and food ingredients are becoming more complex and globalized, increasing the difficulty for quality control of such ingredients. Even though the adulteration of feed and food ingredients is considered as fraud, it seems that it is still a common occurrence. Food fraud can be defined as a collective term that encompasses the deliberate substitution, addition, tampering or misrepresentation of food, food ingredients, or food packaging, or false or misleading statements made about a product for economic gain (Moore *et al*., 2012).

The adulteration of feed ingredients is of great concern due to the risk of economic losses that purchasers may incur, but also due to the potential health risks to the consumers. The main reason for adulteration is solely for economic gain. In most cases, the perpetrators do not necessarily have the expertise to assess whether the adulteration pose any toxicological risks to the purchaser or consumer, which is usually unknown until it's too late (Moore *et al*., 2012). This statement holds true with regards to the whole melamine adulteration crises originating from China.

From 1992 to 2008, China's agricultural product exports to 132 countries increased from 9.7 billion US\$ to 30.1 billion US\$, respectively (Mangeldorf *et al*., 2012). It was also during this time that both the consumption and production of melamine increased considerably in mainland China. However, by 2006, melamine production in China was in surplus and even though the global melamine price remained stable, the increased price of urea (feedstock for melamine) reduced the profitability of melamine manufacturing (Wang, 2006). This scenario may have contributed to the melamine adulteration involved in the pet food recall of 2007. However, in April 2007, the New York Times reported the adulteration of livestock feeds

with melamine wastes to be an "open secret" in many parts of mainland China for quite some time.

Melamine has a high nitrogen content *viz.* 667 g/kg N on a molecular basis (Merck, 2001). The crude protein content of feeds and foods are calculated from its nitrogen content (AOAC, 2000). Theoretically, the crude protein content (N x 6.25) of pure melamine would be 4167 g/kg. Therefore, the high nitrogen content of melamine makes it an attractive adulterant for protein feedstuffs as it can artificially increase the apparent protein content when added to feed and food ingredients. It is of interest to note that the use of melamine as an adulterant for high protein content ingredients can be traced back to the early 1980's (Cattaneo & Cantoni, 1982). During this period, potato meal in Germany and meat and fish meals in Italy were found to be adulterated with melamine (Dorne *et al*., 2012).

Whether it was the increased pressure of international demand for high protein feedstuffs, the surplus melamine production in China, the pure greediness of the feedstuff suppliers or all of the above scenarios which led to the exportation of melamine adulterated protein feedstuffs from China is unclear, but it severely impacted the world when the massive pet food recall was announced early in 2007.

2.3 The international pet food recall of 2007

Early in 2007, US authorities were alerted of various reported cases of illness, renal failure and mortalities in dogs and cats exposed to melamine contaminated pet foods (FDA, 2007). Symptoms exhibited by pets included vomiting, lethargy, polyuria and anorexia (Puschner *et al*., 2007). The estimated total number of dog and cat mortalities ranged between 2 000 and 7 000 (Puschner & Reimschuessel, 2011). It was soon discovered that wheat gluten and rice protein concentrates (Dobson *et al*., 2008) imported from China was fraudulently adulterated with melamine waste and subsequently used in the manufacturing of pet food by various companies. The melamine concentrations of the protein ingredients and pet food ranged between 2 000 – 80 000 mg/kg (EFSA, 2010) and 9.4 – 1952 mg/kg (Bhalla *et al*., 2009), respectively. Even though no deleterious animal and human health issues were reported, soybean meal, intended for feed, were also reported to have been adulterated with melamine (EFSA, 2010).

South Africa was also affected by the melamine adulterated feedstuffs. However, the feed ingredient identified as the main culprit was maize gluten 60 imported from China (Cruywagen & Reyers, 2009). Melamine analysis revealed the melamine concentration of the maize gluten to have been 15 117 g/kg (Cruywagen *et al*., 2009).

The feedstuffs used in the manufacturing of the pet foods (mainly wheat and maize gluten meal) were adulterated with melamine waste. During the manufacturing of melamine and depending on the purification process, the resultant residues (melamine waste/"scrap") contain variable levels of melamine and its analogues. Therefore, melamine and melamine analogues (ammelide, ammeline and cyanuric acid) were also present in the pet foods. Autopsies done on the dogs and cats that succumbed after the ingestion of melamine tainted pet foods revealed insoluble melamine cyanurate crystals in the kidneys and renal tubules (Brown *et al*., 2007). It was concluded that the cause of death was due to renal failure. Due to the simultaneous ingestion of melamine and cyanuric acid, the risk for kidney stone formation is increased (Kobayashi *et al*., 2010). This is due to the fact that melamine reacts with cyanuric acid on a 1:1 basis to form melamine cyanurate, a crystalline complex held together by an extensive two-dimensional network of hydrogen bonds (Perdigão *et al*., 2006). The toxicosis observed in the 2008 Chinese infant milk scandal was different from that observed in the pet mortalities.

2.4 The 2008 Chinese infant milk scandal

In September 2008, pure melamine was discovered to have been fraudulently added to infant milk formula and other milk products produced in China. The melamine concentration of the adulterated infant formula was reported to range from 0.09 to 2563 mg/kg (Zhang *et al*., 2009). This resulted in detrimental health effects of infants and young children who consumed those products. Only two months later, Chinese authorities confirmed that six babies had died and more than 294 000 infants and young children were hospitalised and diagnosed with urinary tract stones (WHO, 2008). As infants are rarely diagnosed with urinary tract calculi resulting in renal failure (Sun *et al*., 2010), it is clear that the consumption of melamine adulterated infant formula was the main causative agent resulting in renal dysfunction and failure.   Some of the clinical symptoms in the affected infants and children included pain during urination, vomiting, stone discharge during urination, fever due to urinary tract infection and kidney failure (Langman *et al*., 2009). The kidney stones were identified as containing melamine-urate (Dorne *et al*., 2012).

In humans, uric acid is the final product of nucleic acid metabolism as humans lack the enzyme urate-oxidase (uricase) to convert uric acid to allantoin (Wu *et al*., 1989). Melamine and uric acid share some structural similarities due to the N-formylformamide group in the 4, 5 and 6 positions in the six-membered ring (Ogasawara, 1995). Therefore, melamine may form closely related hydrogen-bonded complexes with uric acid, resulting in the formation of kidney stones. In fact, the kidney stones that were obtained from children affected by melamine adulterated products were identified as melamine-urate where uric acid and melamine were bound in a molar ratio of 1.2:1 to 2:1 (Sun *et al*., 2010).

Compared to adults (which were also exposed to melamine adulterated foods), infants and young children were especially affected by the melamine adulteration purely due to physiological and nutritional differences. Compared to adults, infants and small children consume a larger percentage of food per unit of body weight, due to their higher growth rates (Mifflin *et al*., 1990), which consists predominantly of milk and milk products (including infant formula).   Infants and small children also consume their source of nutrition more frequently (up to every 2 hours) throughout the day compared to adults. Physiologically, infants and small children have higher urinary and serum uric acid concentrations and urinary uric acid clearance rates (38 - 61% *vs*. 10%) when compared to adults (Stapleton, 1983). In addition, infant boys would be more susceptible than infant girls due to higher uric acid concentrations in males and differences in male urethra anatomy (e.g. urethra length, stegnosis and arcuations). This was confirmed by Sun *et al*. (2010) in which their affected subjects represented a male to female ratio of 2.1:1. Therefore, the high levels of melamine in the adulterated infant formulae and inert high uric acid concentrations contributed to the increased melamine-urate precipitations. Even though the majority of the Chinese population were exposed to melamine adulterated food products, it was the infants and small children who were the most susceptible.

It is well known that melamine was added to the final milk products to artificially increase the product's apparent crude protein content as Hau *et al*. (2009) demonstrated that the addition of 1 g of melamine to 1 L of milk would increase the apparent protein content by 0.4%. It is also well known that one of the large dairies in China, Sanlu, adulterated milk by the addition of water to increase the volume, followed by the addition of melamine to restore the apparent protein content (Bradsher, 2008). Apart from milk and milk products, melamine was also detected in frozen desserts, powdered milk and cereal products, cakes and biscuits,

protein powders and processed foodstuffs (Gossner *et al*., 2009). Certain non-dairy products (e.g. ammonium bicarbonate, eggs and non-dairy creamer) originating from China were also reported to be contaminated with melamine. In addition, some pet food industries were also victims of melamine adulteration. This led to the identification of the possible risks that the commercial animal (i.e. cattle, pigs and poultry) feed industry may incur by the fraudulent addition of melamine to protein feedstuffs. The Rapid Alert System for Food and Feed (RASFF) confirmed the presence of melamine in animal feed and feed ingredients in 2008. Ultimately, this raised the question as to the possibility of melamine transfer from the animal's feed to edible animal products.

2.5 Melamine transfer from feed to edible animal products

In total, 47 countries (including South Africa) were affected by melamine adulterated products. Near the end of 2006, South Africa (unknowingly) imported 600 metric tons of melamine-tainted maize gluten 60 from China. Microscopic analysis (Cruywagen & Reyers, 2009) revealed that the product was, in fact, not maize gluten 60 with added melamine, but a mixture of various ingredients, blended in such a way to vaguely simulate maize gluten 60 in terms of appearance and basic chemical composition. Ingredients included wheat starch, wheat bran, maize bran, maize gluten 20, maize gluten 60, urea, melamine and colourants. Following the 2007 pet food recall, the South African Department of Agriculture could only manage to quarantine 308 metric tons of the tainted feed, as the remaining amount had already found its way into the animal feed industry. Subsequently in 2008, reports were made that melamine was found in the milk of some South African dairies. This was the initial inspiration for Cruywagen *et al*.'s (2009) study to determine whether the possibility exists for melamine to be transferred from feed to cow's milk.

2.5.1 Melamine transmission from feed to milk and milk products

Cruywagen *et al*. (2009) were the first to confirm the transmission of dietary melamine to cow's milk. The cows received an experimental diet which resulted in a daily intake of 17.1 g of melamine for eight consecutive days, followed by a melamine withdrawal period where the diet contained no melamine. Melamine appeared in the milk as soon as 8 hours after the initial ingestion.   The milk melamine concentration increased rapidly and reached a maximum within 3 d after first ingestion of the experimental diet. This trend was also observed in the studies of Shen *et al*. (2010) and Sun *et al*. (2011). Cruywagen *et al*. (2009)

reported the mean excretion efficiency of melamine (g of melamine ingested / g of melamine excreted into milk x 100) as 2.1%. Upon melamine withdrawal, the milk melamine concentration dropped rapidly by 39% and 85% for 8h and 32h, respectively. No melamine could be detected in the milk 7 d (152 h) after melamine withdrawal. These results were later confirmed by Sun *et al*. (2011).

Baynes *et al*. (2010) provided dairy goats with a single melamine oral bolus (40 mg/kg) in order to determine the pharmacokinetics of melamine in a ruminant model in an attempt to estimate melamine depletion time from blood and milk. They reported the plasma half-life of melamine to be 11.12 ± 2 h, the apparent volume of distribution as 4.09 ± 1.05 L/kg and the clearance rate of melamine as 0.26 ± 0.04 mg/h per kg milk. In addition, Baynes *et al*. (2010) reported the melamine residues in the milk 12 h post melamine administration, to have ranged between 8 - 12 μg/mL, which then rapidly declined over the following 72 h. Contradictory to the findings of Cruywagen *et al*. (2009) and Battaglia *et al.* (2010), Baynes *et al*. (2010) reported the average total amount of the melamine dose excreted in milk to have been 0.31%. This may be ascribed to the fact that Baynes *et al*. (2010) provided dairy goats with a single dose of melamine, as it appears from various literature reports (Cruywagen *et al*., 2009; Battaglia *et al.*, 2010; Shen *et al*., 2010 and Sun *et al*., 2011) that melamine excretion may be influenced by melamine dosage and duration of exposure. The non-compartmental model proposed by Baynes *et al*. (2010), accurately predicted melamine depletion (time at which melamine concentration was below 0.01 μg / mL) of 120 h and 108 h for plasma and milk, respectively.

Battaglia *et al*. (2010) also investigated the transfer of melamine to cows' milk. These authors fed four doses of melamine as a single oral bolus to four groups of cows. The selected melamine doses were 0.05 g, 0.50 g, 5.00 g or 50.00 g per cow. In agreement with the findings of Cruywagen *et al*. (2009), Battaglia *et al*. (2010) reported a rapid appearance of melamine in the milk only 6 h after ingestion of the bolus. They calculated the melamine excretion efficiency via milk to range between 2.3 and 3.3%. Owing to the fact that these authors observed different times when maximum milk melamine concentrations were observed in the milk (6 and 18 h after melamine intake), as well as different times when melamine reached undetectable levels (102 and 174 h after melamine withdrawal), it appears that the excretion pattern of melamine to milk may be dose dependent. Shen *et al.* (2010) reported no detectable levels of melamine in the milk at 4 d after melamine withdrawal. This

16

could be ascribed to the relatively low melamine doses *viz.* 90, 270 and 450 mg used in their trial. Both Shen *et al.* (2010) and Sun *et al.* (2011) reported a significant effect of dietary melamine intake on milk melamine concentration, but neither observed dose effects on the transfer efficiency to cows' milk. However, Shen *et al.* (2010) reported milk yield to significantly affect the transfer efficiency of melamine from feed to milk and proposed that high-producing cows may be more efficient in excreting melamine via milk than low-producing cows.

Battaglia *et al.* (2010) also investigated the possible transfer of melamine from tainted milk to cheese. They observed increased melamine concentrations in the milk, cheese and whey as the melamine dosage increased. Their results indicated that melamine partitioning from milk was mainly to whey (approximately 85 %), while only 1.9 % was partitioned to cheese. The balance of approximately 13% could not be accounted for. Because melamine analyses were only done in cheese after a curing period of two weeks, the authors speculated that some of the melamine in the cheese may have been degraded during the 14 d conditioning period. However, no literature could be found to support this hypothesis.

2.5.2 Melamine transfer from feed to meat and organ tissues

Since the confirmation that a pathway exists for dietary melamine to be transmitted to milk, the possibility of dietary melamine transmission to animal tissues (e.g. meat, liver and kidneys) also received research attention. Mast *et al.* (1983) reported melamine to be rapidly absorbed from the rat's gastrointestinal tract, with little or no metabolism and then rapidly excreted via urine. Owing to the rapid excretion of melamine via urine, 90% of the ingested melamine was recovered within 24 h, with total excretion in rats recorded at 96 h (Mast *et al.*, 1983). Following the administration of melamine as a single oral dose to rhesus monkeys, Liu *et al.* (2010) confirmed the rapid excretion of melamine via urine and faeces at 36 h.

Yang *et al.* (2009) reported melamine to be restricted to blood and extracellular fluid, which, according to them, explained the limited distribution of melamine to animal tissues and the lack of melamine accumulation in animal tissues. This was in agreement with Baynes *et al.* (2008) who determined melamine distribution in pigs after intravenous administration, and found it to be similar to total body water. This led them to conclude that melamine is restricted to the extracellular fluid compartment with limited distribution to organ tissues.

The lack of melamine residues in animal tissues observed by the above mentioned authors may be ascribed to the administration of a single melamine dose. Due to the short half-life of melamine and rapid excretion via urine, it appears that the kidneys are quite capable in eliminating single doses of melamine from the body. However, the kidney's ability to eliminate melamine via urine may differ when animals consume melamine on a continuous basis. Dominigues-Everez et al. (2010) calculated the average steady-state concentration of melamine in urine as the absolute total daily melamine intake divided by the daily urinary volume. Therefore, continuous exposure to dietary melamine may prolong the presence of melamine in plasma, which may promote the distribution of melamine to other tissues and ultimately result in melamine deposition in certain tissues.

Melamine residues in various tissues (including muscle, liver and kidneys) were indeed observed in many animal species by various authors. A study on various fish species that received melamine tainted feeds was done by Anderson et al. (2008) as it was noted that some fish feeds were also affected by the melamine adulteration scandal that involved the animal feed industries. In this study, catfish, trout, tilapia and salmon received approximately 400 mg of melamine per kg body weight (BW) for three consecutive days. Melamine residues were detected in the meat of all the fish with maximum concentrations reported as 210, 177, 94 and 80 mg/kg for catfish, tilapia, salmon and trout, respectively. After a six day withdrawal period, the residual meat melamine concentration was considerably lower in tilapia (0.02 mg/kg) compared to that in trout (34 mg/kg), salmon (58 mg/kg) and catfish (81 mg/kg). Even though it was observed that tilapia refused some of the melamine containing gel feed which may have resulted in lower feed intake, Anderson et al. (2008) still proposed tilapia species to be more capable to excrete melamine compared to the other fish species. This was based on the fact that the tilapia and catfish had similar melamine concentrations when the peak concentrations were observed. Therefore, the tilapia species were able to eliminate melamine more efficiently when compared to the catfish.

Broiler chickens (Lü et al., 2009a) and ducks (Lü et al., 2009b) were provided with feeds containing graded levels (0, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 mg/kg) of melamine for 42 d, followed by a 7 d withdrawal period. These studies were conducted to determine melamine residues in the tissues of broilers and ducks after consuming graded levels of melamine. No adverse effects on both broiler and duck health and performance were reported at any of the inclusion levels. In the broiler study, melamine residues were detected

in the breast muscle and liver tissues on day 28 for all groups receiving a treatment of 200 mg of melamine/kg feed or more. Interesting enough, no melamine could be detected in the muscle and liver samples of the 200 mg/kg treatment group on day 42. In addition, all melamine concentrations of the higher dosage groups were reported to have lower values on day 42 compared to day 28. From the results of Lü *et al*. (2009*a*), it appears that some kind of physiological adaptation occurs in the body to improve the elimination of melamine from the body as the birds aged. However, there is no literature available to support this hypothesis and warrants further investigation. On day 42 of the duck study (Lü *et al*., 2009*b*), melamine residues were detected in the tissues (liver, kidney and muscle) of all ducks receiving treatment diets containing more than 50 mg melamine/kg BW. In both studies, melamine partitioning was significantly ($P \leq 0.05$) higher to the kidneys, followed by the liver and muscle tissues. After the 7 d withdrawal period, no melamine was detected in any broiler and duck tissues for all treatment groups.

Sirilaophaisan *et al*. (2010) also fed melamine at various inclusion levels to broiler chickens for 42 d followed by a withdrawal period of 7 d. The selected melamine inclusion levels were 2500, 5000, 7500 and 10 000 mg/kg. Sirilaophaisan *et al*. (2010) reported significant reductions ($P < 0.05$) in body weight gain (BWG) and feed conversion ratios (FCR) in birds receiving the highest melamine treatment. The survival percentage decreased significantly as the level of melamine inclusion increased with the most severe effect observed at the highest melamine level (10 000 mg/kg). These results are contradictory to the findings of Lü *et al*. (2009*a*) who reported no effects on production parameters. This may be ascribed to the relatively higher levels of melamine inclusion in the broiler diets of Sirilaophaisan *et al*. (2010), as the highest melamine inclusion level in the study of Lü *et al*. (2009*a*) was 1000 mg/kg. Melamine was detected in the liver and muscle tissues of all treatment groups, with melamine concentrations being higher in the liver than in the muscle tissues. In agreement with Lü *et al*. (2009*a*), no melamine residues could be detected in any tissues after a 7 d withdrawal period.

Brand *et al*. (2012) provided graded levels of melamine (0, 5 000, 10 000, 15 000, 20 000, 25 000 and 30 000 mg/kg) to broiler chickens for 21 days. In agreement with Sirilaophaisan *et al*. (2010), BWG and FCR were also significantly reduced in the treatment group receiving $\geq$ 10 000 mg/kg. However, the survival rate was only significantly reduced when treatments $\geq$ 25 000 mg/kg were fed, while Sirilaophaisan *et al*. (2010) noticed severe reductions in

19

survival rate at 10 000 mg/kg. Melamine was detected in the kidney, liver and muscle tissues of all treatment groups, with the pattern of melamine partitioning following the same trend as reported by Lü *et al*. (2009*a*).

Bai *et al*. (2010) provided graded levels (125, 250, 500, 1000 and 2000 mg/kg) of melamine to layer hens for 34 d followed by a 20 day withdrawal period. In agreement with Lü *et al*. (2009*a*), melamine tainted feeds did not influence any production parameters of the hens, but histopathology revealed some histological alterations in the kidneys of hens receiving the 250 mg/kg and higher doses of melamine. In addition, some crystals were observed in the kidneys of hens receiving 1000 mg/kg and 2000 mg/kg. Melamine was detected in various tested tissue samples, including the kidneys, livers, muscles, stomachs and duodena of all treatment groups. The partitioning of melamine to various tissues followed the same distribution pattern as observed by Lü *et al*. (2009*a*), with the kidneys having the highest melamine concentration, followed by the liver and muscle. Upon the 10 d melamine withdrawal period, melamine was detected at low concentrations only in the livers and stomachs of treatments groups receiving treatments 1000 mg/kg and higher. This is contradictory to the findings of Lü *et al*. (2009*a*) and Sirilaophaisan *et al*. (2010) who reported no detectable levels of melamine in any tissues following a 7 d withdrawal period. In addition, Sirilaophaisan *et al*. (2010) fed melamine levels up to 5 times higher than that of Bai *et al*. (2010) and still reported no detectable levels of melamine in any tissues on day 7 of the melamine withdrawal period.

Valat *et al*. (2011) fed two levels of melamine (*viz*. 50 and 500 mg/kg) to laying hens for 8 consecutive weeks. Melamine residues were detected in kidney, liver and muscle tissues. Interesting to note, is that three weeks after the initial feeding of melamine tainted feed, melamine residues were detected in the kidney, liver and muscle tissues of the low (50 mg/kg) treatment group, but not in the higher (500 mg/kg) treatment group. However, melamine was present in all tissue samples of all treatment groups 6 and 8 weeks after initial melamine feeding. Another interesting observation from this study is that the melamine concentrations in the tissues were lower after 8 weeks, compared to the values observed at 6 weeks. This observation may support the hypothesis of Lü *et al*. (2009*a*) that advanced age of the birds may result in an increased ability to eliminate melamine from the body.

Gallo *et al*. (2012) also studied melamine transmission from feed to tissues in laying hens. The selected melamine inclusion levels were 2.5, 25 and 250 mg/kg, and were offered to the

20

hens for 13 consecutive days. No melamine could be detected in any tissues of the lowest treatment group. For the remaining two treatments, melamine was detected in the kidney, liver and muscle tissues. Regarding the 25 and 250 mg/kg melamine treatments, the melamine concentrations in the kidney, liver and muscle tissues were 0.396 vs. 2.952 mg/kg, 0.056 vs. 0.936 mg/kg and 0.078 vs. 0.799 mg/kg, respectively. From these results, it can be noted that the increasing melamine dose resulted in increased melamine concentrations in the tissues by approximately the same factor. Gallo *et al*. (2012) reported that the pattern of melamine partitioning to the various tissues was similar to that observed by Lü *et al*. (2009*a*).

In addition to poultry studies pertaining to dietary melamine transmission to meat, some work on pigs were also reported. Before the 2007 melamine tainted protein feedstuffs made international headlines, it appears that some areas in the western parts of Spain were affected by feed adulterated with melamine. González *et al*. (2009) reported that between November 2003 and June 2006, a total of 300 to 400 piglets had developed anorexia, depression, lethargy and polydipsia several days after weaning. González *et al*. (2009) reported the estimated morbidity and mortality rates of the total population of post-weaning piglets as 40-60% and 20-40%, respectively. Upon further investigation, it was discovered that all of the affected farms fed identical protein concentrates to which piglets had access from birth to weaning. Upon elimination of the protein concentrate, no new cases were reported. At that time, the possibility of melamine adulteration was not even considered as there was no data available on melamine toxicosis and the associated clinical symptoms. It was only after the adulteration of protein feedstuffs with melamine made international headlines that González *et al*. (2009) suggested melamine to have been the causative agent. Post-mortems performed on nine of the affected piglets, revealed gross abnormalities in the kidneys as a result of crystalluria. The crystals were reportedly very similar to that observed by Brown *et al*. (2007). Even though the implicated protein concentrate could not be attained, LC/MS analyses on the kidneys revealed concentrations of melamine, ammeline, ammelide and cyanuric acid, which confirmed the suspicion that the piglets had consumed feeds adulterated with melamine and its analogues.

Following the intravenous administration of melamine (6.13 mg/kg) in pigs, Baynes *et al*. (2008) determined the volume of melamine distribution in pigs to be close to total body water, *viz*. 0.61 L/kg, which suggested the restriction of melamine to the extracellular fluid compartment and limiting its distribution to organ tissues. Baynes *et al*. (2008) also reported

rapid clearance of melamine from the body, predominantly via renal filtration, with a half-life of 4.07 h. Baynes *et al*. (2008) reported rats to have an approximate 5-fold greater renal clearance compared to pigs, resulting in the 1.5 times longer melamine half-life in pigs than in rats. Approximately 99% of the administered melamine was cleared from the pigs' blood by 28 h.

Buur *et al*. (2008) developed a physiologically based pharmacokinetic (PBPK) model in order to predict an appropriate meat withdrawal interval if pigs were to be exposed to melamine adulterated feed. This was achieved by developing a PBPK model for rats with information from various authors and then extrapolating the model to pigs. Even though the pig model under-predicted melamine plasma concentrations at early time points, it resulted in greater accuracy at later time points which are indicative of relevant withdrawal times. In addition, Buur *et al*. (2008) estimated a withdrawal interval of 19.2 and 20.9 h if pigs were to receive a single oral dose of 3.0 and 5.12 mg/kg of melamine, respectively.

Based on the above mentioned observations, it is clear that melamine toxicity and partitioning differs between animal species which could be ascribed to species-specific pharmacokinetics. Apart from monogastric animals (e.g. poultry and pigs), one should keep in mind that the distribution kinetics of melamine to tissues may differ between monogastric and ruminant animals. In addition, no melamine metabolism was reported in either rat (Mast *et al*., 1983) or pig (Baynes *et al*., 2008) studies. This may not be the case in ruminants as Newton & Utley (1978) reported increased rumen ammonia concentrations with the addition of melamine in an *in vitro* trial. The *in vitro* hydrolysis of melamine to melamine analogues (e.g. cyanuric acid) by an anaerobic strain of *Pseudomonas* have been reported by Jutzi *et al.* (1982), but it remains uncertain whether that strain occurs in the rumen. However, Duncan *et al*. (1999) reported the presence of *Pseudomonas aeruginosa* in the rumen of sheep. Unpublished data from an *in vitro* study (Chapter 3) at Stellenbosch University, South Africa, revealed that the incubation of melamine in rumen liquor for 48 h resulted in 13.6% of the melamine being degraded.   Therefore, it may be possible that melamine metabolism occurs in the rumen of ruminants, which could result in different melamine partitioning patterns than observed in monogastric animals.

Lv *et al*. (2010) were the first to report melamine deposition in lambs fed varying levels of melamine for 60 days. Lv *et al*. (2010) reported melamine residues in the gluteal muscle, *Longissimus dorsi* muscle, liver and kidneys for all fed treatments containing $\geq$ 10 mg

22

melamine per kg BW. The reported melamine residues were similar in both the gluteal and longissimus dorsi muscle, with melamine concentrations ranging between 0.23-0.37 mg/kg in lambs fed the highest melamine dose (*viz*. 100 mg/kg). Lv *et al*. (2010) observed the highest melamine residues in the kidneys, followed by the liver, with muscle tissues having the lowest melamine residues. This melamine partitioning pattern to the different tissues were observed in all of the fed treatments. Upon melamine withdrawal, Lv *et al*. (2010) reported melamine concentrations in all tissues to be below 0.02 mg/kg after 108 h.

In another sheep study, Cruywagen *et al*. (2011) fed 0.69 g melamine (which equates to approximately 11.5 mg melamine per kg BW) daily to rams for 8 consecutive days, followed by the slaughtering at the end of the trial. Tissue samples of the *Longissimus dorsi* muscle, liver, kidneys and abdominal fat were collected for melamine analyses. From the results, Cruywagen *et al*. (2011) suggested the apparent digestibility of dietary melamine to be 76.7%. Melamine analysis on the various tissue samples revealed a melamine concentration of 9.6 mg/kg in the longissimus dorsi muscle, which accounted for approximately 3.5% of the dietary melamine (Cruywagen *et al*., 2011). Other melamine concentrations of the liver, kidney and fat tissues were reported as 4.0, 9.63 and 0.20 mg/kg, respectively. Contrary to the findings of Lv *et al*. (2010), the results from Cruywagen *et al*. (2011) suggested melamine partitioning to the muscle and kidneys to be similar. However, the majority of studies pertaining to dietary melamine partitioning to tissues, suggests melamine distribution predominantly to the kidneys, followed by the liver, with the muscle tissues having the lowest residual melamine concentration (Lü *et al*., 2009; Bai *et al*., 2010; Brand *et al*., 2012; Gallo *et al*., 2012). Cruywagen *et al*. (2011) also quantified the excretion of dietary melamine via urine and faeces. The reported urine and faecal excretions accounted for 53.2 and 23.3% of the dietary melamine, respectively. In agreement with the early rat study of Mast *et al*. (1983), the majority of melamine was excreted via urine. However, Mast *et al*. (1983) reported the contribution of faecal melamine excretion to be minimal and proposed that the low faecal melamine concentration may have been as a result of urinary contamination.

From the discussed literature, it is clear that the partitioning kinetics differ between and within species. In addition, the melamine dosage and duration of exposure may influence the distribution kinetics of melamine which in turn, may result in the varying results observed between different studies. It is also possible that age may influence the capability of animals to eliminate melamine more efficiently, as was suggested by Lü *et al*. (2009*a*). It also

appears that melamine partitioning and excretion from the body may be influenced by gender. Apart from physiological differences between males and females, Stapleton (1983) reported human males to have higher uric acid concentrations than females. Therefore, males may be more susceptible to renal stone formation should they be exposed to melamine. This may also be true in animals, but further research would be required to confirm this. Contributing to the melamine concentration variations reported within and between various studies, it should be kept in mind that melamine has a short half-life of approximately 3 h and is excreted rapidly from the body via urine. Therefore, the experimental feeding regimes and time of sampling may contribute to the variation seen in the reported melamine concentrations. In addition, the type of melamine (pure or waste) used in experiments may also result in variations of melamine concentrations and toxicological effects, especially in cases where melamine waste is used as it may contain additional melamine analogues such as cyanuric acid. Other factors that also require consideration are the methods used for sample preparation, sample extraction as well as methods for detecting and quantifying the melamine present in samples, as these factors may also contribute to the variability of the melamine concentrations reported. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is reported to be the most sensitive and selective analytical method used to measure melamine and its structural analogues (EFSA, 2010).

The distribution of melamine to various animal tissues has been well documented in various animal (e.g. poultry, fish and sheep) studies. It was also shown that even though the majority of dietary melamine is excreted via urine, followed by faeces, some dietary melamine can be excreted into animal by-products, including milk (Cruywagen *et al*., 2009) and cheese (Battaglia *et al*., 2010). Apart from melamine distribution to various tissues in broiler chickens, some studies have proved the carry-over of dietary melamine to eggs.

2.5.3 Melamine transfer from feed to eggs

Various authors have confirmed the transfer of dietary melamine to eggs. The highest inclusion level of 500 mg melamine/kg BW had no effect on daily feed intake and egg production (Valat *et al*., 2011). This was in agreement with Bai *et al*. (2010), Gao *et al*. (2010) and Gallo *et al*. (2012) who reported no observed effects on layer production parameters when various levels of melamine were included in the feed. Upon the initiation of melamine administration, melamine was detected in the eggs on the same day (d1) of melamine exposure (Valat *et al*., 2011; Gallo *et al*., 2012). This was also observed by Gao *et*

24

al. (2010) who fed melamine to ducks at a low level of 1 mg melamine/kg BW.   However, Bai *et al*. (2010) only reported melamine residues in the eggs on day 2 of melamine consumption. The egg melamine concentrations reportedly peaked on day 2 when treatments contained ≥ 150 mg melamine/kg BW (Bai *et al*., 2010; Gao *et al*., 2010). The transmission of dietary melamine to eggs at a steady state was reported as 0.50, 0.70 and 0.84% for hens receiving treatments containing 2.5, 25 and 250 mg melamine/kg BW, respectively (Gallo *et al*., 2012). Chen *et al*. (2010) reported the transfer rate for melamine to the egg yolk after continuous melamine exposure to vary between 1.5 and 3.2 % when hens received 0.5, 25, 50 and 100 mg melamine/kg feed. Gao *et al*. (2010) proposed the dose of melamine exposure to influence the time at which eggs will contain undetectable levels of melamine upon withdrawal. Upon melamine withdrawal, authors were in agreement that the melamine concentration in eggs decline rapidly. The withdrawal period required to ensure undetected levels of melamine in eggs, were reported for layers fed 100 mg of melamine/kg BW as 7 days (Bai *et al*., 2010) and 8.52 days (Gao *et al*., 2010).

2.5.4 Melamine transfer from fertilizer to pastures

Introductory to this review, it was mentioned that melamine was used as a slow nitrogen releasing fertilizer for crops and pastures (Hauck & Stephenson, 1964). Melamine proved to release nitrogen slowly when used as fertilizer. However, the nitrogen is released too slowly into the soil (Mosdell *et al*., 1987) in order to benefit the growth of crops and pastures. With only 1% of melamine nitrogen converted to nitrate (Scholl *et al*., 1937), it is not surprising that grass showed a low growth response when melamine-fertilizer was applied (Wehner & Martin, 1989).

These early studies focussed on the efficiency of melamine to supply plants with nitrogen and did not consider the uptake of intact melamine. Therefore, a study done at Stellenbosch University (Botha, 2010) investigated the possible transfer of melamine to pastures. This study consisted of a pilot pot plant trial and an applied pasture trial. In both trials, Kikuyu grass (*Pennisetum clandestinum*) was fertilized with melamine. In the pot plant trial, Kikuyu grass was fertilized with melamine adulterated maize gluten 60 (as was imported to South Africa from China) that contained 15 117 mg/kg of melamine. The application rate was equivalent to 8.8 kg of melamine/ha. Seven days after application, melamine was detected in the grass leaf material at levels of 228 mg/kg. In the applied pasture trial, three Kikuyu grass camps of 0.3 ha each were used. In the Control treatment, pastures were fertilized with lime

25

stone ammonia nitrate (LAN) to provide 40 kg of N/ha. In the melamine treatments, either 10% (Treatment 1) or 20% (Treatment 2) of the LAN-N was replaced by melamine-N, which related to 5.97 and 11.94 kg of melamine/ha, respectively. One week after fertilizer application, melamine was detected in the pasture grass at levels of 10 and 32 mg/kg for Treatments 1 and 2, respectively. Four weeks after fertilizing the pastures, cows were allowed to graze the respective pastures for 9 consecutive days, 10 h/d. Melamine was detected in the milk of all cows approximately 8 h after initial consumption of melamine fertilized pastures. The highest milk melamine concentrations recorded were 0.02 and 0.042 mg/kg for cows grazing Treatment 1 and Treatment 2, respectively. As a result, the mean transfer efficiencies (calculated as the ratio between melamine ingestion and melamine excretion via milk) were 3.02 and 2.10% for Treatments 1 and 2, respectively. These reported transfer efficiencies were well in the range as reported by Cruywagen *et al.* (2009) and Battaglia *et al.* (2010). After the 9 d grazing period, cows were removed from the fertilized pastures and grazed on melamine free pastures. After 6 d, melamine could not be detected in the milk, which is in agreement with the findings of Cruywagen *et al.* (2009) and Battaglia *et al.* (2010).

The initial confirmation of melamine transfer from fertilizer to grass which consequently resulted in melamine transfer to cow's milk, raised the concern as to the implications of pasture recontamination via cow's urine and faeces following melamine contaminated pasture consumption. By now it is clear that the major excretion route of dietary melamine is via urine and faeces. Faecal samples were collected on the grazed pastures of Treatment 1 and 2 after the daily 10 h grazing period from day 3 to 9. Only faecal samples were collected as the collection of the cows' urine on pastures proved to be too difficult. The mean faecal melamine content was 0.51 and 1.02 mg/kg for Treatments 1 and 2, respectively. These results confirmed that metabolic excretions (i.e. urine and faecal) could result in recontamination of pastures with melamine (Calitz *et al.*, 2012). Even though urine samples were not collected, it can be expected that urine would result in higher levels of pasture recontamination compared to faeces, as urine is the primary route of melamine excretion.

2.6 Other sources of melamine contamination

Apart from deliberate adulterations, certain food sources (e.g. leafy vegetables, melons, mushrooms and potatoes) may be contaminated with melamine. This may result from the application of a pesticide, cyromazine, which acts as a systemic insect growth regulator

(WHO, 2008). When plants metabolize cyromazine, melamine is a resultant metabolite. The presence of melamine has been detected on fruits (Lim *et al*., 1990), vegetable crops (Patakioutas *et al*., 2007) and beans (Karras *et al*., 2007) after the application of cyromazine. The European Union established a Maximum Residue Limit (MRL) for the application of cyromazine of 15-20 mg/kg for leafy vegetables and beet leaves, respectively (EFSA, 2010).

Cyromazine is also an approved veterinary drug used in the sheep and poultry industry, which might result in the presence of melamine in animal tissues when cyromazine is metabolised by the animals. In sheep, cyromazine is used as a dip to prevent blow-fly strikes. The European Union proposed MRL of 0.3 mg/kg for cyromazine when used as a veterinary drug. According to EFSA (2010), a cyromazine MRL of 0.3 mg/kg may result in a maximum melamine concentration of 0.004 mg/kg in sheep meat. In poultry, cyromazine is added to the feed in order to control dipteral larvae in chicken manure. According to EFSA (2010), the amount of melamine resulting from the metabolism of cyromazine in poultry feed, would not exceed 10% of the cyromazine dosage, which may ultimately result in melamine residues of 0.03 and 0.036 mg/kg in the meat of layers and broilers, respectively. In a study by Basson (2011), cyromazine was included in broiler and layer diets at a level of 4 mg/kg. No melamine was detected in meat or eggs in these trials and it was concluded that cyromazine in poultry diets was not a reason for concern in terms of resultant melamine transfer to edible products.

In the USA and other countries, the use of trichloromelamine, is approved for use in sanitizing solutions used on food processing equipment, utensils and washing solutions for fruit and vegetables, with the exception of milk containers or equipment (WHO, 2008). Melamine is a product of decomposed trichloromelamine. The US FDA estimated an approximate 0.14 mg/kg melamine contamination in foods when assuming that all sanitizers used during processing contain melamine (WHO, 2008).

Qin *et al*. (2010) assessed the extent of environmental melamine contamination by analysing various samples of water, soil and crops from 21 provinces in China. Waste and irrigation water samples, as well as soil samples near melamine-manufacturing factories and on farmlands (150 km away from such factories) were collected and analysed. The sample analyses revealed melamine concentrations higher than 0.02 mg/L in 6.38 and 35% of all irrigation and waste water samples, respectively. The highest reported melamine concentrations were 0.198 and 226.77 mg/L for irrigation and waste water, respectively. Of

the 124 farmland soil samples, melamine was detected in only one sample with a concentration of 0.176 mg/kg. Soil samples taken in the vicinity of melamine factories resulted in 43.7% of the samples with melamine concentrations higher than 0.1 mg/kg, with the highest concentration reported as 41.14 mg/kg. The results from Qin *et al*. (2010) suggests that melamine manufacturing plants should be monitored more extensively by the Chinese government as their inappropriate disposal of waste water and products results in environmental contamination of melamine.

2.7 Human health risks

Brown & Brown (2010) discussed the impact the global melamine contamination had on society and the lessons learned from such a disaster. The first lesson these authors pointed out was the devastating consequences that occurred as a result of melamine adulteration before the problem was identified. It is unfortunate that so many infants, children and animals had to pay the price for a group of negligent fraudsters seeking financial gain, with no knowledge of the potential threat being posed to society. Since the identification of food and feed adulteration with melamine which resulted in various toxicological effects in animals and humans, including mortalities, Yang & Batlle (2008) pointed out the vulnerability of mankind to contaminated foods and the great responsibility health authorities have in controlling and limiting such health risks. Subsequent to the melamine adulteration incident, all major international health regulatory authorities (including the World Health Organization (WHO), United States Food and Drug Association (US FDA) and European Food Safety Authority (EFSA) implemented various strategies in an attempt to ensure public health will not be at risk in the future.

This was achieved by developing risk assessments of melamine in which the potential effects and/or risks, the consumption of foods (not of animal origin) and animal products would pose to human health should those foods be adulterated with melamine and the slaughtered animals have consumed melamine tainted feeds, respectively. In addition, new regulations with regards to food and feed adulterations, including melamine migration from contact materials of food packaging, have been revised. The different regulatory authorities have also reviewed past set limits pertaining to the amount of melamine that is allowed (taking into consideration inevitable environmental contaminations and excluding possible adulteration levels) in food and feed ingredients, as well as the Tolerable Daily Intake (TDI) for melamine in an attempt to ensure human health. The maximum melamine concentration allowed in

infant foods and other food and feed sources are 0.1 and 2.5 mg/kg, respectively (WHO, 2008; EFSA, 2010). In 1984, The Scientific Committee for Food (SCF) established a TDI for melamine of 0.5 mg/kg BW. Upon revision and new scientific data, EFSA (2010), the WHO (2008) and FDA (2007) recommended a TDI for melamine of 0.5, 0.2 and 0.63 mg/kg body weight, respectively. These recommendations by EFSA (2010) and WHO (2008) were considered appropriate for the whole population including infants, provided infants are not born prematurely due to their reduced kidney function. However, the FDA (2009) adjusted the TDI of 0.63 mg/kg for infants due to their higher susceptibility to melamine exposure by the addition of a tenfold safety factor which results in a recommended TDI for infants of 0.063 mg/kg body weight.

EFSA (2010) proposed the use of melamine as monomer or additive in plastics (as food contact materials) with a specific migration limit of 30 mg melamine/kg food to pose no risk for human health. However, it should be noted that this proposed "safety" limit is based on a 60 kg adult consuming 1 kg packaged food per day. In addition, the specific migration limit of 30 mg/kg is presumably the standard accepted limit for the use of melamine in non-plastic materials (e.g. paper and can coatings), yet no specific regulation is set in place. Therefore, should manufacturers produce any product with non-plastic materials that results in melamine migration to exceed the limit of 30 mg/kg, they would not be violating any laws. Another factor that needs consideration with reference to melamine-based can coatings is the fact that canned foods and beverages are subjected to high temperatures during the pasteurisation process prior to long-term storage of the product, which may jeopardize the stability of the coating resulting in possible increased melamine migration.

The fact that modern society have become accustomed to the convenience of canned, packaged and pre-prepared foods, drinks, and snacks (including take-away meals), may result in higher levels of melamine consumption than was originally estimated by health authorities. Infants and small children may be more susceptible to melamine exposure (via food contact materials) as modern society predominantly relies on the convenience of infant formula and "ready-to-eat" baby foods, which are canned and/or packaged and less time consuming to prepare. Also, the method used in preparing (i.e. heating of food or milk) such products may contribute to increased melamine migration, should these foods be heated (e.g. via microwave) in the packaged material or plastic ware. The tableware (e.g. bottles, plates, bowls, cups, spoons) used for administering meals to infants and children, as well as keeping

in mind the frequency of infant feedings, are predominantly plastic ware (or melaware). EFSA (2010) reported that small children (aged 1 – 2 years) are theoretically more prone to significant melamine exposure as the sources with the highest potential of melamine exposure include melaware and canned foods. Another factor that should be considered is the fact that melamine degradation may occur or may even increase when subjected to high temperatures and acidic foods (e.g. tomato soup, apple purée) and drinks (e.g. tea with lemon). According to the WHO (2008), the concentration of melamine in foods as a result of migration would not exceed 1 mg/kg. However, Bradley *et al*. (2005) and Lund & Petersen (2006) reported melamine migration from plastic ware of up to 5 mg/kg when articles were subjected to 70°C for 2 h containing 3% acetic acid which simulated acidic foods. Additionally, these authors all reported the continuous migration/degradation of melamine throughout the lifetime of the melamine containing articles. Subsequently, the question that arises from these confounding factors is whether the specific migration limit takes all these factors into consideration to ensure the health of infants and small children.

EFSA (2010) recognized the susceptibility of infants and small children to melamine exposure, which resulted in the establishment of the "Individual food consumption data and exposure assessment studies for children" (EXPOCHI) project. The EXPOCHI project assessed the possible melamine concentration to which small children may be exposed to (via food and beverages which came into contact with melaware which was considered to have high migration levels) by creating two hypothetical scenarios. Scenario A ("typical migration levels") was assessed by assuming all foods and beverages came into contact with melaware under normal time and temperature conditions. In turn, Scenario B ("high migration levels") was assessed by assuming that any food item could come into contact with melaware under severe time and temperature conditions. The mean estimated total melamine exposure from Scenario A and Scenario B were 0.03-0.08 mg/kg BW/day and 0.04-0.11 mg/kg BW/day, respectively. However, it should be noted that the melamine migration levels of Scenario B did only account for one food item and did not consider the total daily exposure from all other food item contributions as was done in Scenario A.

The fact that one country's lack of proper control resulted in a vast international food and feed recall and simultaneously causing an international population health threat, indicates the shortcomings and deficiencies in ensuring food safety throughout the food supply chain which is becoming increasingly complex. Food and feed safety control and regulation, with

special reference to the globalization of raw material import and export, has also been reviewed, updated and improved by implementing laws and regulations by health authorities and countries. The initial response of health authorities were to physically analyse all products susceptible to melamine adulteration, especially food and feed containing high protein levels. As of October 2008, the importation of milk or milk products, soya or soya products originating from China, and intended for use in infants and young children's nutrition were prohibited in Europe. However, in November 2009, this decision was revoked based on reduced notifications of melamine residues by the Rapid Alert System for Food and Feed (RASFF; EFSA, 2010). Also contributing to the improved regulations, different regulatory agencies across different borders agreed to co-operate by improving the monitoring and regulation of raw material production prior to exportation (Brown & Brown, 2010). However, Yang & Batlle (2008) suggested that the importation of products produced in countries which are known to have less strict regulatory and quality control systems and lax standards (especially some third world countries), are required to be tested, even though the label may guarantee a specific pure product and approved by that country's regulatory authority.

2.8 Conclusion

The fraudulent adulteration of nutritional sources for humans and animals may have devastating consequences resulting in financial losses and health concerns as was evident in the melamine adulteration scandal. The unfortunate increase in child and animal morbidity and mortalities during the melamine scandal, should be regarded as an important indicator of where health regulatory authorities have neglected to ensure the supply of safe foods. In addition, this whole incident of melamine adulteration have proved how vulnerable human society is to food associated risks and how dependant we are of our governments and international health authorities, as our lives are literally in their hands.

Through international cooperation between manufacturers and suppliers, the provision of accurate information, adequate communication, increased quality and safety control and assistance from health authorities on latest health concerns and risks, practices of fraudulent adulterations and incidences of health related threats may decrease. Health authorities have implemented baseline limits for the amount of melamine allowed in feed and food sources, accounting for inevitable environmental contribution. However, these limits are calculated from estimates and hypotheses with information obtained from animal studies. And even

31

though safety factors were included to account for any uncertainties, the fact remains that there is no data available from human subjects (obviously for ethical reasons) to support the recommended baseline levels. In addition, from all the cited literature of animal studies, the rate of melamine toxicity still remains undetermined. Therefore, to ensure the health of our society, we must strive for zero melamine levels in the foods we eat and produce, compared to baseline melamine levels which are regarded as "safe".

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34

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CHAPTER 3

In vitro degradation of melamine in rumen liquor

Abstract

An in vitro *study was conducted to determine the extent of melamine degradation in rumen liquor. Approximately 1 L of rumen liquor was collected from each of two ruminally cannulated Holstein cows, blended separately and filtered through four layers of cheese cloth. Erlenmeyer flasks (250 mL) were prepared for incubations by adding 1000 mg substrate, 100 mg melamine and 100 mL incubation medium, purged with CO_2 and fitted with rubber stoppers equipped with one-way gas release valves. The initial melamine concentration was thus 1000 mg/L. The substrates consisted of 600 mg of a commercial dairy concentrate, 200 mg lucerne hay and 200 mg oat hay. The incubation medium consisted of 19 mL rumen liquor from the respective cow, 77 mL Van Soest buffer solution and 4 mL reducing solution. The flasks were incubated at 39°C for 0, 6, 24 or 48 hours. The 0 h incubation served as a control treatment to enable the calculation of melamine recovery values. For the control treatment (0 h), fermentation was terminated at the onset of the trial. Fermentation termination involved aeration of rumen liquor and submerging the flasks in 50 mm ice. Upon termination of the incubations, 100 mL of 0.2 M perchloric acid was added to each flask in order to dissolve any undegraded melamine, thus ensuring that no melamine would remain in the flasks during sampling. Melamine concentrations were determined by LC/MSMS. Melamine degradation was low after 6 and 24 h of incubation (3.2 and 5.5%, respectively) and increased to 13.6% after 48 h of incubation. It was concluded that melamine has a low degradability in rumen liquor.*

Key words: Melamine, *in vitro*, rumen liquor

3.1 Introduction

The industrial chemical melamine (C3H6N6), or 1,3,5-triazine-2,4,6-triamine, is used in the manufacturing of plastic ware, laminates and paints. Pure melamine has a high nitrogen content (667 g/kg) (Merck, 2001) which theoretically equates to a crude protein content (N x 6.25) of 4167 g/kg. Based on the fact that the crude protein content of feed and food ingredients is calculated from their nitrogen content (AOAC, 2002), no distinction can be made between the nitrogen contributions from true protein or non-protein nitrogen (NPN) sources. Therefore, the high nitrogen content of melamine makes it an attractive adulterant for protein feedstuffs.

Following the reports by Loosli *et al*. (1949) that ruminants (e.g. sheep and goats) are capable to efficiently utilize NPN sources (e.g. urea) to produce essential amino acids, Colbey & Mesler (1958) patented the use of melamine as a NPN source for ruminants. By recognizing the high nitrogen content of melamine and its structural similarities to cyanuric acid which, according to Clark *et al.* (1965) proved to be a safe and effective NPN source for sheep, MacKenzie (1966) investigated the potential use of melamine as a NPN source for ruminants. MacKenzie (1966) concluded that melamine was an inefficient NPN source due to observed reductions in apparent nitrogen digestibility, reduced feed intake and the inexplicable deaths of five sheep fed 10 g melamine per day. Clark (1966) reported that sheep fed 7 g of melamine daily showed no signs of morbidity, but mortalities were observed when 10 g of melamine was administered daily. He observed crystalluria in the sheep that died and ascribed the mortalities to renal failure as a result of crystalluria. Newton & Utley (1978) also investigated the effectiveness of melamine as a NPN source in steers. Even though they managed to prove an increase in rumen ammonia concentration in an *in vitro* trial, they reported that the rate of melamine hydrolysis in the rumen was insufficient to promote maximum ruminal protein synthesis and concluded that melamine was not an acceptable NPN source for ruminants.

The 2008 pet food recalls and the 2009 melamine tainted infant formula incidents brought renewed interest to melamine research. The health related concerns expressed by the international population was heightened when reports were made that animals consuming melamine tainted feeds, could result in melamine residues in the products (i.e. meat, milk and eggs) produced for human consumption.

Cruywagen *et al.* (2009) were the first to report that a pathway exists for the transmission of melamine from feed to milk. The excretion efficiency of ingested melamine to milk was reported to be approximately 2.1%. This finding was supported by Battaglia *et al.* (2010) who reported that the efficiency of melamine excretion via milk is dose dependant and may vary between 2.3-3.3%. Various studies pertaining to dietary melamine transmission confirmed the deposition of melamine in meat (Lü *et al.*, 2009; Sirilaophaisan *et al.*, 2010; Brand *et al.*, 2012) and eggs (Bai *et al.*, 2010; Chen *et al.*, 2010; Gao *et al.*, 2010; Valat *et al.*, 2011; Gallo *et al.*, 2012). Lv *et al.* (2010) reported melamine residues in the muscle, liver and kidney of lambs fed ≥ 10 mg melamine per kg body weight (BW) with melamine concentrations in the muscle samples ranging between 0.23-0.37 mg/kg for lams fed 100 mg melamine/kg BW. In another study by Cruywagen *et al.* (2011), sheep received 0.69 g melamine (approximately 11.5 mg melamine per kg BW) daily for 8 consecutive days and were slaughtered at the end of the trial. From the results, Cruywagen *et al.* (2011) reported the apparent digestibility of dietary melamine to be 76.7%. They found that the melamine residue in muscle tissues (*viz.* 9.6 mg/kg) accounted for approximately 3.6% of ingested melamine. Melamine excretion via urine and faeces were quantified and reported as 53.2 and 23.3% of the dietary melamine, respectively. The excreted and residual melamine concentrations were expressed as percentage of melamine intake. The authors reported that 18.6% of the melamine intake was not accounted for. This may include melamine in the abdominal fat and blood which Cruywagen *et al.* (2011) did not quantify as a proportion of melamine intake. As these authors only sampled muscle, liver and kidney tissues, it is possible that some unaccounted for melamine may be present in other organs as Bai *et al.* (2010) reported melamine residues in the stomach, duodenum and reproductive organs of hens. However, one should keep in mind the anatomical and physiological differences between monogastric and ruminant digestive tract systems.

The digestion of nutrients in ruminants involves microbial digestion through fermentation in the rumen prior to enzymatic digestion in the abomasum whereas monogastric animals only make use of enzymatic digestion in the stomach. Microbial digestion in the rumen enables ruminants to utilize fibrous feed and NPN sources to the benefit of the animal. The rumen environment is complex as it provides adequate conditions for various microbes to proliferate. Included in the consortium of microbes are vast amounts of different bacteria, fungi and protozoa. Bacteria represent the majority of the microbial population which can amount to 10^{10}-10^{11} cells per mL of rumen contents (Madigan *et al.*, 2003). Diversity within

the bacterial population is extensive and complex (Russell & Hespell, 1981) with a wide range of substrate affinities. Therefore, one could speculate that the great diversity of microbes present in the rumen may be able to degrade melamine and may result in different melamine partitioning pathways when compared to monogastric animals.

Newton & Utley (1978) reported increased rumen ammonia (NH_3) concentrations with the addition of melamine via an *in vitro* trial. In addition, the *in vitro* metabolism of melamine to melamine analogues (e.g. ammeline, ammelide, cyanuric acid) by aerobic microorganisms, such as *Arthrobacter* spp., *Klebsiella terragena* and *Pseudomonas* spp., were reported by Strong *et al*. (2002), Shelton *et al*. (1997) and Jutzi *et al*. (1982), respectively. Even though neither the *Arthrobacter* spp. nor *Klebsiella* spp. were isolated from the rumen, Duncan *et al*. (1999) did report the presence of *Pseudomonas aeruginosa* in the rumen of sheep.

Based on the observations of Newton & Utley (1978) who reported increased rumen NH_3 concentrations in steers that ingested melamine and the 18% of ingested melamine that was unaccounted for in the sheep trial of Cruywagen *et al*. (2011), it was hypothesized that melamine may at least partly be degraded in the rumen. It was thus decided to conduct an *in vitro* study to determine whether or not melamine is degraded in rumen liquor.

3.2 Materials and Methods

3.2.1 Rumen liquor collection and preparation

Rumen liquor (~ 1 L) was collected from two ruminally cannulated, lactating Holstein cows housed at the Welgevallen Experimental Farm of Stellenbosch University. The cows had free access to oat hay and received 15 kg of a commercial semi-complete lactation diet daily. The rumen liquor was collected from the centre of the rumen and transferred to thermos flasks. The flasks were filled to the brim before screwing on the lid to prevent aeration. In the laboratory, the rumen liquor of each thermos flask was transferred to a commercial blender and blended for 20 seconds under a continuous stream of CO_2 in order to maintain anaerobic conditions. The blended rumen liquor was then filtered through 4 layers of cheesecloth to remove course particulate matter. The filtered rumen liquor was collected in a 1 L Erlenmeyer flask, purged with CO_2, fitted with a rubber stopper and placed in a 39°C water bath.

3.2.2 Sample preparation for in vitro incubation

Each 250 mL Erlenmeyer flask contained 100 mg of melamine (melamine 99%, Sigma-Aldrich, St. Louis, MO), 1000 mg substrate and 100 mL incubation medium.   The initial melamine concentration was thus 1000 mg/L. This significant concentration was decided on to ensure that any degradation of melamine would be readily detected.   The substrate consisted of 600 mg dairy concentrate, 200 mg lucerne hay and 200 mg oat hay in order to simulate a typical dairy ration of 60:40 concentrate to roughage ratio.   All substrate ingredients (dairy concentrate, lucerne hay and oat hay) were ground with a laboratory hammer mill (Scientec, South Africa) fitted with a 2 mm screen. Chemical analyses of the substrate ingredients were performed in duplicate according to AOAC (2002) methods for dry matter (DM; method 934.041), ash (method 942.05), crude protein (CP; method 990.03) and ether extract (method 920.39).   The ANKOM Fiber analyzer (Ankom® Technology Corp., Macedon, NY, USA) was used for the determination of ADF and NDF. Heat stable alpha amylase and sodium sulfite was added to the neutral detergent solution. The chemical composition of the substrate is presented in Table 3.1. The incubation medium consisted of 19 mL of the prepared rumen liquor, 77 mL buffer solution and 4 mL reducing solution. The buffer and reducing solutions were prepared as described by Van Soest & Robertson (1985). The flasks were purged with CO_2 and fitted with rubber stoppers equipped with one-way gas release valves.

Table 3.1 Chemical composition (g/kg DM) of the substrate ingredients used in the *in vitro* melamine degradability trial.

	Substrate ingredients		
Nutrient	**Dairy concentrate**	**Lucerne hay**	**Oat hay**
Crude protein (CP)	221.7	115.8	96.7
Ether extract (EE)	18.3	10.1	16.4
Acid detergent fibre (ADF)	58.7	448.1	398.9
Neutral detergent fibre (NDF)	117.1	561.1	659.9
Ash	64.6	75.2	75.8

3.2.3 Sample incubation

The flasks containing the incubation medium, melamine and substrate, were placed in an incubator and incubated at 39°C for 0, 6, 24 or 48 hours. The 0 h served as a control treatment to enable the calculation of melamine recovery. In the latter treatment, fermentation was terminated at the onset of the trial by aeration and submerging the Erlenmeyer flasks in 50 mm ice. Fermentation of the incubated samples was terminated in the same way after the predetermined incubation times.   Upon the termination of fermentation, 100 mL of 0.2 M perchloric acid was added to each Erlenmeyer flask in order to dissolve the undegraded melamine. Representative rumen liquor samples were taken, diluted with 0.2 M perchloric acid (1:1) and stored in airtight containers at -20°C pending melamine analysis.   The incubation trial was repeated four times in four separate runs.

3.2.4 Melamine analysis of rumen liquor samples

An adapted method from Shai *et al.* (2008) was used for melamine analyses. Thawed rumen liquor samples were centrifuged at 4 500 x g for 5 min. The conditioning of the Cation-exchange solid-phase extraction cartridges (Phenomenex Strata SCX; 55 μm, 70 A, 500 mg / 3 mL, supplied by Separations, Randburg, South Africa) was done with 6 mL methanol, followed by 6 mL distilled water. The supernatants of the rumen liquor samples (3 mL) were loaded onto the cartridges together with 100 μL of a 0.5 mg/L stable isotope-labelled melamine ($^{13}C3H6^{15}N3$) internal standard solution (Cambridge Isotope Laboratories Inc., Andover, MA). Therefore, 0.05 μg of the labelled melamine was loaded onto each cartridge. The cartridges were then washed with 6 mL 0.1 *N* HCl followed by 6 mL methanol and allowed to aspirate under vacuum for 1 min. The melamine was eluted with 6 mL ammonium hydroxide: methanol: dichloromethane (1:5:5) and collected into clean glass tubes. The resulting extracts were then dried under a stream of nitrogen, resuspended with 1 mL 50% acetonitrile and transferred to individual vials to be loaded into the liquid-chromatograph triple quadruple mass spectrophotometer.

3.2.5 Liquid Chromatography Tandem Mass Spectrometry (LC/MSMS) Analysis

Rumen liquor samples were analyzed for melamine concentration by LC/MSMS on a Water API Quattro Micro triple quadruple mass spectrometer coupled to a Waters 2690 HPLC (Waters Corp., Milford, MA). For this method, the limit of detection for rumen liquor samples was 0.001 mg/kg.

3.2.6 Calculations

Melamine recovery values were determined from the control (0 h) treatment (with an initial melamine concentration of 1000 mg/L) and adjusted to 100%. The melamine concentrations of the incubated samples were then adjusted accordingly.

3.2.7 Statistical analysis

A randomized complete block design was used and a Main Effects ANOVA was performed on the melamine concentration data with the aid of Statistica version 10 (2011) Main effects were Fermentation time, Cow and Block (run). Significance was declared at $P < 0.05$.

3.3 Results and Discussion

Melamine concentration over time is indicated in Fig. 3.1. It can be seen that the melamine concentration in rumen liquor gradually decreased as incubation time increased. The melamine concentration at 48 h (864 mg/kg) was significantly ($P = 0.008$) lower compared to previous hours (1000, 968 and 944 mg/kg at 0, 6 and 24 h, respectively). As no previous research on ruminal melamine degradation could be found in the literature, these values could not be compared with other documented results.
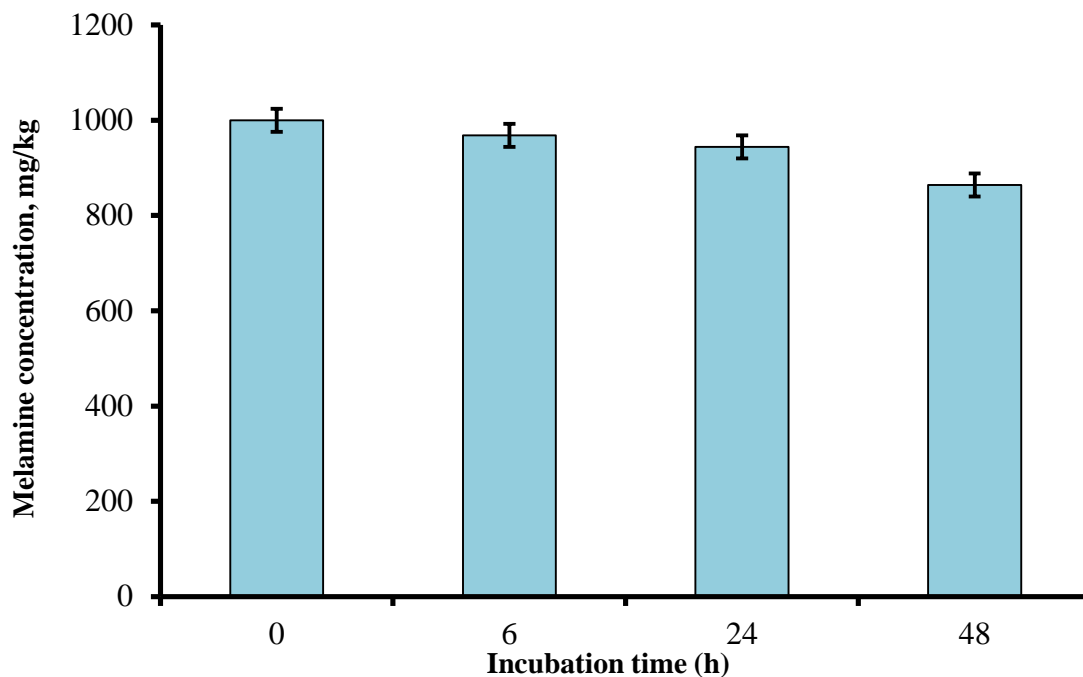
Figure 3.1. Melamine concentration after *in vitro* incubation of 100 mg of melamine in 100 mL buffered rumen liquor.

47

The extent of melamine degradation is indicated in Fig. 3.2. It is evident that melamine degradation increased gradually from 0 h to 24 h and then at a faster rate until 48 h. By 48 h, the amount (%) of melamine that was degraded in rumen liquor (13.6%) was significantly (P = 0.008) higher than at previous hours (3.2 and 5.5%) at 6 and 24 h, respectively. When the accepted rumen passage rate of k_p=.08 for lactating dairy cows is applied, the rumen retention time of a high concentrate feed would be 12.5 h (100/8), which would be the corresponding time point for the calculation of effective degradability. In the current study, samples were not incubated specifically for 12.5 h, but the effective degradability of melamine would be a value between 3.2% (6 h incubation) and 5.5% (24 h incubation). It can thus be accepted that the effective degradability of melamine is very low.
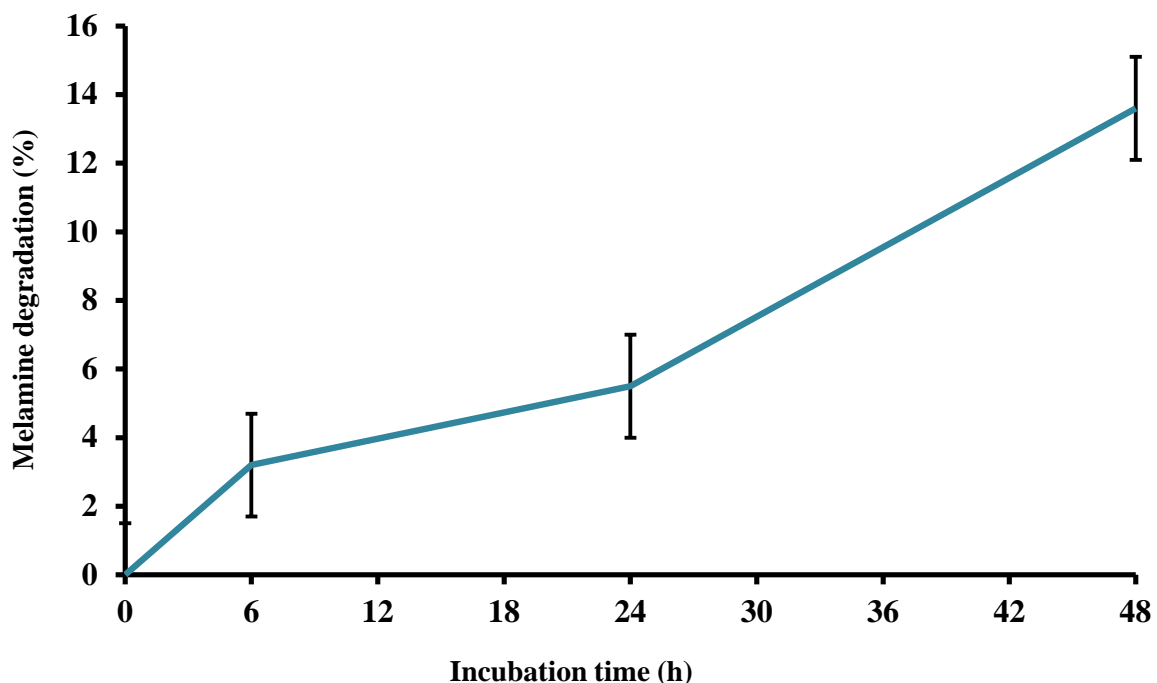
Figure 3.2. *In vitro* degradation of melamine (%) incubated in 100 mL buffered rumen liquor.

Some degradation of melamine in rumen liquor was expected as Jutzi *et al*. (1982) reported the hydrolytic cleavage of melamine into ammonia by *Pseudomonas sp*. strain A *in vitro*. It should be noted that the presence of *Pseudomonas sp.* strain A in rumen liquor is unclear. The production of ammonia following melamine cleavage (Jutzi *et al.*, 1982) may also explain the increased ammonia concentration (even though not significant) observed in the *in vivo* and *in vitro* trials of Newton & Utley (1978). Of interest to note from Newton & Utley's (1978) *in vitro* study, is that higher ammonia concentrations were observed after 24 h of

incubation when the melamine substrate was incubated in rumen liquor obtained from a steer fed melamine compared to rumen liquor from a steer fed cottonseed. This observation may infer that a period of adaption may be required by rumen microbes in order to hydrolyze melamine more efficiently.   This may support the results of this study as significant melamine degradation was only observed after 48 h of incubation and the microbes might have had an increased period of time to adapt in order to degrade melamine in the substrate.

The amount of 13.6% degraded melamine observed at 48 h in this study may partly explain the unaccounted for melamine observed in the trial of Cruywagen *et al*. (2011) who investigated excretion routes of melamine in sheep. However, care should be taken when interpreting *in vitro* fermentation results as Ørskov & McDonald (1970) and Pienaar *et al*. (1989) reported *in sacco* and *in vivo* techniques to be more comparable when determining protein degradability and organic matter fermentation, respectively. Due to the small particle size of pure melamine, the use of artificial-fibre (Dacron) bags in an *in sacco* trial would have resulted in great losses as melamine would have escaped through the bag pores. This would have resulted in an over-estimate of the amount of melamine degraded in the rumen. Hence, it was decided in the current study that an *in vitro* trial would be more appropriate than an *in sacco* trial to determine whether or not melamine degradation would occur in the rumen.

McDonald (1981) reported the percentage degradability of protein supplements in the rumen to be dependent on the course of degradation and retention time in the rumen. McDonald (1981) added that the percentage of degradable protein would decrease when the passage rate from the rumen increase. Although melamine is not a true protein, this statement applies as the percentage of melamine degradation would depend on the duration of exposure to rumen microbes as this study reported significant melamine degradation (13.6%) at 48 h. Yet, the feasibility of this result must be evaluated with reference to the expected retention time of melamine in the rumen. Due to the small particle size of melamine, it can be expected that the retention time in the rumen may be short. Small feed particles pass through the rumen with the liquid-phase via rumen contractions at a higher rate compared to larger particles (Poppi *et al*., 2000). Therefore, melamine may not reside in the rumen for periods as long as 48 h, which may greatly reduce the microbes' ability to adapt and degrade melamine.

3.4 Conclusion

Results of the current trial showed that melamine has a low, but significant, degradability in rumen liquor. As melamine degradation in the rumen has been postulated by Newton & Utley (1978), the extrapolation of the *in vitro* results observed in the current trial to *in vivo* conditions warrants further investigation.

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CHAPTER 4

The effect of melamine on *in vitro* rumen liquor volatile fatty acid and ammonia concentrations

Abstract

An in vitro *study was conducted in three separate runs to determine the effects of melamine on ruminal ammonia (NH_3) and volatile fatty acid (VFA) concentrations. In each run, approximately 1 L of rumen liquor was collected from each of three ruminally cannulated Holstein cows, blended separately and filtered through four layers of cheese cloth. Five Erlenmeyer flasks (250 mL) were prepared for each cow at each incubation time (6, 24 and 48 h). Two flasks contained no substrate and served as Blanks. Substrate added to the other flasks consisted of 600 mg of dairy concentrate, 200 mg of lucerne hay and 200 mg of oat hay. The substrate was the same for all three treatments and the difference between treatments were attributed to the amount of melamine added, viz. 0, 0.2 and 0.4 mg for the Control, T1 and T2 treatments, respectively. The incubation medium (100 mL) consisted of 20 mL of rumen liquor from each respective cow, 76 mL buffer medium and 4 mL reducing solution. The flasks were purged with CO_2 and fitted with rubber stoppers equipped with one-way gas release valves. The flasks were incubated for 6, 24 or 48 h. Fermentation termination at the predetermined time points involved aeration of rumen liquor and submerging the flasks in 50 mm ice. Samples were collected for NH_3 and VFA analyses. The NH_3 concentrations were determined as described by Broderick & Kang (1980) and the VFA concentrations were determined using a HPLC method. The melamine treatments had no effect on the acetate, butyrate, propionate, valerate and total VFA concentrations. The different melamine treatments also had no effect on the NH_3 concentrations at 6 and 48 h. At 24 h, treatment T2 showed a significant increase in NH_3 concentration which could not readily be explained as it could not have been ascribed to the amount of melamine degraded alone. It was concluded that melamine is an inefficient non-protein nitrogen source as rumen microbes are limited in hydrolysing melamine to NH_3.*

Key words: *In vitro*, ammonia, volatile fatty acid, melamine

4.1 Introduction

The potential benefits of supplementing ruminant diets with non-protein nitrogen (NPN) sources have been an important component of ruminant nutrition since the early 1950's. Early research reported the ability of the microbial population in the rumen of sheep and goats to produce amino acids which are then made available to the ruminant animal (Loosli *et al*., 1949; Duncan *et al*., 1953; Virtanen, 1966; Oltjen, 1969). The subsequent studies to follow these reports aimed to determine optimal conditions required that would enable the efficient use of NPN sources in order to benefit the productivity of ruminant animals to the maximum.

The presence of the complex consortium of microorganisms in the rumen is what enables ruminants to potentially use NPN sources efficiently. With approximately 10^{11} bacterial cells/ mL of rumen fluid (Ishler *et al*., 1996)), the rumen microorganisms hydrolyse the NPN source to ammonia (NH_3) which represents the primary source of nitrogen used by the microbial population (Bryant & Robinson, 1963; Ishler *et al*., 1996). However, peptides and amino acids are also required for microbial growth (Sniffen & Robinson, 1987). In addition to nitrogen, the microorganisms also require energy sources which are obtained from readily fermentable carbohydrates. The amount of NH_3 produced depends on the extent to which rumen degradable proteins (RDP) and NPN sources can be hydrolysed by the rumen microorganisms (Mackie & White, 1990) as well as the amount and type of carbohydrates supplied (Mugerwa & Conrad, 1971; Sannes *et al*., 2002; Broderick, 2003). The nitrogen from the NH_3 is utilized by the microorganisms for maintenance and protein synthesis. The minimum required NH_3 concentration required for maintaining microbial growth is 5 mg NH_3-N/ 100 mL (3.57 m*M*) of rumen liquor (Satter & Slyter, 1974). The increased production of microbial proteins (MP) in the rumen results in increased MP flow to the small intestines where it provides a valuable source of protein to the ruminant animal for maintenance, growth and production.

When evaluating a NPN source as a potential supplement to a ruminant diet, it is important to determine the extent and rate at which the rumen microorganisms can degrade it to ensure sufficient NH_3 production required for microbial maintenance and growth. Urea is the most popular NPN source studied and used commercially in ruminant nutrition. The incorporation of urea into ruminant diets has proved to be efficient owing to its high nitrogen content and high rate of degradation to NH_3 by rumen microorganisms. Chalupa *et al*. (1964) reported the

rumen NH_3 concentration to peak at 1 – 2 h after the ingestion of a urea purified diet. The addition of starch to a diet supplemented with urea has proved to be more beneficial for microbial growth compared to sugars (Kertz, 2010). Other NPN sources have also been studied in an attempt to improve nitrogen utilization by the ruminant animal. Oltjen (1969) reported no significant differences in nitrogen retention values when urea was compared to urea phosphate, biuret and uric acid in steers fed purified diets. Clark *et al*. (1965) also evaluated potential NPN sources for sheep and found no differences in nitrogen retention values between urea, biuret, triuret and cyanuric acid for sheep fed a low protein roughage diet. However, cyanuric acid in the diet resulted in a rapid change over from a negative to positive nitrogen balance upon which Clark *et al*. (1965) concluded the effective and safe use of cyanuric acid as a NPN source.

Based on the structural similarities between cyanuric acid and melamine and melamine's high nitrogen content (*viz*. 667 mg/kg), Mackenzie (1966) evaluated the potential of melamine as a NPN source in sheep roughage diets that were deficient in protein. From the results, Mackenzie (1966) concluded melamine to be an inefficient source of NPN and toxic as a number of sheep died during the trial. As Mackenzie (1966) failed to explain the cause of death, Clark (1966) subsequently conducted a toxicological study to determine the effects of melamine ingestion. Clark (1966) reported melamine to be an unsafe NPN source following the fatal uraemia observed in sheep as a result of crystalluria. In agreement with Clark (1966), Newton & Utley (1978) also reported melamine to be an inefficient NPN source for ruminants.

From the early studies of the abovementioned authors, it is understandable why no further studies pertaining to melamine supplementation as NPN source for ruminants were executed. Melamine in animal feeds only made headlines again in 2006, when melamine containing pet foods resulted in numerous mortalities in household pets worldwide (Brown *et al*., 2007; Puschner *et al*., 2007). Then again in 2008, reports were made that protein ingredients used for animal feeds were also adulterated with melamine (WHO, 2008). Various countries were affected by the adulterated ingredients exported from China. Melamine was detected in soya products imported to Europe and France (EFSA, 2010), rice proteins and wheat gluten imported to the US (FDA, 2007), fishmeal imported to Canada (CBC News, 2007) and maize gluten 60 imported to South Africa (Cruywagen & Reyers, 2009). It was also during 2008 that the Chinese government announced the deaths of six babies and numerous infants

hospitalized with kidney related illnesses as a result of consuming melamine adulterated infant formula (WHO, 2008).   Consequently, with the health risks associated with melamine consumption, the people's increased concerns related to the consumption of animal products exposed to melamine contaminated feeds required various health authorities (EFSA, US FDA, WHO) to improve current regulations and perform risk assessments to ensure safe food.

Numerous studies pertaining to dietary melamine transmission to cow's milk (Cruywagen *et al*., 2009; Battaglia *et al*., 2010; Shen *et al*., 2010; Sun *et al*., 2011; Zheng *et al*., 2011; Sun *et al*., 2012), cheese (Battaglia *et al*., 2010), sheep meat (Lv *et al*., 2010; Cruywagen *et al*., 2011), fish meat (Anderson *et al*., 2008), poultry meat (Lü *et al*., 2009; Sirilaophaisan *et al*., 2010; Brand *et al*., 2012) and eggs (Bai *et al*., 2010; Chen *et al*., 2010; Valat *et al*., 2011; Gallo *et al*., 2012) provided valuable information on the partitioning of melamine to animal products used for human consumption. Hence, the potential health risks to both animal and humans can be evaluated. However, there is still limited information available on the metabolism of melamine in ruminants.

It is clear from monogastric animal (e.g. pig and rat) studies that melamine is not metabolised in the body and is rapidly excreted via urine (Mast *et al*., 1983; Baynes *et al*., 2008). Based on the physiological differences between monogastric and ruminant animals, as well as the fermentative digestion in the rumen prior to enzymatic digestion, it may be possible that melamine degradation occurs in the ruminant. Various authors have reported the degradation of melamine by various strains of bacteria. However, these bacterial strains were obtained from soil (Strong *et al*., 2002; Takagi *et al*., 2012) and sewage water (Cook & Hütter, 1981; Shelton *et al*., 1997). The early studies of Clark (1966) and Mackenzie (1966) reported elevated blood urea nitrogen following the consumption of melamine by sheep. In both the *in vivo* and *in vitro* studies of Newton & Utley (1978), increased rumen NH_3 concentrations were observed when melamine was fed to steers and added as a substrate, respectively. The study of Cruywagen *et al*. (2011) could not account for approximately 18% of the dietary melamine fed to sheep and the authors hypothesized that some melamine degradation may have occurred in the rumen. This hypothesis was confirmed by an *in vitro* study conducted at Stellenbosch University (Chapter 3) that reported 13.8% of the melamine substrate to have been degraded in rumen liquor after 48 h.

Zhuang *et al*. (2010) proposed melamine to have an inhibitory effect on rumen microbes after observing significant reduced total VFA concentrations in rumen liquor with a melamine concentration of 2 mg/kg. However, there is no literature to support Zhuang *et al*.'s (2010) hypotheses.

A previous *in vitro* study (Chapter 3) confirmed the degradation of melamine in rumen liquor. Therefore, it was decided to conduct a second *in vitro* trial in order to determine the effects of melamine on ruminal NH_3-N. The current trial also evaluated the effect of melamine on *in vitro* rumen VFA concentrations to determine if melamine has an inhibitory effect on rumen microbes.

4.2 Materials and Methods

4.2.1 Rumen liquor collection and preparation

Rumen liquor (~ 1 L) was obtained in three consecutive runs from three ruminally cannulated lactating Holstein cows housed at Welgevallen Experimental Farm of Stellenbosch University. The cows had free access to oat hay and received 15 kg of a commercial semi-complete lactation diet daily. The collected rumen liquor was immediately transferred to thermos flasks. The flasks were filled to the brim before screwing on the lid to prevent aeration during transportation to the laboratory. The *In Vitro* laboratory of the Department of Animal Science (Stellenbosch University, South Africa) is equipped with a temperature controlled 9 m^2 room. The room temperature was adjusted to 39° C the day prior to the incubations to ensure temperature equilibrium at the onset of the trial.   All the required rumen liquor and sample preparations were performed in the 39° C room to prevent any temperature fluctuations during the preparation and incubation of the samples. The rumen liquor was transferred to a commercial blender and blended for 20 seconds under a continuous stream of CO_2 to maintain anaerobic conditions. The blended rumen liquor was then filtered through 4 layers of cheesecloth to remove course particulate matter. The filtered rumen liquor was subsequently collected in a 1 L Erlenmeyer flask, purged with CO_2 and fitted with a rubber stopper until it was mixed with the buffer solution and added to the inoculation vessels/flasks.

4.2.2 Substrate preparation

In a previous study conducted at Stellenbosch University (to be discussed in a later chapter), cows were provided with dairy concentrate pellets adulterated with melamine-tainted corn gluten 60. As the cows used in that trial were not equipped with cannulae, it was decided to simulate the effects that the 5000 (M3) and 10000 mg (M4) of melamine/d treatments would have on certain rumen parameters *in vitro*. In that trial, the dairy concentrates were formulated to ensure that cows would consume the required amount of melamine in 15 kg pellets. Thus, the melamine concentrations in the concentrate pellets were 333 and 667 mg/kg for treatments M3 an M4, respectively. In addition to the melamine containing dairy concentrates, cows were also provided with lucerne and oat hay. For the current trial, the substrate consisted of 600 mg dairy concentrate with a melamine concentration of 0 (Control), 333 or 667 mg/kg, plus 200 mg lucerne hay and 200 mg oat hay. Since 600 mg of the dairy concentrate was incubated in 100 mL incubation medium, the initial melamine concentrations were 2.0 and 4.0 mg/kg for Treatments T1 and T2, respectively. In other words, 0.2 mg and 0.4 mg of melamine were added to the substrates of Treatments T1 and T2, respectively.

The source of melamine was pure melamine (melamine 99%, Sigma-Aldrich, St. Louis, MO). For the *in vitro* simulation it was decided to incorporate pure melamine and not melamine-tainted maize gluten (Chapter 5). The reason for this decision was based on the fact that the maize gluten was adulterated with melamine waste which is known to contain melamine and its analogues (including cyanuric acid). The presence of cyanuric acid in the adulterated maize gluten might have complicated the interpretation of the results as cyanuric acid has been reported to be an effective NPN source for ruminants (Clark *et al.,* 1965).

All substrate constituents (dairy concentrate, lucerne- and oat hay) were ground to pass through a 2-mm screen prior to incubation and chemical analyses. For chemical analyses, representative samples of the substrate constituents were collected prior to the incubation trial and stored at -20° C pending chemical analyses.

All chemical analyses of the substrate constituents were performed in duplicate as described by AOAC (2002) for dry matter (DM; method 934.041), ash (method 942.05), crude protein (CP; method 990.03) and ether extract (EE; method 920.39). Acid detergent fibre (ADF) and neutral detergent fibre (NDF) was determined with the aid of an ANKOM 2200 fibre

59

analyzer (ANKOM Technology, Macedon, NY). Heat stable amylase and sodium sulfite was added to the neutral detergent solution. The chemical composition of the substrates is presented in Table 4.1.

Table 4.1 Chemical composition of substrate constituents

Chemical Composition	Substrate Constituents		
(g/kg DM)	Dairy concentrate	Lucerne hay	Oat hay
Crude protein (CP)	221.7	115.8	96.7
Ether Extract (EE)	18.3	10.1	16.4
Acid detergent fibre (ADF)	58.7	448.1	398.9
Neutral detergent fibre (NDF)	117.7	561.1	659.9
Ash	64.6	75.2	75.8

4.2.3 In vitro volatile fatty acid and ammonia concentrations

Five Erlenmeyer flasks (250 mL) were prepared for each of the three cows at each incubation time (6, 24 and 48 h). Two flasks contained no substrate, whereas the remaining three flasks contained 1000 mg substrate. The flasks containing no substrate served as Blanks (in duplicate) in order to correct for changes in rumen parameters resulting from the rumen liquor alone. The substrate added to the other three flasks consisted of 600 mg of dairy concentrate (Control, T1 or T2), 200 mg lucerne hay and 200 mg oat hay. The composition of the substrate was chosen as such to represent a typical concentrate to roughage ratio (60:40) provided to dairy cows.   The incubation medium (100 mL) consisted of 20 mL prepared rumen liquor from each respective cow, 76 mL buffer solution and 4 mL reducing solution. The buffer and reducing solutions were prepared as described by Van Soest & Robertson (1985). The flasks were purged with CO_2 and fitted with rubber stoppers equipped with one-way gas release valves.

Fermentation of the incubated samples were terminated at the predetermined times of 6, 24 or 48 h by aeration and submerging the Erlenmeyer flasks in 50 mm ice. Upon terminating

fermentation, the contents of the flasks were mixed and two sub-samples (20 mL) of the incubated sample were collected in air-tight containers for volatile fatty acid (VFA) and ammonia (NH_3) analysis. The samples were immediately stored at -20° C pending analysis. This incubation trial was repeated twice.

The thawed rumen liquor samples were prepared for VFA and NH3 analysis. The respective rumen liquor samples for VFA analysis were subjected to a clean-up procedure (Siegfried *et al*. (1984) prior to analysis. VFA concentrations were determined using a HPLC method with the aid of a Waters 717 autosampler (Empower 2 software) equipped with a RI Detector. The column used was a Biorad Aminex HPX 87H (65 °C). Rumen liquor samples for NH_3 analysis were prepared as described by Broderick & Kang (1980) and analysed with the aid of a spectrophotometer set at 630 nm.

4.2.4 Statistical analysis

A randomized complete block design was used and a Main Effects ANOVA was performed on the melamine concentration data with the aid of Statistica version 10 (2011) Main effects were Fermentation time, Cow and Block (run). Significance was declared at $P < 0.05$.

4.3 Results and Discussion

4.3.1 Ammonia concentrations

Results of the *in vitro* NH_3 concentrations for the different treatments are presented in Figure 4.1. From the results it can be observed that there were no significant differences between treatments after 6 and 48 h of incubation. After 24 h of incubation, treatment T2 had a significantly ($P < 0.01$) higher NH_3 concentration compared to the other treatments. Newton & Utley (1978) reported increased NH_3 concentrations (although they were not significant) after incubating melamine in rumen liquor for 24 h and proposed slow hydrolysis of melamine in the rumen environment. One might argue that the degradation of melamine to NH_3 could be responsible for the increase in NH_3 concentration observed at 24 h for the T2 treatment. This is, however, unlikely. From the *in vitro* melamine degradation study (Chapter 3), 5.5% of the original melamine was degraded after 24 h and 13.6 % after 48 h. Even if 10% of the melamine had degraded after 24 h, it would have related to 0.04 mg. If melamine is hydrolysed completely, 6 mol of NH_3 would be released per mol of melamine and NH_3 contributes 71.4% towards the molecular weight of melamine. Therefore, 0.029 mg (or 0.017

mM) of NH_3 would have been released from the hydrolisation of 0.04 mg melamine, relating to 0.17 mmol/L. This is an insignificant amount compared to the 25.3 mM/L. Zhuang *et al.* (2010) observed significant reductions in NH_3 concentrations of rumen liquor with a melamine concentration of 2 mg/kg after a 72 h incubation period. Zhuang *et al.* (2010) proposed melamine to have an inhibitory effect on microbial fermentation. In this trial, melamine was incubated for 48 h. From Figure 4.1 it appears that the NH_3 concentration decreases from 24 h to 48 h for treatment T 2. The higher NH_3 concentration of T 2 at 24 h can thus not be readily explained.
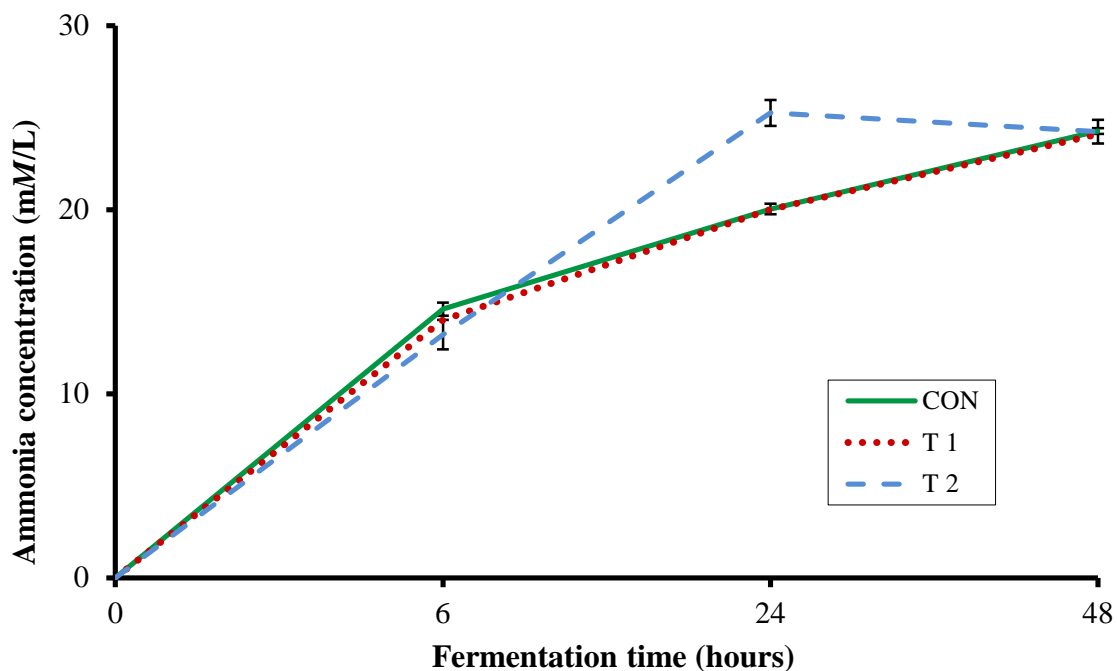
Figure 4.1 *In vitro* ammonia concentrations over time for treatments Control (CON) = 0 mg, T1 = 0.2 mg and T2 = 0.4 mg of melamine.

4.3.2 Volatile fatty acid concentrations

Results of the *in vitro* VFA concentrations of the different treatments over time are presented in the following graphs. The concentrations of acetate, butyrate, propionate, valerate and total VFA's are presented in Figures 4.2, 4.3, 4.4, 4.5 and 4.6, respectively.

The different melamine treatments had no effect on the acetate, butyrate, propionate, valerate and total VFA concentrations, suggesting that up to 48 h, melamine did not inhibit microbial fermentation. According to Zhuang *et al.* (2010), rumen liquor with a melamine concentration of 2 mg/kg significantly reduced the total VFA concentration without altering the molar proportions of the VFA pattern after a 72 h incubation period. In this trial, the

samples were incubated for only 48 h. During the 48 h incubation period, the individual and total VFA concentrations for all treatments increased, indicating no inhibition of microbial fermentation.
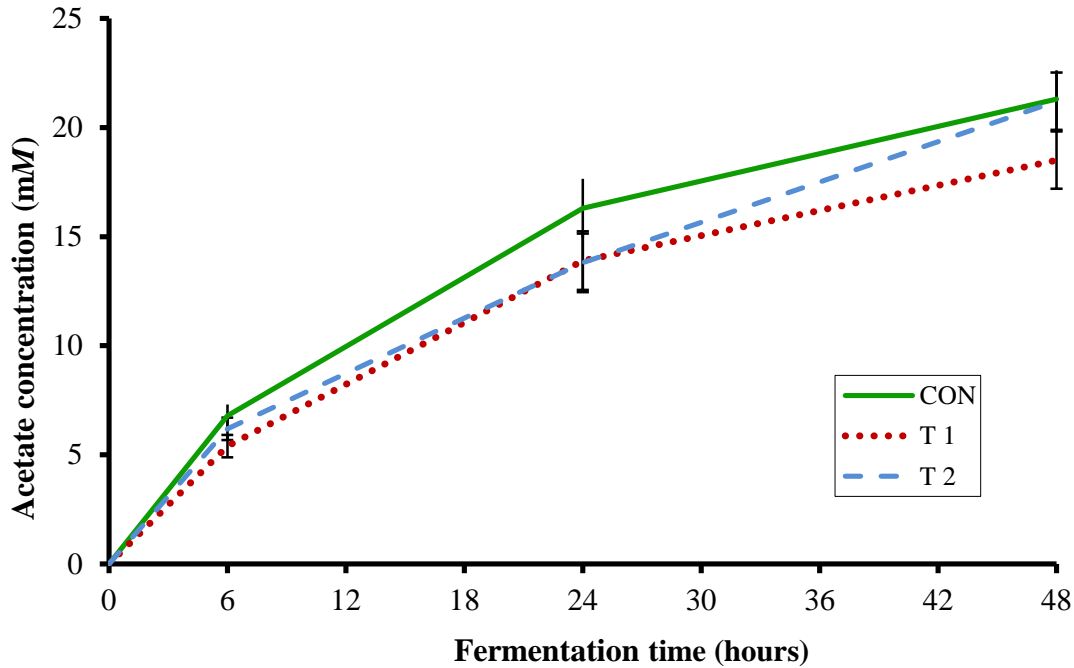
Figure 4.2 *In vitro* acetate concentrations over time for treatments Control (CON) = 0 mg, T1 = 0.2 mg and T2 = 0.4 mg of melamine.
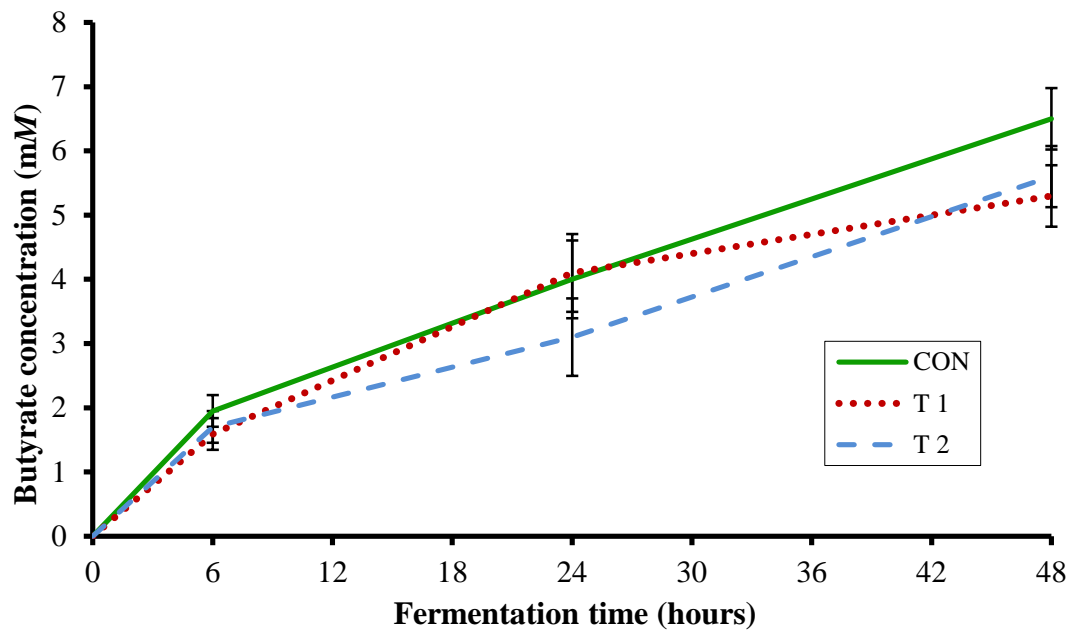
Figure 4.3 *In vitro* butyrate concentrations over time for treatments Control (CON) = 0 mg, T1 = 0.2 mg and T2 = 0.4 mg of melamine.

63
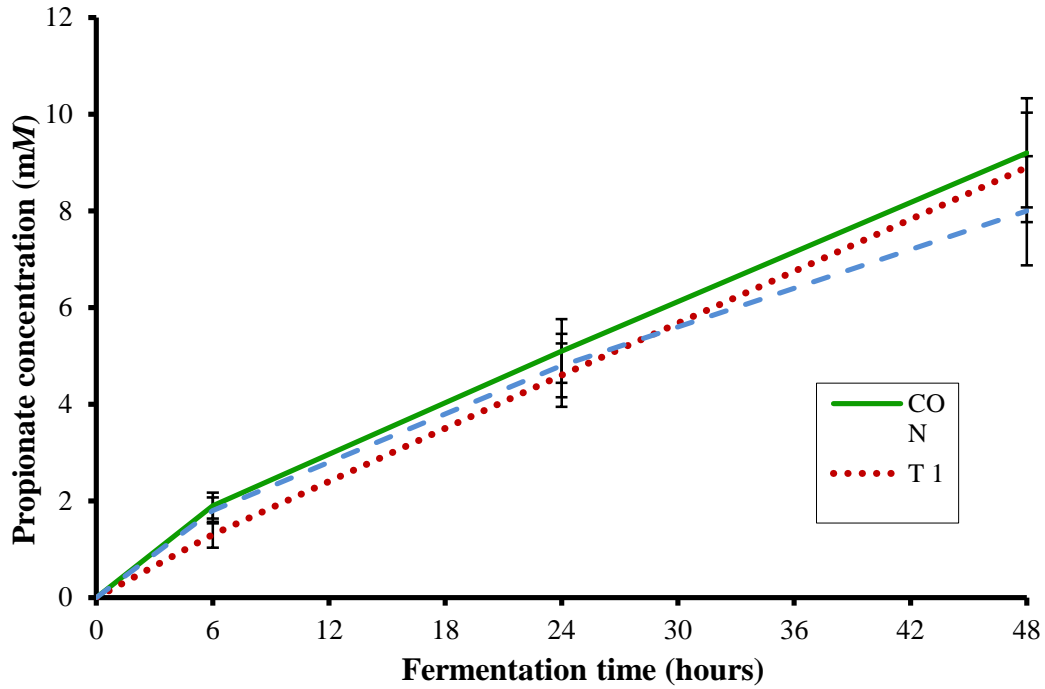
Figure 4.4 *In vitro* propionate concentrations over time for treatments Control (CON) = 0 mg, T1 = 0.2 mg and T2 = 0.4 mg of melamine.
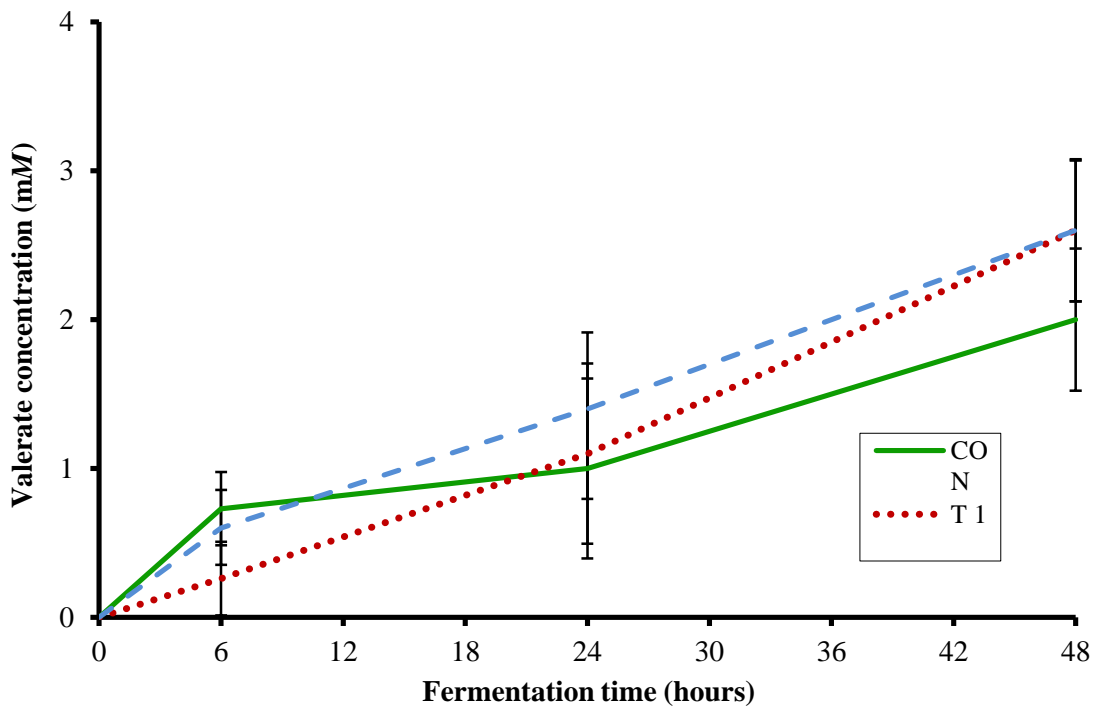
Figure 4.5 *In vitro* valerate concentrations over time for treatments Control (CON) = 0 mg, T1 = 0.2 mg and T2 = 0.4 mg of melamine.

64
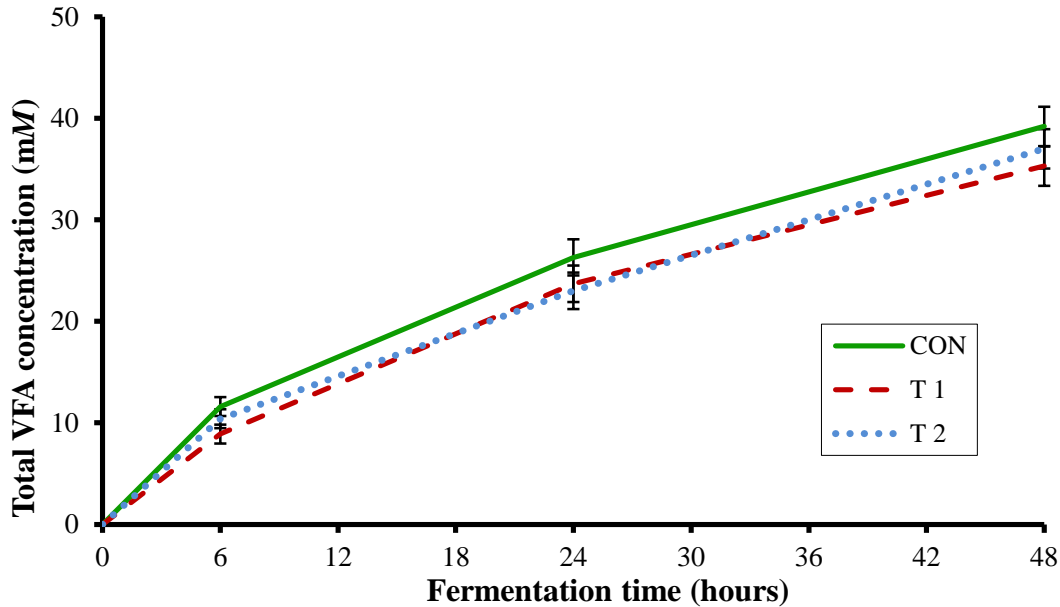
Figure 4.6 *In vitro* total volatile fatty acid concentrations over time for treatments Control (CON) = 0 mg, T1 = 0.2 mg and T2 = 0.4 mg of melamine.

4.4 Conclusion

In this study, the addition of melamine to substrates for *in vitro* incubation resulted in an inexplicable increase of NH_3 at 24 hours, but no differences were observed after 48 hours of incubation. Melamine had no effect on the individual and total VFA concentrations. It was concluded that melamine does not affect rumen NH_3 and VFA concentrations after incubating samples for 48 h. Due to the limited selected time points in this study, it remains unknown what the effects of melamine would have on rumen NH3 and VFA concentrations at different time points and warrants further research.

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CHAPTER 5

Dietary melamine excretion via milk, urine and faeces in lactating dairy cows

Abstract

Five Holstein cows in mid-lactation, producing 20.8 ± 1.24 (SE) kg milk/d, were randomly allocated to treatments according to a 5 x 5 Latin square design to determine melamine excretion via milk, faeces and urine. At the onset of the trial, all cows received the Control diet (no added melamine) for 7 d which served as an initial adaptation period. This was followed by five experimental periods of 15 days each, during which the cows received their respective experimental diets for 7 d and the Control diet for another 8 d. The latter 8 d will be referred to as the withdrawal period. The five experimental treatments had the same basal composition and the only difference between treatments pertained to the level of melamine inclusion. The source of melamine was adulterated maize gluten 60 of Chinese origin (MEL-gluten) with a melamine concentration of 16630 mg/kg. The experimental treatments were formulated to provide a daily melamine intake of 0 (M0), 500 (M1), 1000 (M2), 5000 (M3) or 10000 mg (M4) via 15 kg of dairy concentrate pellets. Milk samples were collected on d 1, 2 and 3 of the experimental periods and on d 1, 2 and 8 of the withdrawal periods. Faecal and urine samples were collected on d 3 and 8 of the experimental and withdrawal periods, respectively. Melamine treatments had no effect on DMI, milk yield and milk composition. Melamine was detected in the milk of all cows receiving melamine treatments 8 h after initial ingestion. Milk melamine concentrations increased rapidly and reached a peak on d 3 of the trial. Melamine excretion efficiency was not affected by dietary melamine level and values were 1.5, 1.9, 2.1 and 1.7 % for treatments M1, M2, M3 and M4, respectively. Upon melamine withdrawal, milk melamine concentrations decreased rapidly and reached undetectable levels 8 d after withdrawal. Mean apparent digestibility of melamine for all treatments was 78 % and faecal and urinary melamine excretion 22 and 54 %, respectively. It was concluded that melamine appears in the milk very soon after first ingestion, reaches a peak on d 3 and takes 8 d to reach undetectable levels after withdrawal. It was further confirmed that urine and faeces are the primary excretion routes of melamine from the body.

Keywords: Melamine, milk, urine, faeces

5.1 Introduction

Melamine ($C_3H_6N_6$) results from a urea distillation process. A large amount of waste water is produced during the process and, in an attempt to limit environmental pollution and to enable convenient disposal thereof, the water is concentrated into a solid product, also known as melamine waste. Melamine is commonly used in the manufacturing of plastic-ware, laminates and paints. On a molecular weight basis, melamine contains 667 g/kg nitrogen (Merck, 2001). In turn, depending on the purification methods used by manufacturers, the resultant melamine waste may vary in composition with regards to the melamine and its analogues' content. Kirk-Othmer (1978) reported melamine waste to typically consist of approximately 70% melamine, 23% oxytriazines (including cyanuric acid, ammeline and ammelide) and 7% polycondensates (including melem, melam and melon). Hence, melamine waste is expected to have a high nitrogen content.   Due to the high nitrogen content of melamine waste and the fact that it is a waste product, fraudulent feed manufacturers realized the potential economic gain they may achieve when using the melamine waste product as an adulterant for protein feedstuffs. Unfortunately, their negligent greediness had resulted in numerous morbidities and mortalities in the animal industry, of which pets (including dogs and cats) were most severely affected.

The cause of the high morbidity and mortalities observed in pets were ascribed to the formation of renal stones, resulting in kidney damage and ultimately kidney failure (Brown *et al*., 2007; Puschner *et al*., 2007). The kidney stones were analysed and found to contain triazine compounds of which melamine and cyanuric acid were the principle compounds (Dobsen *et al*., 2008). Melamine reacts with cyanuric acid on a 1:1 basis to form melamine cyanurate which is described as a crystalline complex (Perdigão *et al*., 2006). Upon analysis of the implicated pet feeds, the melamine concentrations reportedly ranged between 9.4 – 1952 mg/kg (Bhalla *et al*., 2009). Following the pet food recall, some of adulterated pet food was used to supplement the feeds administered to pigs (FDA, 2007). In turn, this resulted in the public expressing their concern with regards to health risks when consuming meat from animals exposed to melamine tainted feeds prior to slaughtering.

The public's increased concern was not unjust following the reports made by the Chinese government in 2008 that 296 000 infants had been diagnosed with kidney-related illnesses and six have died following the consumption of melamine adulterated infant formula (WHO, 2008). In an attempt to avoid melamine tainted food products to protect the public, various

countries banned the importation of milk and milk-containing products originating from China.   This was also the case in South Africa. Even though the import of Chinese protein products used for human consumption was banned, melamine was soon detected in the milk of some South African dairies by the end of 2008. During 2006, reports were made that melamine was detected in protein ingredients used in animal feeds (WHO, 2008).

Adulterated protein ingredients exported from China included wheat gluten and rice proteins to North America (Dobsen *et al.,* 2008), soy products to Europe (EFSA, 2010) and maize gluten to South Africa (Cruywagen & Reyers, 2009). As the deliberate addition of melamine to milk in South Africa seemed highly unlikely due to the high cost of melamine, Cruywagen *et al.* (2009) hypothesized that the presence of melamine in the milk of dairy cows may have resulted from the consumption of melamine tainted feeds.

Cruywagen *et al.* (2009) managed to obtain some of the melamine adulterated maize gluten imported from China from a source which would remain anonymous due to a confidentiality agreement. Upon analysis, the melamine concentration of the Chinese maize gluten was reportedly 15117 mg/kg. Cruywagen *et al*. (2009) incorporated 69 g/kg Chinese gluten into the total mixed ration of dairy cows which resulted in a daily intake of 17.1 g of melamine. Melamine analysis of the milk confirmed that a pathway exists for the transmission of dietary melamine to milk. Astonishingly, the melamine appeared in the milk as soon as 8 h after initial ingestion by cows. The excretion efficiency of melamine into milk was reported at 2.1% when the maximum melamine concentration in milk was reached.   Upon the withdrawal of melamine, milk reached undetectable levels of melamine at 152 h.

In a similar study, Battaglia *et al*. (2010) also investigated the transfer of melamine to cows' milk. These authors provided cows with graded levels of melamine (*viz*. 0.05 g, 0.50 g, 5.00 g or 50.00 g) as a single oral bolus. In agreement with Cruywagen *et al*. (2009), Battaglia *et al*. (2010) reported a rapid appearance of melamine in the milk only 6 h after the ingestion of the melamine containing bolus. Melamine excretion efficiency via milk reportedly ranged between 2.3 and 3.3% (Cruywagen *et al*., 2009; Battaglia *et al.,* 2010).

Information pertaining to the excretion pathways of dietary melamine in lactating dairy cows still remains limited. The studies of Cruywagen *et al*. (2009), Battaglia *et al*. (2010), Shen *et al*. (2010) and Sun *et al*. (2011) provide valuable information on the excretion of melamine into cows' milk. However, little is known about the excretion of dietary melamine via other

routes, including urine and faeces in lactating dairy cows. Cruywagen *et al.* (2009) only took spot urine and faecal samples on the last day of their trial. In addition, only two articles were found where the excretion of melamine in cow's urine, faeces and milk were reported (Zheng *et al.*, 2011; Sun *et al.*, 2012).

Therefore, the current study was conducted to determine the excretion of melamine via milk, urine and faeces in lactating dairy cows. In addition, the study aimed to determine whether or not the dietary melamine dosages influence melamine excretion efficiency into milk. The withdrawal period required to ensure the absence of melamine in milk was also determined. The current study also investigated the excretion of melamine via urine and faeces to confirm the major excretion routes of ingested melamine.

5.2 Materials and Methods

5.2.1 Animals and housing

Cows were housed and cared for according to current ethical norms and did not experience any discomfort for the duration of the trial. The study was conducted at the Welgevallen Experimental Farm of Stellenbosch University, South Africa. The trial was approved by the Stellenbosch University's Animal Ethics Committee (Ref:10LV_CRU02).

Five lactating Holstein cows, 155 ± 14.6 (SE) days in milk (DIM) and producing 20.8 ± 1.24 (SE) kg milk/day were used in the trial. The cows were housed individually in well-ventilated, semi-open pens (6- x 4-m) with concrete floors. Each pen was equipped with two feed troughs to enable separate feeding of roughage and experimental treatments. Each cow had access to a sand-bedded sleeping crate and fresh water was available *ad libitum* via a ball valve-controlled water bowl. The pens were cleaned twice daily throughout the duration of the trial by hosing and sweeping away accumulated manure while the cows were at the milking parlour to prevent interference with their normal eating behaviour and to minimize stress.

5.2.2 Experimental design and treatments

Cows were allocated to treatments according to a 5 x 5 Latin square design with five treatments and five periods. Therefore, all cows received each of the five treatments during the course of the trial. At the onset of the trial, all cows received the Control diet (no added

melamine) for 7 d which served as an initial adaptation period. This was followed by five experimental periods of 15 days each, during which the cows received their respective experimental diets for 7 d and the Control diet for another 8 d. The latter 8 d will be referred to as the withdrawal period. The duration of the total trial was thus 82 d.

The experimental treatments were manufactured according to our specifications by Tanqua Feeds (Riviersonderend, South Africa). The five experimental diets had the same basal composition. The differences between the treatments were attributed to the level of melamine inclusion. The source of melamine was melamine adulterated maize gluten 60 (MEL-gluten) of Chinese origin. It was decided to incorporate the MEL-gluten as the source of melamine in the experimental treatments, due to the fact that this was the means by which South African dairies were exposed to melamine. The experimental treatments were formulated as such to allow for a daily melamine intake of 0 (M0), 500 (M1), 1000 (M2), 5000 (M3) or 10000 mg (M4) via 15 kg of dairy concentrate pellets. The control treatment was represented by M0. The basal diet included corn gluten meal at a rate of 50 g/kg as this is the average rate of maize gluten inclusion in dairy concentrates in South Africa. Therefore, in order to obtain the required melamine dosages, MEL-gluten and locally produced maize gluten free of MEL (local gluten) had to be adjusted accordingly. The melamine concentration of the MEL-gluten was 16630 mg/kg (as determined by LC/MSMS).   Melamine analyses confirmed no detectable levels of melamine in the local gluten, control treatment, lucerne hay and oat hay. Table 5.1 presents the ingredient composition of the experimental treatments.

Table 5.1 Ingredient composition of the experimental treatments on a DM basis

Ingredient (g/kg)	Treatments*				
	M0	M1	M2	M3	M4
Ground corn	535	535	535	535	535
Soybean meal	213	213	213	213	213
Cottonseed meal	67	67	67	67	67
MEL-gluten	-	2	4	20	40
Local-gluten	50	48	46	30	10
Molasses syrup	50	50	50	50	50
Molasses meal	40	40	40	40	40
Fish meal	25	25	25	25	25
Limestone	10	10	10	10	10
Salt	5	5	5	5	5
Mono Calcium Phosphate	3	3	3	3	3
Premix[1]	2	2	2	2	2

[1]Trace mineral premix supplied by Advit Animal Nutrition SA (Pty) Ltd, Kempton Park, South Africa
*Amount of daily melamine ingestion in 15 kg dairy concentrate pellets: M0 = 0 mg (Control), M1 = 500 mg, M2 = 1000 mg. M3 = 5000 mg, M4 = 10 000 mg.

5.2.3 Feeding and milking program

Each cow received 15 kg/d of the appropriate treatment pellets. A 50:50 forage mixture (lucerne- and oat hay) were provided *ad libitum*. The experimental treatment pellets and forages were provided in separate feed troughs. Feed was provided twice daily at 07h00 (8 kg pellets) and again at 16h00 (7 kg pellets). Feed troughs were thoroughly cleaned every morning before fresh feed was allocated for that day. Feed troughs were cleaned by collecting all the refusals from the previous day. Cows were milked twice daily at 06h00 and again at 15h00. Proper milking procedures were employed during each milking session to maintain udder health.

5.2.4 Feed samples

Feed samples of the individual experimental treatments, lucerne- and oat hay were collected every day throughout the 7-d experimental period. The daily collections of each individual feed were composited and thoroughly mixed. A representative sample was then taken, ground to pass through a 2-mm screen and stored at -20°C pending chemical analyses. An additional sample of each of the individual experimental treatments was collected for melamine analysis by LC/MSMS. All chemical analyses of the individual feeds were performed in duplicate as described by AOAC (2002) for dry matter (DM; method 934.041), ash (method 942.05), crude protein (CP; method 990.03) and ether extract (EE; method 920.39). Acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined with the aid of an Ankom 2200 fibre analyzer (Ankom Technology, Macedon, NY). Heat stable alpha amylase and sodium sulfite were added to the neutral detergent solution. The chemical compositions of all feed constituents are presented in Table 5.2.

Table 5.2 Chemical composition of experimental treatments and roughages

Feeds*	Chemical composition (g/kg DM)				
	CP[1]	EE[2]	NDF[3]	ADF[4]	Ash
M0	218.4	18.3	138.9	57.8	64.6
M1	225.1	26.1	103.2	56.0	61.0
M2	217.8	20.6	113.2	58.2	64.0
M3	218.2	21.3	110.3	62.8	61.2
M4	221.2	21.4	123.1	58.8	62.8
Lucerne hay	115.8	10.1	519.7	415.0	75.2
Oat hay	94.7	16.4	607.9	367.4	75.8

[1]CP = Crude protein; [2]EE = Ether extract; [3]NDF = Neutral detergent fibre; [4]ADF = Acid detergent fibre; * Amount of daily melamine ingestion in 15 kg dairy concentrate pellets: M0 = 0 mg (Control), M1 = 500 mg, M2 = 1000 mg. M3 = 5000 mg, M4 = 10 000 mg.

5.2.5 Dry matter intake

Total dry matter intake was recorded daily throughout the 7-d experimental period by weighing the daily feed allocation and feed refusals. By administering the experimental treatment and roughages in separate feed troughs, the daily feed intake of the treatments and forages could be determined individually. Therefore, it was possible to determine the individual melamine intake of each cow when treatment refusals were observed.

5.2.6 Milk yield, composition and milk melamine concentration

Daily milk yields from each cow were manually recorded at every milking (morning and afternoon) throughout the trial. Before commencement of the trial, milk from all cows was tested to confirm the absence of melamine in all milk samples. Throughout each of the 7-d experimental periods, milk samples were collected individually for milk composition and melamine analysis. Milk samples collected for milk composition were immediately preserved with potassium dichromate ($K_2Cr_2O_3$). Representative samples for milk component analysis from each cow during each experimental period were obtained by compositing the daily collected milk samples in amounts proportional to yield (10 mL/L milk produced). Milk samples were analyzed for fat, protein, lactose, total solids and milk-urea nitrogen (MUN) with the aid of a Milk-O Scan 605 analyzer (Foss Electric, Hillerod, Denmark) at the Dairy Laboratory of the Agricultural Research Council at Elsenburg, Stellenbosch.

The milk samples collected for melamine analysis were frozen and stored at -20° C pending analysis. Milk samples selected for melamine analysis from each cow during each period were as follows: day 1 (afternoon milk), day 2 (morning and afternoon milk) and day 3 (morning and afternoon milk pooled) for the experimental period and day 1 (morning and afternoon milk), day 2 (morning and afternoon milk pooled) and day 8 (morning and afternoon milk pooled) for the withdrawal period. The reasons for pooling some samples were due to the high cost of melamine analysis (LC/MSMS) and the large number of milk samples collected throughout the trial for melamine analysis. For the duration of the trial, the cows selected for the trial were milked last. The milk from cows receiving the melamine containing treatments were collected separately and discarded in accordance with South African regulations. Therefore melamine contaminated milk could not contaminate other milk produced on the farm. After the cows had been milked, the milking machine was

thoroughly cleaned to avoid any melamine contaminated milk to reside in the pipelines, thus preventing contamination of the bulk tank.

5.2.7 Faecal and urine samples

Faecal and urine samples were collected from each cow during each period on the following days: Days 1 and 3 of the experimental periods, and days 1, 2 and 8 of the subsequent withdrawal period. Faecal and urine samples were collected 5 h (~ 12h00) after morning feed allocation.

Melamine excretion via faeces and urine could not be quantified in this trial as faecal and urinary output was not collected quantitatively. However, daily faecal output values were estimated using an equation (Equation 1) reported by Nennich *et al.* (2005). These values were used to calculate apparent melamine digestion (AMD) and excretion via faeces.

Equation 1:

$$DM_E = [DMI \times 0.356(\pm0.011)] + 0.8(\pm0.34)$$

<u>Where</u>:

DM_E = daily faecal DM excretion (kg) and

DMI = daily DM intake (kg)

5.2.8 Sample preparation for melamine analysis

An adapted method from Shai *et al.* (2008) was used for melamine analyses. Cation-exchange solid-phase extraction cartridges (Phenomenex Strata SCX; 55 µm, 70 A, 500 mg / 3 mL, supplied by Separations, Randburg, South Africa) were used for solid phase extraction of all samples. As various samples were collected for melamine analysis during the trial, different preparation methods were required prior to solid phase extraction (SPE). The different preparation methods required for the respective samples were as follows:

Feed and faecal samples were dried for 48 h at 60°C. The dried samples were ground through a 1 mm screen. The ground samples (1 g) were extracted with 50% acetonitrile, 0.1% formic acid (10 mL) under sonication for 2 h in an ultrasonic bath (Branson 2210, Connecticut, USA). Due to the high melamine concentration of the experimental treatments,

the extracts of treatments M1 and M2 were diluted 10-fold, while treatments M3 and M4 were diluted 100-fold. The extracts (0.5 mL) were then loaded onto the SPE cartridges.

Thawed urine samples were centrifuged at 4 500 x g for 5 min. The resultant supernatants (0.5 mL) were subsequently loaded onto the SPE cartridges.

Thawed milk samples were diluted on a 1:1 basis with 0.2 M perchloric acid, vortexed for 1 min and subsequently centrifuged at 4 500 x g for 5 min. A volume of 3 mL from the resultant supernatant were loaded onto the SPE cartridges.

5.2.9 Melamine extraction

The SPE cartridges were conditioned with 6 mL methanol, followed by 6 mL distilled water. The respective volume of sample extracts (as specified in sample preparation methods) were loaded onto the SPE cartridges together with 100 µL of a 0.5 mg/L stable isotope-labelled melamine ($^{13}C_3H_6{}^{15}N_3$) internal standard solution (Cambridge Isotope Laboratories Inc., Andover, MA). Therefore, 0.05 µg of the labelled melamine was loaded onto each cartridge. The addition of the internal standard enabled corrections to be made should incomplete extractions occurred. The cartridges were then washed with 6 mL 0.1 *N* HCl followed by 6 mL methanol and allowed to aspirate under vacuum for 1 min. The melamine was eluted with 6 mL ammonium hydroxide: methanol: dichloromethane (1:5:5) and collected into clean glass tubes. The resulting extracts were dried under a gentle stream of nitrogen, re-suspended with 1 mL 50% acetonitrile and transferred to individual vials to be loaded onto the liquid-chromatograph triple quadruple mass spectrophotometer.

5.2.10 Liquid Chromatography Tandem Mass Spectrometry (LC/MSMS)

The melamine concentration of all samples were analyzed by LC/MSMS on a Waters API Quattro Micro triple quadruple mass spectrometer coupled to a Waters 2690 HPLC (Waters Corp., Milford, MA). For this method, the limit of detection was 0.005 mg/kg for feed and faecal samples and 0.001 mg/kg for urine and milk samples.

5.2.11 Statistical analysis

All the data were subjected to a Main Effects ANOVA with the aid of Statistica 10 (2011). Main effects were Treatment, Cow and Period. Differences were declared significant at $P < 0.05$, whereas tendencies were considered at $P < 0.1$.

5.3 Results and Discussion

5.3.1 Melamine Concentration of Experimental Treatments

The source of melamine incorporated into the experimental diets was melamine-adulterated maize gluten 60 of Chinese origin (MEL-gluten). Melamine analysis revealed the MEL-gluten to contain 16630 mg melamine/kg MEL-gluten. The amount of MEL-gluten added to the respective treatments was calculated according to the required dosage. The experimental treatments were formulated as such to ensure that cows would consume 0 (M0), 500 (M1), 1000 (M2), 5000 (M3) or 10000 (M4) mg melamine/day in 15 kg concentrate pellets. Hence, the desired melamine concentrations were 0, 33.3, 66.7, 333.3 and 666.7 mg/kg for treatments M0, M1, M2, M3 and M4, respectively. Following melamine analysis, the actual melamine concentrations of the experimental treatments were 0, 29.24 ± 0.94 (SE), 61.63 ± 3.61, 175.12 ± 1.59 and 591.05 ± 12.11 for M0, M1, M2, M3 and M4, respectively.

Prior to the trial, various samples throughout the MEL-gluten batch were taken. This would have ensured that a good representation of the melamine concentration (*viz.*16630 mg/kg) would be obtained to allow for accurate calculations of MEL-gluten inclusion into the experimental treatments. Unfortunately, the melamine concentrations of M3 and M4 were lower than anticipated. This may have resulted from the fact that the practice of adulteration does not ensure an even distribution of the adulterant throughout the product. As a result, this may have contributed to the incorporation of a MEL-gluten batch with a lower melamine concentration during the formulation process of both the M3 and M4 treatments.

5.3.2 Dry matter intake, Milk yield and Milk composition

The results for DMI, milk yield and milk composition are presented in Table 5.3. Treatments did not affect DMI ($P > 0.05$). This is in agreement with the results of other reported studies with dairy cows (Zheng *et al.*, 2011) and dairy goats (Baynes *et al.*, 2010). MacKenzie (1966) reported the feed intake of sheep to have markedly decreased when melamine (~10 g/d) was provided in addition with low quality hay. Clark (1966) also reported anorexia in sheep when > 10 g of melamine was provided, followed by subsequent death. However, when Clark (1966) supplemented maize meal with 7 g of melamine/d in addition with teff hay, no ill effects were observed, but did report sheep to refuse much of the melamine-maize mixture. It should be kept in mind that the relative high dosages of melamine offered to sheep by Clark (1966) were provided in a single application, which may have resulted in

81

toxicological symptoms and subsequent anorexia due to morbidity. In the current study, the dietary melamine was thoroughly mixed into the basal diet to ensure even distribution throughout the experimental treatments. In addition, the melamine dosages were well below the proposed "safety" value of 0.16 g melamine/kg live weight for ruminant animals as calculated from the work of Newton and Utley (1978). Therefore, the experimental treatments (i.e. melamine concentrations) would not have affected DMI as was reported in the current trial.

Neither milk yield nor milk composition was influenced by the treatments. This is in agreement with all the reported studies pertaining to melamine consumption by dairy cows (Battaglia *et al*., 2010; Cruywagen *et al*., 2009; Shen *et al*., 2010; Sun *et al*., 2011). Treatments also had no effect on milk urea nitrogen (MUN). Some researchers (Cruywagen *et al*., 2009; Shen *et al*., 2010) mentioned that they had expected the MUN content of the milk to have been influenced following the addition of melamine to dairy cow diets. The rationale for expecting the MUN to be influenced by melamine consumption is based on literature reporting at least some melamine hydrolysis in ruminants. Newton & Utley (1978) observed increased NH_3 concentrations when melamine was added as a substrate in an *in vitro* trial, but concluded that the slow rate of melamine hydrolysis (hence slow rate of NH_3 production) would not benefit microbial growth and protein synthesis. Anaerobic hydrolysis of melamine by a *Pseudomonas* strain in an *in vitro* study (Jutzi *et al*., 1982) and the presence of *Pseudomonas aeruginosa* in the rumen of sheep (Duncan *et al*., 1999) were also reported. In addition, the results of the previously mentioned *in vitro* degradation study (Chapter 3) showed a13.6 % melamine degradation in rumen liquor, which may lead one to expect the MUN content to be affected by melamine administration. The lack of effect of melamine on MUN observed by abovementioned authors, as well as in this current trial, may be ascribed to the fact that the melamine is degraded at a very slow rate, *viz.* 5.5 % degradation after 24 h (Chapter 3). The MUN content of milk is more likely to be affected when rumen degradable proteins (RDP) and non-protein nitrogen sources (NPN) and/or rumen undegradable proteins (RUP) are readily degraded to NH_3 or amino acids, respectively (Cunningham *et al*., 1996; Sannes *et al*., 2002). This is especially true when nitrogen sources (i.e. RDP, NPN and RUP) are fed to dairy cows in excess (Baker *et al*., 1995; Huber, 1975; Mugerwa & Conrad, 1971) which is why MUN data can be used to determine the efficiency of nitrogen utilization in dairy cows (Nousiainen *et al*., 2004).

Table 5.3 Effect of treatments on DMI, milk yield and milk composition of Holstein cows consuming diets containing different levels of melamine

Item	Treatments[1]					SEM[2]	*P*
	M0	M1	M2	M3	M4		
DMI[3] (kg/d)	17.6	17.5	18.2	18.4	18.7	0.369	0.181
Milk yield (kg/d)	18.2	18.1	20.2	19.6	19.4	0.787	0.306
Fat (g/kg)	33.2	33.1	34.2	33.6	33.2	2.213	0.857
Protein (g/kg)	36.7	36.9	36.1	37.1	37.1	0.631	0.787
Lactose (g/kg)	46.7	47.9	47.7	46.5	47.0	0.813	0.681
TMS[4] (g/kg)	123.9	123.9	124.9	124.5	123.7	2.201	0.871
MUN[5] (mM/L)	18.6	18.4	18.3	18.9	18.1	0.491	0.456

[1]Treatments: M0 = 0 mg melamine/ d, M1 = 500 mg melamine/ d, M2 = 1000 mg melamine/d, M3 = 5000 mg melamine/d, M4 = 10000 mg melamine/d.

[2]SEM = Standard error mean; [3]DMI = Dry matter intake; [4]TMS = Total milk solids; [5]MUN = Milk urea nitrogen

5.3.3 Melamine Excretion in Milk

The milk melamine concentration results of the different treatments over the experimental and withdrawal periods are presented in Figure 5.1.

From the results it is clear that melamine rapidly appeared in the milk following initial ingestion of the melamine containing diets. No literature could be found to indicate the exact time at which melamine first appears in milk. Battaglia *et al*. (2010) detected melamine in the milk 6 h after initial ingestion. Due to the design of the current trial and feasibility of the milking program, cows were milked 8 h after morning feed allocation at which time melamine was detected in the milk. This is in agreement with the findings of Cruywagen *et al*. (2009). Both Cruywagen *et al.* (2009) and Sun *et al.* (2011) reported the maximum melamine concentration in milk to be reached on the third day of the trial after which the melamine concentration fluctuated for the following days. Contradictory to the previously mentioned authors, Battaglia *et al*. (2010) reported maximum milk melamine concentrations to have been reached between 6 and 18 h after consuming a single melamine containing

bolus. However, it should be noted that administration of a single bolus may result in different melamine pharmacokinetics when compared to a continuous melamine feeding regime. As melamine transportation is limited to blood and extracellular fluid (Yang *et al*., 2009), the short half-life of melamine (Baynes *et al*., 2008) and rapid excretion via urine (Mast *et al*., 1983) indicate that the kidneys may be capable in eliminating a single melamine dose from the body. Therefore, cows receiving melamine on a continuous basis may prolong the presence of melamine in plasma and milk, as melamine excretion via urine is restricted to daily urinary volume (Dominigues-Everez *et al*., 2010). In addition, results from various studies (Cruywagen *et al*., 2009; Shen *et al*., 2010; Sun *et al*., 2011) suggest that melamine excretion via milk may be influenced by melamine dosage and duration of exposure which might explain the peak milk melamine concentration to be reached on the third day of continuous melamine consumption.
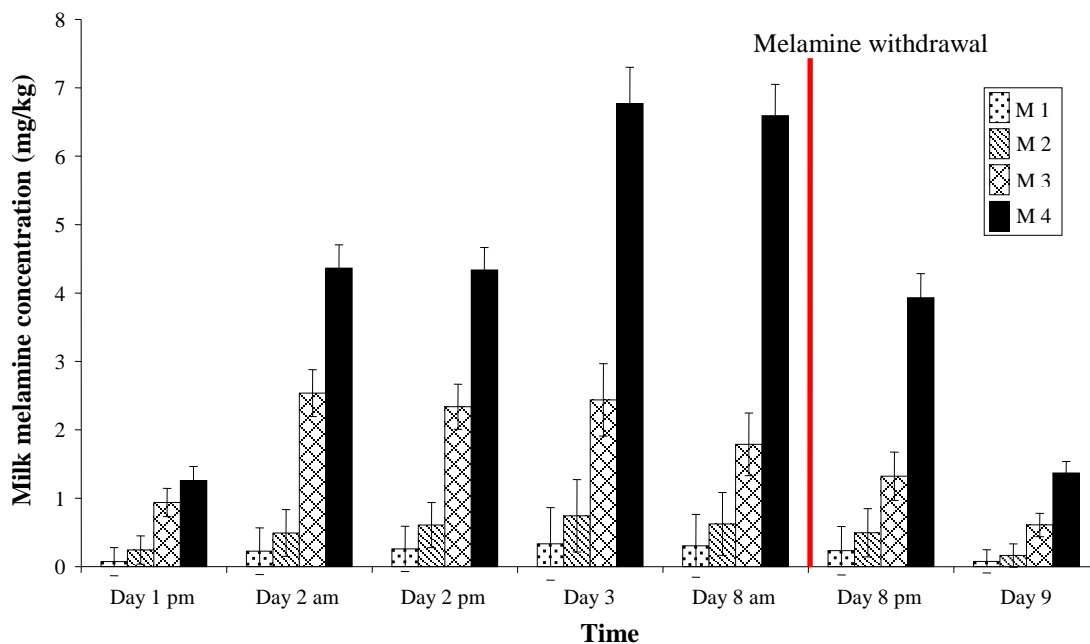
Fig 5.1. Milk melamine concentration following the ingestion of different levels of melamine by dairy cows. Melamine containing feeds were first fed on the morning of Day 1 and withdrawn on Day 8 after morning milking. Total daily melamine intake for treatments were: M1 = 500 mg melamine; M2 = 1 000 mg melamine; M3 = 5 000 mg melamine; M4 = 10 000 mg melamine.

The milk melamine concentration for all treatments increased rapidly during the second day with the peak concentration observed on the third day of the experimental period. It should be noted that, on the morning of d 2 (approximately 23 h after initial melamine consumption), all milk samples from cows on treatments M3 and M4 had melamine concentrations

84

exceeding the safety level of 2.5 mg/kg as recommended by the WHO (2008). The peak milk melamine concentrations were 0.33, 0.74, 2.44 and 6.77 mg/kg for treatments M1, M2, M3 and M4, respectively. In a similar study, Sun *et al*. (2011), provided 500 mg and 1000 mg of melamine/d to cows and reported the peak milk melamine concentrations as 0.27 and 0.51 mg/kg, respectively. From Figure 5.1 it can be seen that higher milk melamine concentrations were observed as the melamine concentration in the treatment diets increased. This is in agreement with other authors reporting the mean milk melamine concentrations to be significantly influenced by the dietary melamine dose (Shen *et al*., 2010; Sun *et al*., 2011).

Melamine excretion efficiency in this trial was calculated by expressing the amount of melamine excreted in the milk as a percentage of that ingested. Contradictory to the speculations made by Cruywagen *et al*. (2009), the different levels of melamine in the treatment diets did not significantly affect melamine excretion efficiency. Similar results were also reported by Shen *et al*. (2010) and Sun *et al*. (2011). Melamine excretion efficiencies at peak concentration on d 3 of this trial were 1.5, 1.9, 2.1 and 1.7 % for treatments M1, M2, M3 and M4, respectively, with SEM = 0.138. There was, however, a tendency (P = 0.091) for the melamine excretion efficiency of M3 to be higher than M1. Shen *et al*. (2010) proposed the excretion efficiency to increase as milk yield increase, suggesting that higher producing cows may be more efficient in excreting melamine via milk compared to low producing cows. In this trial, the mean milk yields of M1 and M3 were very similar which does not explain the observed tendency. However, upon reviewing the individual data sets of the M1 and M3 treatments, it was noted that two of the five cows had higher milk yields when they received treatment M3 compared to their milk yields when they received treatment M1. As a result, their individual melamine excretion efficiencies increased as Shen *et al*. (2010) suggested. For those two cows, their excretion efficiencies ranged between 1.5 to 1.8% and 2.4 to 2.7% for treatments M1 and M3, respectively. This may explain the observed tendency for the excretion efficiency for M3 to be higher compared to M1. The melamine excretion efficiencies obtained from this study fall well within the range of 1.7 to 2.1% as reported by Cruywagen *et al*. (2009).

On the morning of d 8 of the trial, all cows were switched back to the control (M0) treatment. Following melamine withdrawal, the milk melamine concentrations for all treatments rapidly decreased. Interesting to note is that the milk melamine concentration of the highest melamine treatment (M4) decreased almost 2-fold more after 8 h following melamine

withdrawal compared to the other treatments. Eight hours after melamine withdrawal, the milk melamine concentrations decreased by 23.2, 21.0, 26.0 and 40.4% for treatments M1, M2, M3 and M4, respectively. This observation confirms the fact that the dietary melamine concentration influences the milk melamine concentration and that continuous melamine consumption by the cows is required to maintain peak levels of melamine in the milk. There is a general agreement amongst authors (Battaglia *et al*., 2010; Cruywagen *et al*., 2009; Shen *et al*., 2010; Sun *et al*., 2011) reporting a 7 d withdrawal period to be required for milk to reach no detectable levels of melamine. In this trial, the exact time at which milk reached undetectable levels was not determined. However, on the last day (d 15) of the each period, no melamine was detected in any of the milk samples of cows that received the melamine treatments. Therefore, following a withdrawal period of 8 d would ensure milk free from melamine and safe for human consumption.

5.3.4 Melamine in Urine and Faeces

Table 5.4 presents the faecal and urine melamine concentrations of the different treatments, as well as the estimated melamine digestibility and faecal melamine excretion. From Table 5.4 it is clear that both faecal and urine melamine concentrations increased as melamine intake increased. This trend was also reported by Zheng *et al*. (2011). It therefore appears that the excretion of melamine via milk, urine and faeces in this and other trials (Shen *et al*., 2010; Sun *et al*., 2011; Zheng *et al*., 2011) are influenced by the dietary melamine dosages.

The average apparent melamine digestibility value of 78% for the four treatments is quite comparable with the value reported by Cruywagen *et al.* (2011) for sheep, *viz.* 76.3%. Correspondingly, the average apparent faecal melamine excretion value of 22% across the treatments agrees with the value of 23.7% reported by Cruywagen *et al.* (2011). In the study of Sun *et al*. (2012), faeces and urine were quantitatively collected over a period of 3 days. From their results, the reported mean faecal melamine excretion value (*viz.* 10.98 %) for cows receiving 800 mg of melamine/d was approximately 2-fold lower compared to the values estimated in this trial. However, the current trial confirmed that melamine has a fairly high apparent digestibility (or apparent absorption) and from the high urine melamine concentrations it can be concluded that urine is the major excretion route of absorbed melamine.

Table 5.4 Faecal and urine melamine concentrations and amount (%) excreted

Item	Treatment*				SEM[1]	P
	M1	M2	M3	M4		
Faecal melamine (mg/kg)	14.6[a]	26.8[a]	92.6[b]	184.9[c]	13.8	0.00008
Urine melamine (mg/kg)	24.4[a]	41.3[a]	131.3[b]	202.7[c]	11.5	0.00001
AMD (%)[2]	75.2	78.3	74.3	84.2	0.28	0.128
Melamine excreted via faeces (%)[3]	24.8	21.7	25.7	15.8	2.78	0.128

*Treatments: M1 = 500 mg melamine/d, M2 = 1000 mg melamine/d, M3 = 5000 mg melamine/d, M4 = 10000 mg melamine/d.

[a-c]Means within rows with different superscripts differed significantly ($P < 0.01$).

[1]SEM = Standard error mean

[2]AMD = Apparent melamine digestibility

[3]Estimated melamine excretion via faeces expressed as percentage of melamine intake.

The implications of the high melamine concentrations present in faeces and urine have yet to be discussed with regards to the contamination of pastures. In some dairies, cows are provided with concentrates in the milking parlour and allowed to graze pastures for the remainder of the day. Keeping in mind that concentrate raw materials, especially protein feedstuffs, are more likely to be adulterated as was apparent in the 2008 melamine adulteration incident. If a scenario occurred where cows were provided with a melamine adulterated dairy concentrate in the milking parlour, up to approximately 80% of the ingested melamine would then be excreted via urine and faeces on the pastures. Calitz *et al*. (2012) proposed that faecal and urinary melamine may contaminate pastures which may result in the pasture grass containing melamine. Botha (2010) reported the transmission of melamine from fertilizer to pasture grass within 7 d after fertilization. Should the cows then consume such melamine-contaminated pastures in addition to the melamine tainted concentrate, melamine may be present in the milk at concentrations exceeding the recommended safety limits of 2.5 mg/kg. In addition, should the producer become aware of the presence of melamine in the concentrate and immediately remove the concentrate, the recommended withdrawal period required to ensure melamine free milk is 7 d. However, due to the recontamination of pastures, melamine may still be detected after the 7 d withdrawal period, as melamine in pasture grass would reach undetectable levels only 10 weeks after a single

application. It is unclear whether a repeated application of melamine to pastures (as would occur during defecation and urination of cows exposed to melamine tainted feeds) would result in melamine accumulation in the soil. However, it can be speculated that repeated melamine excretion on pastures may prolong the time needed for pasture grass to reach undetectable melamine levels.

5.4 Conclusion

In this study, melamine was rapidly detected in the milk of all cows receiving the melamine containing treatments with the peak milk melamine concentrations observed on the third day of exposure. Higher dietary melamine concentrations resulted in higher melamine concentrations in the milk, urine and faeces indicating that melamine excretion are influenced by melamine dosage. This trial confirmed that urine and faeces are the primary routes for eliminating melamine from the body. No levels of melamine could be detected in all milk, urine and faecal samples eight days following melamine withdrawal.

The hypothetical scenario discussed previously with regards to pasture contamination via urine and faeces indicate the chain of events that may occur as a result of a single melamine adulteration. However, further research is required to determine the extent to which melamine excreted via urine and faeces may recontaminate pastures and the implications thereof.

Even though adulterations of feedstuffs are prohibited by law, the reality remains that it still occurs more often than we think. The melamine adulteration incidents did not only result in financial implications, it had devastating consequences with regards to animal and human health. With the safety limits set by health regulatory authorities in an attempt to ensure public health, it does not ultimately ensure the absence of melamine in food and feed. One should keep in mind that the recommended safety limits are estimates from animal studies and therefore, to ensure absolute consumer safety regarding melamine exposure, all feeds and animal products should be free of melamine.

5.5 References

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CHAPTER 6

Melamine absorption in the mammary gland of lactating dairy cows

Abstract

Five docile Holstein cows producing 39 ± 10.6 (SD) kg milk/d were used in a trial to determine melamine absorption by the mammary gland. Cows received 10 g of melamine daily, provided via treatment boluses (5 g of melamine/bolus) twice daily for three consecutive days. Day 3 of the trial was used for collecting blood and milk samples to determine melamine absorption by the mammary gland through arterio-venous (A-V) difference. On the morning of day 3, catheters were inserted into the caudal superficial epigastric vein (milk vein) and the caudal auricular artery, following administration of a local anaesthetic. After confirming catheter patency, 1.0 mL oxytocin was injected via the auricular artery to remove residual milk from the udder during subsequent milking. Arterial and venous blood samples were collected hourly for the following 9 hours. Cows had access to fresh water, lucerne hay and semi-complete dairy pellets throughout the 9 hour period. Catheter patency was maintained by flushing the catheters with heparinised saline solution between blood collections. After the final blood collection, oxytocin was administered again, the catheters were carefully removed and cows were milked immediately thereafter. After each blood collection, samples were centrifuged (15 min, 1800 x g), the resultant plasma was decanted and stored at -20° C. Milk yield was recorded and milk samples were collected for milk content and melamine analyses. Plasma samples were analysed for melamine and amino acid content. Phenylalanine and tyrosine contents of milk and plasma were used to calculate mammary blood flow which was required to calculate melamine absorption in the udder. Melamine in milk and plasma was determined by LC/MSMS. A net positive melamine flux was observed, indicating net absorption of melamine by the mammary gland. Melamine absorption efficiency by the mammary gland was 0.29% and melamine excretion efficiency into milk was 1.47%. It was concluded that melamine ingested by cows will result in net absorption of melamine by the mammary gland, but that absorption efficiency is low.

Keywords: Melamine, milk, mammary gland, absorption

6.1 Introduction

Feeding ruminant animals requires indebt knowledge about the animals' requirements for growth, maintenance and production. Ruminant diet formulation is rather complex due to the microbial fermentation of nutrients in the rumen prior to enzymatic digestion in the abomasum and small intestines. Dairy cow nutrition especially poses great challenges, because not only should the dairy cow's nutritional requirements be met, one must also consider the impact that the diet would have on milk production. Milk yield and milk composition are the determining factors of the producer's profitability. In addition, milk should be free of hormones, antibiotics and foreign substances as consumers demand healthy and safe milk.

The diet consumed by the lactating dairy cows will ultimately influence the composition of milk as various metabolic processes involved in digestion, absorption and transportation will determine the supply of nutrients to the mammary gland. However, the relationship between feed constituents and milk composition is complex due to nutrient transformation in the rumen and the biochemical and physiological processes involved in the way milk solids are synthesized and secreted by cells in the mammary gland (Miller *et al*., 1991; Sutton, 1989). Apart from dietary nutrients, it is possible for non-nutrient substances transported in arterial blood to be taken up by the mammary gland and excreted into the milk. In some cases, non-nutrients excreted into the milk may pose a risk to human health when consumed. For this reason, the United States Food and Drug Association (FDA) have set maximum residue limits (MRL) to enforce dairy producers to comply with the regulations in an attempt to ensure public health and safety. Non-nutrient substances reported to be excreted into cows' milk upon administration include antibiotics (Koesukwiwat *et al*., 2007; Passchyn *et al.,* 2009; Tang *et al*., 2009), toxins present in feeds such as aflatoxins (Battacone *et al*., 2009), hormones (Chalupa & Galligan, 1989; Guyer & Juskevich, 1990; Etherton *et al*., 1993) and even industrial chemicals such as polybrominated diphenyl ether (Lake *et al*., 2011). Melamine, another industrial chemical, received much attention in the feed and food chain since 2006, which has also been reported to be excreted into cow's milk (Cruywagen *et al.,* 2009). The reason for the sudden international interest in melamine resulted following reports of melamine adulteration in pet food (Brown *et al.,* 2007; Puschner *et al.,* 2007), infant formula (WHO, 2008; Yang & Batlle, 2008) and protein ingredients used in the animal feed industry (WHO, 2008) which had a catastrophic impact on animal and human health.

The transmission of dietary melamine to cow's milk has been confirmed by various authors (Cruywagen *et al*., 2009; Battaglia *et al*., 2010; Shen *et al*., 2010; Sun *et al*., 2011; Zheng *et al*., 2011). Upon initial consumption, melamine is rapidly excreted into cow's milk and can be detected in milk as soon as 6 – 8 h after first exposure (Cruywagen *et al*., 2009; Battaglia *et al*., 2010). The continuous consumption of melamine by cows, results in a peak melamine concentration in the milk to be reached on the third day of exposure (Cruywagen *et al*., 2009; Shen *et al*., 2010; Sun *et al*., 2011). The mean excretion efficiency of melamine is reported to range between 2.1- 3.3 % (Cruywagen *et al*., 2009; Battaglia *et al*., 2010). Upon melamine withdrawal, the concentration of melamine in milk rapidly decreases and is not detected after approximately 7 d (Cruywagen *et al*., 2009; Sun *et al*., 2011).

Since the announcement of melamine adulterated animal feeds, a vast amount of studies pertaining to dietary melamine transfer to various animal products (including milk, meat and eggs) have been reported (Cruywagen *et al*., 2009; Bai *et al*., 2010; Chen *et al*., 2010; Cruywagen *et al*., 2011). However, these studies mainly focused on understanding and determining melamine partitioning patterns, as well as the potential risks posed to human health should such products be consumed. Infants and small children are more susceptible to melamine exposure (WHO, 2008) and the fact that their diets consist mainly of milk and milk products, resulted in emphasis being placed on melamine transfer to milk. Miller *et al*. (1991) proposed the supply of nutrients to the mammary gland to be dependent on the concentration of the nutrients in the arterial blood and the rate of mammary blood flow (MBF). This could also be applied to melamine, as the melamine concentration in arterial blood and the rate of MBF would determine the amount of melamine supplied to the mammary gland and in turn determine the amount of melamine excreted in the milk. Even though the information obtained from studies pertaining to melamine excretion in milk are valuable, no information on the metabolism or absorption kinetics of melamine in the mammary gland is available.

Studies on amino acid metabolism in the mammary gland were made possible by measuring arterio-venous (A-V) differences across the udder and MBF (Cant *et al*., 1993; Lapierre *et al*., 2009). It was decided to use similar techniques to study melamine absorption by the mammary gland in lactating dairy cows.

6.2 Materials and Methods

6.2.1 Animals and treatment

The trial was conducted at the Welgevallen Experimental Farm of Stellenbosch University and was approved by the Stellenbosch University's Animal Ethics Committee (Ref: 10LV_CRU02).

Five lactating Holstein cows producing 39 ± 10.6 (SD) kg milk/d were selected from the herd based on temperament to ensure docile animals during catheterization and blood sampling. Two weeks prior to commencement of the trial, the selected cows were moved to an in-house facility adjacent to the milking parlour for 2 h after morning and afternoon milking to familiarize them with the environment. The facilities were equipped with individual stalls (1- x 2-m) and each stall contained feed and water troughs. Wheat straw was provided as bedding. During the 2 h periods after each milking, the cows received small portions of semi-complete dairy pellets to condition them in consuming the allocated feed in preparation of the treatment bolus. The cows were also conditioned to human interaction by touching and stroking the cows in preparation of the contact required for repetitive blood sampling during the trial and to reduce stress by the animals. After the 2 h period, the cows were moved back to the free stall barns to join the rest of the herd for the remainder of the day. Milk samples collected from all the cows the day before commencement of the trial confirmed the absence of melamine in the milk.

The treatment boluses were provided to the cows for three consecutive days, twice daily (07h00 and 16h00) after milking. Each bolus contained 5 g of pure melamine (melamine 99%, Sigma-Aldrich, St. Louis, MO) mixed with 10 g molasses syrup and diluted with 20 mL warm water. The melamine containing liquid mixture was then poured over 200 g of semi-complete dairy pellets, thoroughly mixed and oven-dried at 60°C for 1 h. This preparation ensured that the boluses were readily eaten by the cows and resulted in a daily melamine intake of 10 g/cow.

6.2.2 Sample collection

The third day of the trial was used for collecting blood and milk samples to determine melamine absorption by the mammary gland through arterio-venous (A-V) difference. On the morning of the third day, catheters were inserted into the blood vessels. The locations of

catheter placement were shaved and sterilized prior to insertion. The subcutaneous administration of local anaesthetic lignocaine enabled the insertion of one catheter into the caudal superficial epigastric vein (milk vein) and one catheter into the caudal auricular artery. All procedures were performed by a veterinarian and qualified technician. The catheter (20 gauge, 0.9 x 80 mm) selected for the milk vein was fitted with an extension tube (3.0 x 4.1 mm) and routed to the cow's back to allow easy and safe sampling. The catheter (24 gauge, 0.7 x 19 mm) inserted into the auricular artery was fitted with a 152 mm extension set equipped with a needle-free valve port. Catheters and extension equipment were supplied by Stelmed CC, Stellenbosch, South Africa.

Following catheter placement and confirmation of catheter patency, 1.0 mL oxytocin was injected through the auricular artery to ensure removal of residual milk from the udder when cows were milked immediately thereafter. Cows were then moved to the adjacent tie-stalls where they received their final treatment boluses, followed by the commencement of the blood collection period. Arterial and venous blood samples were taken hourly into 10 mL heparinised tubes for the following 9 hours. Throughout the blood collection period, cows had access to fresh water, lucerne hay and semi-complete dairy pellets containing no melamine. Catheter patency was maintained by flushing the catheters with heparinised saline solution between blood collections. After the final blood samples had been taken, oxytocin was administered again, the catheters were removed carefully and the cows were milked immediately thereafter.

After each blood collection, blood samples were immediately centrifuged (15 min, 1800 x g) and the resultant plasma was decanted and frozen at - 20°C pending analysis. Plasma samples were analysed for melamine and amino acid concentrations. At both milkings, milk yield was recorded and milk samples were collected for milk content and melamine analyses. Milk samples collected for milk content analysis were immediately preserved with potassium dichromate ($K_2Cr_2O_3$) after collection. Milk samples collected for melamine analysis were frozen and stored at -20° C until analysed. Milk samples of both milkings were analysed for melamine in order to calculate melamine excretion efficiency over 24 h, but only milk that was synthesised during the 9 h blood collection period (second milking samples) was analysed for milk components, as these were used in calculations to determine plasma flow through the mammary gland.

6.2.3 Arteriovenous Difference and Mammary Uptake

Determining the arteriovenous (A-V) concentration differences of melamine across the mammary glands require representative blood samples of the arterial supply and venous drainage of the mammary glands (Cant *et al*., 1993). Blood samples collected to represent the arterial supply to the mammary gland can be obtained from any arterial source as arterial blood is considered to be sufficiently mixed. In the current study, the intermediate auricular artery was selected for obtaining arterial blood samples due to simplicity of catheterization (Muylle *et al*., 1996). Mammary venous drainage occurs via two routes: the external pudic- and caudal superficial epigastric veins. In goats, the pudic vein has been reported to be contaminated with abdominal blood as a result of valvular incompetence (Linzell, 1960). Even though this condition is less prevalent in cows (Linzell, 1960), blood samples in the current trial were obtained by catheterization from the caudal superficial epigastric vein (milk vein) to be representative of the mammary glands' total venous drainage. In addition, the milk vein is readily accessible and relatively easy catheterized, limiting invasive procedures.

For the calculation of melamine uptake by the mammary gland by A-V differences, the Fick principle was employed (tissue uptake = A-V difference x mammary blood flow). Mammary blood flow (MBF) was determined by using Phenylalanine (Phe) and Tyrosine (Tyr) concentrations in the arterial and venous blood and in the milk. Phe and Tyr represent indicator compounds as they are stoichiometrically transferred from blood to milk so that mammary tissue uptake can be equated with milk output which enables calculation of MBF (Cant *et al.,* 1993). For the amino acid concentration (Phe and Tyr) output in milk protein, a 3.5% allowance was incorporated to account for blood-borne protein contributions to milk protein (Cant *et al.,* 1993; Lapierre *et al.*, 2009). Therefore, MBF (L/h) was calculated as follows:

$$ MBF = \frac{[\,(Milk\ Protein\ Phe + Tyr\,)\ x\ 0.965] + Free\ Milk\ Phe + Tyr}{Phe + Tyr\ \ A\text{-}V\ differences} $$

The output values for Phe and Tyr in milk protein were calculated from the casein and whey yield of the milk produced over the 9 h collection period. The Phe and Tyr contents of casein N and whey N were obtained from Mepham (1987). Therefore, the Phe content of casein N and whey N were 334 and 224 mg/g, respectively. The Tyr content of casein N and whey N were 371 and 214 mg/g, respectively.

98

6.2.4 Amino Acid analysis

For each cow, all hourly plasma samples collected during the 9 h period, were proportionally composited per cow and per location (arterial or venous) and analysed in triplicate. All plasma samples were prepared for amino acid analysis as described by Armenta *et al*. (2010). Samples were analysed for amino acid contents by mass spectrometry. Milk samples were not analysed for amino acids, since the widely accepted values of Mepham (1987) were used in the calculations.

6.2.5 Melamine analysis

For melamine analysis, an adapted method from Shai *et al*. (2008) was used. The melamine concentration of all milk and plasma samples was analyzed by liquid chromatography tandem mass spectrometry (LC/MSMS) using a Water API Quattro Micro triple quadruple mass spectrometer coupled to a Waters 2690 HPLC (Waters Corp., Milford, MA).

Determining milk melamine concentrations required solid phase extraction (SPE) by using cation-exchange solid-phase extraction cartridges (Phenomenex Strata SCX; 55 μm, 70 A, 500 mg / 3 mL, supplied by Separations, Randburg, South Africa). The milk samples were diluted on a 1:1 basis with 0.2 M perchloric acid, vortexed for 1 min and subsequently centrifuged at 4 500 x g for 5 min. The SPE cartridges were then conditioned with 6 mL methanol, followed by 6 mL distilled water. A volume of 3 mL from the resultant milk supernatant was subsequently loaded onto the SPE cartridges together with 100 μL of a 0.5 mg/L stable isotope-labeled MEL ($^{13}C_3H_6^{15}N_3$) internal standard solution (Cambridge Isotope Laboratories Inc., Andover, MA). The cartridges were then washed with 6 mL 0.1 *N* HCl followed by 6 mL methanol and allowed to aspirate under vacuum for 1 min. The melamine was eluted with 6 mL ammonium hydroxide: methanol: dichloromethane (1:5:5) and collected into clean glass tubes. The extracts were dried under a gentle stream of nitrogen, resuspended with 1 mL 50% acetonitrile and transferred to individual vials to be loaded onto the LC/MSMS spectrophotometer.

For plasma melamine analysis, 0.75 mL of acetonitrile and 0.1 mL of the internal standard was added to 0.75 mL of the plasma sample. The samples were centrifuged at 4500 x g for 10 min. The supernatant was transferred to individual vials and loaded onto the LC/MSMS without undergoing SPE. The limit of detection for this method was 0.001 mg/kg for both the milk and plasma samples.

6.2.6 Statistical Analysis

Because only one treatment was applied (10 g of melamine/cow daily), only standard errors were determined to indicate the amount of variation.

6.3 Results and Discussion

Results of the mammary plasma flow and melamine partitioning parameters are presented in Table 6.1. The mean plasma flow through the mammary gland was 402 L/h. With the mean plasma melamine concentration of just over 5 mg/kg, the amount of melamine transported to the mammary gland was 2055 mg/h. The melamine flux (as calculated from A-V difference) was positive, indicating net absorption of melamine into the mammary gland. Excretion of melamine into the milk, as calculated from milk yield and milk melamine concentration, was 5.63 mg/h. Theoretically, the net melamine flux and melamine excretion via milk could be expected to be identical. In practice, identical values can hardly be expected due to the fact that melamine was analysed in different matrices (plasma and milk) and also because of the role that amino acid analysis plays in the calculation of plasma flow. For the calculation of melamine absorption efficiency by the mammary gland, it was decided to use the milk melamine excretion value and not the net melamine flux value because of the sensitivity of the latter value to the various calculation parameters.

Table 6.1 Mammary plasma flow and melamine partitioning parameters in lactating Holstein cows that ingested 10 g of melamine per day

Parameter	Value ± SE
Mammary blood flow (L/h)	402 ± 43
Melamine transported to mammary gland (mg/h)	2055 ± 403
A-V melamine flux (mg/h)[1]	2.65 ± 2.2
Melamine excretion via milk (mg/h)	5.63 ± 0.7
Absorption efficiency of melamine by mammary gland (%)[2]	0.29 ± 0.03
Excretion efficiency of melamine to milk (%)[3]	1.47 ± 0.08

[1]Based on arterio-venous differences

[2]Mammary absorption efficiency (%) = Amount of melamine excreted via milk / Amount of melamine transported to the mammary gland x 100.

[3]Milk melamine excretion efficiency expressed as percentage of melamine intake

The absorption efficiency of melamine by the mammary gland was calculated by expressing the amount of melamine excreted from the mammary gland as a percentage of the plasma melamine transported to the gland. The efficiency of the melamine absorption by the mammary gland was low, indicating that only 0.29 % of the melamine transported to the mammary gland via blood was absorbed. No literature is available to support this result. The biochemical pathway for melamine absorption into the mammary gland is not known, but it is possible that melamine diffuses into and out of the mammary gland, equilibrating with melamine in the blood, as is the case with urea (Broderick & Clayton, 1997). In dairy cows, blood urea nitrogen (BUN) and milk urea nitrogen (MUN) is highly correlated, with BUN concentrations being slightly higher than that of MUN (Roseler *et al*., 1993). In the current study, the mean plasma melamine concentration was 5.2 mg/kg, while that of milk melamine was 3.9 mg/kg.

Melamine excretion efficiency to milk in this trial for cows consuming 10 g of melamine/d was 1.47 %. This is in agreement with the excretion efficiency values (*viz.* 1.5 – 2.1 %) reported in a previous study (Chapter 5) when cows consumed 10 g of melamine/d. In the study of Cruywagen *et al*. (2009), the melamine excretion efficiency in milk was reported as 2.1%. The fact that the excretion efficiency of melamine to milk from this and a previous study (Chapter 5) and other studies (Cruywagen *et al*., 2009; Battaglia *et al*., 2010) are within range of each other, confirm that the excretion of melamine via milk is probably not influenced by the dietary melamine dosage as different levels of melamine was fed in these reported studies.

Shen *et al*. (2010) speculated that high producing cows are more efficient in excreting melamine via milk than low producing cows and attributed this to differences in milk yield. Factors affecting milk yield include nutrition, genetics, body condition and age of the cow (Bryant *et al*., 2007). Of these factors, genetics is probably the key influential factor determining milk yield as Knight (2000) reported the amount of mammary tissue (i.e. milk secreting mammary cells) to be directly proportional to milk yields in cows of low or high genetic merit. Therefore it can be speculated that cows with a high genetic merit for milk production would have increased number of milk-secreting mammary cells which may result in more efficient melamine excretion via milk compared to cows with a low genetic merit. Whether genetic merit (high vs. low producing cows) with regards to mammary gland tissue

influences the efficiency of melamine absorption by the mammary gland is unclear and warrants further investigation.

6.4 Conclusion

This study indicated that the consumption of melamine in lactating dairy cows results in a net absorption of melamine by the mammary gland. The efficiency with which the mammary gland absorbs melamine is, however, low and less than 0.5% of the melamine that flows through the udder is absorbed. The low affinity for melamine absorption results in less than 2% of ingested melamine being excreted via milk. Further studies would be required to determine the biochemical pathway of melamine absorption by the mammary gland.

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CHAPTER 7

Melamine transfer from milk to milk products, including cheese, whey, yoghurt and kefir

Abstract

A study was conducted to determine the effects of different fermentation processes that occur during the manufacturing of cheese, yoghurt and kefir may have on their melamine content when melamine tainted milk was used. The study also aimed to determine if melamine is degraded in cheese during the curing process. An amount of 67.7 mg of melamine was added to 10 kg milk. Thus, the melamine concentration was 6.77 mg/kg. For the cheese, 1 kg of melamine tainted milk was used. Calcium chloride (0.2g) and cheese culture (60 mg) was added to the 32° C milk. After 30 min, 0.2 mL rennet was added and allowed to coagulate for 45 min before the curd was cut and stirred for 1 h. The curd was then strained and transferred to a mould for 18 h. The cheese surface was treated with sodium chloride (20g/kg curd), covered with cling wrap and cured for 2 wk at 6° C. Whey and cheese samples (before and after curing) were collected for melamine analysis. Yoghurt was made with 500 mL melamine containing milk to which 60 mg of yoghurt culture was added and set for 6 h at 45° C, followed by 18 h incubation at 37° C. Kefir was made with 500 mL melamine containing milk to which 60 mg of kefir grains were added and incubated for 18 h at 30° C. All milk products were made in five repetitions on the same day. All cheese (fresh and cured), whey, yoghurt and kefir samples were analysed for melamine by LC/MSMS. The melamine contents of the yoghurt and kefir were 6.76 and 6.78 mg/kg, respectively, indicating that the melamine concentration was not influenced by the fermentation processes during yoghurt and kefir production. The melamine contents of the whey and fresh cheese were 7.89 and 2.68 mg/kg, respectively. The percentages of milk melamine partitioned to whey and cheese were 97.4 and 6.5 %, respectively. The 2 wk curing period had no effect on the melamine concentration of the fresh (2.68 mg/kg) or cured (2.90 mg/kg) cheeses. It was concluded that milk fermented products do not influence the initial milk melamine concentration. The study also confirmed milk melamine to be predominantly partitioned to the whey fraction in cheese making and that melamine is not degraded in cheese during a 2-wk curing period.

7.1 Introduction

Milk and milk products form an integral part in human nutrition owing to its high protein, mineral (e.g. calcium and magnesium) and vitamin (e.g. vitamins A, D and E) content. According to Agricultural Statistics South Africa (2011), the South African population consumed 1.77 million tonnes fresh milk (including yoghurt, pasteurized- and UHT milk) and 38 000 tonnes of cheese (including Gouda, Cheddar and semi-hard cheese) between March 2004 to February 2005. Milk products, such as yoghurt and kefir have proved to have additional health benefits due to the probiotic actions on human gut health (Golowczyc *et al*., 2008; Lee & Lucey, 2010). During yoghurt production, the cultures responsible for fermentation are a mixture of bacterial species, including *Streptococcus* and *Lactobacillus* species, which ferment the milk lactose to lactic acid (Lee & Lucey, 2010). For kefir production, the milk is fermented by kefir grains. Kefir grains are a mixture of different microorganisms, including lactic acid and acetic acid bacteria, as well as different yeast species (Magalhães *et al*., 2011). The kefir grains ferment the milk lactose to produce sour milk (attributed to lactic acid) with a low alcohol content (Garrote *et al*., 2001). Whey is a by-product of cheese production with a high lactose content. In the past, whey has been disposed of and was regarded as an environmental pollutant due to its high organic load (Londero *et al*., 2012). However, in recent years, new technologies have been developed that uses whey as raw material for the production of foods and food additives (Panesar *et al*., 2007).

By now the 2008 Chinese milk scandal is well known. The melamine adulterated milk products had a severe impact on society due to the health related complications associated with its consumption. It is unfortunate that the majority of the people affected by the melamine adulterated products were babies and small children that were most susceptible to melamine exposure and suffered from kidney-related illness. The Chinese milk scandal involved the external addition of melamine to diluted milk in an attempt to restore the milk's apparent protein content (Bradsher, 2008). This was not the case in South Africa when reports were made of melamine contaminated milk due to the high cost of melamine.

Cruywagen *et al*. (2009) hypothesized that melamine may be excreted into cows' milk following the consumption of melamine adulterated feeds as melamine was detected in Chinese maize gluten 60 (Cruywagen & Reyers, 2009) used in animal feeds. Cruywagen *et al*. (2009) confirmed that a pathway exists for the transmission of melamine from feed to

milk. Since Cruywagen *et al.*'s (2009) initial report, other researchers (Battaglia *et al.*, 2010; Shen *et al.*, 2010; Sun *et al.*, 2011; Zheng *et al.*, 2011) also investigated melamine excretion into cows' milk following the consumption of different melamine dosages. In the study of Battaglia *et al.* (2010), the melamine contaminated milk was processed into a semi-soft cheese cured for 2 weeks. From their results it was concluded that approximately 85% of the milk melamine was transferred to the whey fraction, with only 1.9% partitioned to the cheese. Battaglia *et al.* (2010) reported an inexplicable melamine loss of approximately 13% which could not be accounted for. They speculated that some of the lost melamine could have been the result of melamine degradation during the 2 week curing period, as the cheese was not sampled for melamine analysis prior to the curing period. However, there is no literature available to support their hypothesis.

The current study was conducted to determine the effects of fermentation processes that occur when making cheese, yoghurt and kefir on their melamine content if they were made from melamine tainted milk. This study also aimed to investigate the hypothesis made by Battaglia *et al.* (2010) that melamine in cheese might be degraded to some extent during the curing process.

7.2 Materials and Methods

7.2.1 Experimental procedure

In a previous study (discussed in Chapter 5) a daily intake of 10 g of melamine by lactating dairy cows resulted in a mean milk melamine concentration of 6.77 mg/kg. It was thus decided to make cheese, yoghurt and kefir from milk with the same melamine concentration. A 10 L batch of milk with a melamine content of 6.77 mg/kg was used. Of that, 5 L was used to make five semi-soft Gouda-like cheeses (thus 1 L/cheese), 2.5 L was used to make 5 x 500 mL yoghurt and 2.5 L was used to make 5 x 500 mL kefir. To prepare the melamine tainted milk, a total amount of 67.7 mg melamine was accurately weighed and stirred into 100 ml of warm water. This was then added to 9.9 kg of whole milk and stirred for approximately 10 min to ensure even distribution of melamine throughout the milk.

For the cheese, amounts of 1 kg of the melamine containing milk were transferred to 2 L glass beakers and placed in a water bath with the temperature set at 32° C. Calcium chloride was added to the milk (0.2 g/kg milk) to enhance curd formation. After the milk had reached 32° C, 60 mg of a mesophillic cheese culture was added to each of the beakers and the milk

stirred for even distribution. After 30 min, 0.2 mL of rennet was added into each beaker and stirred into the milk for a few seconds. The curds were allowed to set for 45 min before they were cut into small cubes. The curd cubes were gently stirred every 10 min for 1 h. The curds were then separated from the whey be straining them through a 0.5 mm mesh sieve. The whey was collected in buckets to allow samples to be taken for melamine analysis and stored at -20° C pending analysis. The curds were then transferred to moulds with 1 mm holes in the bottom to allow excess whey to drain. Weights were placed on top of the moulds to facilitate whey drainage and for the cheeses to take on the form of the moulds. The cheeses were allowed to drain off the remaining whey for 18 h at room temperature before being removed from the moulds and weighed. Samples of the cheeses were taken and stored at -20° C pending analysis. The surfaces of the remaining portions of cheese were treated with sodium chloride (20 g/kg curd), wrapped with cling wrap and conditioned for 2 wk at 6° C. Following the conditioning period, the cured cheeses were stored at -20° C. All cheese samples were analysed for protein, fat, water and melamine content.

For the yoghurt, 5 x 500 mL amounts of melamine containing milk were transferred to 1 L glass beakers. Sixty milligrams of yoghurt culture was added to each beaker and gently stirred to distribute the culture evenly throughout the milk. The beakers of cultured milk were then stored for 6 h at 45° C and subsequently at 37° C for another 18 hours. All yoghurt samples were analysed for melamine the following day.

For the kefir, 5 x 500 mL amounts of melamine containing milk were transferred to 1 L glass beakers. Sixty milligrams of kefir grains were added to each beaker and gently stirred to distribute the grains evenly throughout the milk. The beakers of cultured milk were then stored for 24 h at 30° C. All kefir samples were analysed for melamine the following day.

All cheese samples were dried at 55° C in a ventilated oven for 48 h prior to chemical and melamine analyses. All chemical analyses of the individual cheeses were performed in duplicate as described by AOAC (2002) for crude protein (CP; method 990.03) and crude fat (method 954.02).

7.2.2 Sample preparation for melamine analysis

An adapted method from Shai *et al*. (2008) was used for melamine analyses. Cation-exchange solid-phase extraction cartridges (Phenomenex Strata SCX; 55 μm, 70 A, 500 mg / 3 mL, supplied by Separations, Randburg, South Africa) were used for solid phase extraction

of all samples. The cheese, whey, yoghurt and kefir samples collected for melamine analysis required different preparation methods prior to solid phase extraction (SPE). The different preparation methods required for the respective samples were as follows:

For the cheese samples, 1 g was extracted with 50% acetonitrile, 0.1% formic acid (10 mL) under sonication for 2 h in an ultrasonic bath (Branson 2210, Connecticut, USA). A volume of 3 mL of the extract was loaded onto the SPE cartridges.

The whey, yoghurt and kefir samples were diluted on a 1:1 basis with 0.2 M perchloric acid, vortexed for 1 min and subsequently centrifuged at 4 500 x g for 5 min. A volume of 3 mL from the resultant supernatant was loaded onto the SPE cartridges.

7.2.3 Melamine extraction

The SPE cartridges were conditioned with 6 mL methanol, followed by 6 mL distilled water. The sample extracts (3 mL) were loaded onto the SPE cartridges together with 100 μL of a 0.5 mg/L stable isotope-labeled melamine ($^{13}C_3H_6^{15}N_3$) internal standard solution (Cambridge Isotope Laboratories Inc., Andover, MA). The addition of the internal standard enabled corrections to be made should incomplete extractions occurred. The cartridges were then washed with 6 mL 0.1 *N* HCl followed by 6 mL methanol and allowed to aspirate under vacuum for 1 min. The melamine was eluted with 6 mL ammonium hydroxide: methanol: dichloromethane (1:5:5) and collected into clean glass tubes. The resulting extracts were dried under a gentle stream of nitrogen, resuspended with 1 mL 50% acetonitrile and transferred to individual vials to be loaded onto the liquid-chromatograph triple quadruple mass spectrophotometer.

7.2.4 Liquid Chromatography Tandem Mass Spectrometry (LC/MSMS)

The melamine concentration of all samples were analyzed by LC/MSMS on a Water API Quattro Micro triple quadruple mass spectrometer coupled to a Waters 2690 HPLC (Waters Corp., Milford, MA). For this method, the limit of detection was 0.005 mg/kg for the cheese samples and 0.001 mg/kg for the whey, yoghurt and kefir samples.

7.2.5 Statistical analysis

Since the purpose of the trial was to examine the partitioning of melamine to milk products and to determine the effect of ageing of cheese on its melamine content, only one treatment

was applied to the batch of milk that was used. Treatment was the addition of melamine to milk at a level of 6.77 mg/kg. For results pertaining to melamine concentration in the final products and the partitioning of melamine to whey and cheese, standard errors were determined. In each case, n = 5. In examining the effect of ageing of cheese on melamine content, curing time (0 and 14 d) was regarded as treatments. A one-way ANOVA was applied to the data with the aid of Statistica 10 (2011) and significance was declared at $P = 0.05$.

7.3 Results and Discussions

Table 7.1 presents the results of the dairy products' melamine content, melamine partitioning from milk to whey and cheese and the composition of the cheeses. The initial melamine content of the milk from which the milk products were made was 6.77 mg/kg. The results show that the melamine concentrations of the yoghurt and kefir were 6.76 and 6.78 mg/kg, respectively. It was decided to make yoghurt and kefir owing to the different fermentation processes required to produce the products. The results showed that the different fermentation processes used in yoghurt and kefir production had no effect on their melamine content and that melamine was not degraded during the short fermentation periods.

The melamine contents of the whey and fresh cheeses were 7.89 and 2.68 mg/kg, respectively. The lower melamine concentration of the cheeses indicated that melamine from milk is predominantly partitioned to whey during cheese making. This was confirmed after calculating melamine partitioning to the two products which were 97.4 and 6.5 % for whey and cheese, respectively. Battaglia *et al*. (2010) also reported the milk melamine to be predominantly partitioned to the whey fraction. They reported melamine partitioning from milk to whey and cheese as 85 and 1.9 %, respectively.

Table 7.1 Melamine content of dairy products, melamine partitioning from milk to whey and cheese, and cheese composition from milk containing 6.77 mg/kg of melamine.

Item	Value[1]
Milk melamine, mg/kg[2]	6.77
Whey melamine, mg/kg	7.89 ± 0.33
Cheese melamine, mg/kg (as is)[3]	2.68 ± 0.05
Cheese melamine, mg/kg (DM)[3]	6.95 ± 0.23
Yoghurt melamine, mg/kg	6.76 ± 0.19
Kefir melamine, mg/kg	6.78 ± 0.41
Partitioning of melamine to whey (%)	97.4 ± 3.80
Partitioning of melamine to cheese (%)[3]	6.5 ± 0.21
Cheese composition:	
Water, g/kg	613.5 ± 5.4
Crude fat, g/kg	342.5 ± 6.6
Crude protein, g/kg	398.4 ± 2.1

[1]Values are Means ± SE.

[2]As a fixed amount of melamine was added to the milk from which the products were made, no variates applied.

[3]Cheese refers to the fresh, uncured cheese.

Cruywagen *et al*. (2009) showed how the milk melamine concentration would increase by 8-fold when converted to milk powder. This important remark should also be kept in mind when whey is processed. The value of whey has increased in recent years as new technologies developed that use whey as raw materials for producing foods and food additives (Panesar *et al*., 2007). Of these whey products, the use of whey powder as a protein supplement for health conscious people, especially for body building, are becoming more popular. The water content of whey is 935 g/kg, but can vary depending on the process used to obtain the whey (de Wit, 2001). This study showed that approximately 97.4 % of the milk melamine concentration was transferred to whey. Therefore, when whey is processed to whey powder, the melamine concentration would increase approximately 9-fold. The

following example serves to illustrate the implications of converting melamine containing whey to whey powder:

If milk with a melamine concentration of 1 mg/kg, which is accepted as "safe" for infant consumption, were used to make Gouda cheese (as was done in this trial), 97.4 % of the melamine would be transferred to the whey. The whey would therefore have a melamine concentration of 0.97 mg/kg. Processing the whey to whey powder would result in the melamine concentration of the whey powder to be 8.77 mg/kg, which is 3.5 times higher than the accepted melamine level recommended for an adult.

Table 7.2 presents the results of the melamine concentrations of the fresh cheese and 2 wk-cured cheese. The melamine concentrations of the fresh and cured cheeses did not differ, suggesting that melamine was not degraded in the cheese during the 2 wk curing period. Battaglia *et al*. (2010) postulated that some melamine might have degraded during the 2 wk curing period as 13% of the milk melamine could not be accounted for after cheese-making. The average melamine partitioning values to cheese and whey reported by Battaglia *et al*. (2010), *viz*. 84.7 % of milk melamine to whey and 1.9% to cheese, were lower than the values obtained in the current study. Both studies, however, showed that a very small portion of milk melamine is partitioned to cheese, with the bulk being transferred to whey. The cheese composition (water, fat and protein) was within the normal range.

Table 7.2 The effect of 2 weeks curing on cheese melamine concentration.

Item	Fresh cheese	Cured cheese	SEM	*P*
Melamine, mg/kg (as is)	2.68	2.90	0.088	0.118
Melamine, mg/kg (DM)	6.95	6.86	0.235	0.796

7.4 Conclusion

Results from the current study indicated that the different fermentation processes involved during the manufacturing of yoghurt and kefir from melamine tainted milk did not decrease the melamine concentration. During the process of cheese-making, the milk melamine was predominantly partitioned to the whey fraction, with little melamine transferred to cheese. It was also concluded that melamine was not degraded in cheese during a 2-wk curing period. However, it is unclear if melamine degradation would occur when cheese is cured for longer

periods, a question that warrants further investigation. It was again confirmed that caution should be taken by manufacturers when processing milk contaminated with melamine to dried milk products.

7.5 References

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CHAPTER 8

General Conclusion

Since 2006, various countries around the world came to learn about the apparently "open secret" of melamine adulteration practices that occurred in China. Melamine adulteration of pet foods, infant formula, protein feedstuffs and other food products made international headlines following reports of kidney-related illnesses and deaths as a result of melamine consumption by animals and humans. The illegal practice of adulteration of food and feed ingredients is a concerning reality and will remain a problem for many years to come. However, in the case of the melamine adulteration incidences, the complications were not only a financial, but also a detrimental health risk to society. Various studies confirmed the transmission of dietary melamine to animal products used for human consumption. This information is extremely valuable for assessing the health risks posed to humans if such melamine contaminated animal products were to be consumed. However, there are still many unknown factors pertaining to melamine pharmacokinetics in animals and partitioning to their products used for human consumption.

A number of trials were done in this study to investigate certain *in vitro* and *in vivo* aspects of melamine in dairy cows that have not been studied before, or where limited information was available. The current study showed that *in vitro* melamine degradability in rumen liquor was low, with only 13.6% of the melamine degraded after 48 h. Melamine degradation in rumen liquor had no effect on ruminal ammonia and volatile fatty acid concentrations after 48 h.

Melamine, ingested at different levels by cows, did not affect their milk production or milk composition.   Melamine was rapidly detected in the milk (8 h after initial ingestion) and increased to reach a peak concentration on the third day.   Melamine excretion efficiencies were not influenced by the different melamine treatments and ranged from 1.5 to 2.1 %. This study also showed that the consumption of 10 g of melamine per day by dairy cows resulted in a net absorption of melamine by the mammary gland with a low (0.29%) efficiency of absorption. The mean apparent digestibility of melamine was 78 %, with urine (54 %) and faeces (22 %) representing the primary routes for eliminating melamine from the body. The melamine concentration of all milk, urine and faecal samples increased as the dietary melamine concentration increased. A withdrawal period of 8 d was required for all milk, faecal and urine samples to reach undetectable levels of melamine.

117

This study also showed that the fermentation processes involved in the making of yoghurt and kefir from melamine tainted milk did not influence their melamine content. During the process of making cheese with melamine tainted milk, melamine was predominantly partitioned to whey (97.4 %) with little transmission to cheese (6.5 %). Curing the cheese for 2 wk did not result in melamine degradation.

It was concluded that ruminants will not benefit from melamine consumption as melamine degradation to ammonia is low. Melamine transmission from feed to milk and milk products were confirmed and it was found that the processing of milk to milk products do not affect the melamine content of such products. Melamine absorption by the mammary gland was confirmed with the efficiency of absorption, however, being low. Elimination of ingested melamine in cows were predominantly via urine and faeces, with little melamine excreted via milk.

As consumers, we depend on food manufacturers to provide healthy and safe foods, which we blindly accept as being safe. With various international health regulatory authorities enforcing laws and regulations with regards to food safety, it will not ensure manufacturers' compliance, as was clearly evident in the melamine adulteration incidences. The health authorities' recommended safety limits for melamine in foods and feeds, should not be interpreted as foods and feeds containing melamine concentrations below the limit to be safe for human consumption. To ensure future food safety regarding melamine, or any adulterant that may cause harm, governments and food manufacturers, local and international, should comply with set laws and regulations and improve quality control measures.

The saying goes that history repeats itself, which poses the question: What will the next "melamine" be?