THE TRANSMISSION OF MELAMINE FROM FEED TO POULTRY PRODUCTS

by

Petronella Elizabeth Basson

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Agriculture (Animal Sciences)

at

Stellenbosch University

Department of Animal Sciences
Faculty of AgriSciences

Supervisor: Prof CW Cruywagen
Co-Supervisor: Dr E Pieterse

Date: *March* 2011

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: February 2011

ABSTRACT

The transmission of melamine from feed to poultry products

Two studies were done to determine the distribution rate and efficiency of dietary melamine (MEL) to poultry meat and eggs. The possibility of MEL distribution into meat and eggs after feeding cyromazine (CYR) was also investigated. Five separate diets were formulated for broiler and layer chickens containing graded levels of MEL. In the broiler trial (Experiment 1), a number of 480 day-old Cobb 500 broiler chickens were divided into five treatment groups. Diets contained 0 (CON), 50 (MEL50), 100 (MEL100), 500 (MEL500) mg/kg MEL or 4 mg/kg CYR (CYR4). The duration of the trial was 36 days and breast muscle, kidney and liver samples were harvested on Days 11, 13, 15, 18, 22, 29 and 36 after the start of the feeding and analyzed for MEL. For the duration of the trial, all experimental diets were presented *ad libitum* and feed intake, weight gain, mortality rate, feed conversion ratio (FCR), protein efficiency rate of birds and the European production efficiency rate were determined. In the layer trial (Experiment 2), 120 Hyline Silver hens (24 weeks of age) were randomly divided into five treatment groups. The treatment diets were the same as for Experiment 1. The duration of the trial was 20 days and layers received the treatment diets for the first 10 days after which the control diet was provided for another 10 days. Feed intake, mortality rate, egg production and egg weights were recorded daily, while live weight was recorded at the beginning and end of the trial. Dietary MEL levels of up to 500 mg/kg did not have any detrimental effect on production parameters for broilers. In the layer trial, feed intake and egg weights were negatively affected by the MEL500 treatment. Dietary MEL was absorbed by broilers and layers and rapidly distributed to the kidneys, livers, muscles and eggs. As the dietary MEL concentration increased from 50 and 100 mg/kg to 500 mg/kg, an increase ($P < 0.01$) was observed in muscle tissue and egg MEL residue concentrations. Melamine concentration for broilers peaked at 22 days of age and decreased until day of slaughter. The kidneys contained the highest MEL residue levels, compared to other organ tissues, such as muscle and liver. In layer hens, a MEL distribution plateau in eggs was reached between Days 1 and 4 and decreased from Day 7 to 10. The distribution of MEL in eggs was higher to albumin than to the yolk. Upon withdrawal, MEL concentration in these tissues declined to undetectable levels within seven days. No MEL could be detected in meat or eggs when birds received the CYR4 treatment. The distribution efficiency (DE_f) of MEL to meat and eggs did not appear to be dose dependant. For meat, the DE_f varied between 1.2 and 2.7% and for eggs it varied between 0.7 and 0.8%.

UITTREKSEL

Die transmissie van melamien vanaf voer na pluimvee produkte

Twee studies is uitgevoer om die verspreidings tempo en effektiwiteit van melamien (MEL) na hoender vleis en -eiers te bepaal. Die moontlikheid van MEL verspreiding na vleis en eiers deur die voeding van cyromazien (CYR) is ook ondersoek. Vyf aparte diëte is geformuleer vir braaikuikens en lê-henne wat verskillende MEL insluitings vlakke bevat het. Vir die braaikuiken proef (Eksperiment 1), is 480 dag oud Cobb 500 braaikuikens ingedeel in vyf behandelings groepe. Diëte het 0 (CON), 50 (MEL50), 100 (MEL100), 500 (MEL500) mg/kg MEL en 4 mg/kg CYR (CYR4) bevat. Die tydsduur van hierdie proef was 36 dae en bors-, spier-, nier- en lewer monsters is ingesamel op Dae 11, 13, 15, 18, 22, 29 en 36 wat geëvalueer is vir MEL. Tydens die verloop van die proef is alle eksperimentele diëte *ad libitum* gevoer en voerinname, massa toename, mortaliteit, voeromsettings verhouding, proteïen effektiwiteits tempo asook die Europese produksie effektiwiteits tempo is bepaal. Vir die lê-hen proef (Eksperiment 2), is 120 Hyline Silver henne (24 weke oud) ewekansig verdeel in vyf behandelings groepe. Die behandelings diëte het dieselfde MEL en CYR konsentrasies bevat as Eksperiment 1. Die tydsduur van hierdie proef was altesaam 20 dae waarvan henne behandelings diëte vir die eerste 10 dae ontvang het, waarna henne vir die daarop volgende 10 dae ‘n kontrole dieët wat 0 mg/kg MEL bevat gevoer is. Voerinname, mortaliteit, eier produksie en eier gewig is daagliks opgeteken, terwyl lewende massa aan die begin en einde van die proef gemeet is. Melamien dieët vlakke tot 500 mg/kg het geen negatiewe effek op braaikuiken produksie parameters gehad nie. Vir lê-henne, is slegs voerinname en eier gewig negatief beïnvloed vir MEL500. Melamien is na inname geabsorbeer deur braaikuikens en lê-henne en het vinnig versprei na die niere, lewer, spiere en eiers. Soos die MEL vlakke van die behandelings diëte toegeneem het van 50 en 100 mg/kg na 500 mg/kg, het ‘n beduidende ($P < 0.01$) toename in spierweefsel en eier MEL residu konsentrasies voorgekom. Melamien konsentrasies vir braaikuikens het gepiek op 22 dae en geleidelik afgeneem tot op dag 36. In lê-henne het ‘n MEL verspreidings platu in eiers plaasgevind tussen Dag 1 en 4 en geleidelik afgeneem tydens Dag 7 en 10. Die niere het die hoogste MEL residu vlakke bevat in vergelyking met die lewer- en spierweefsels. Die verspreiding van MEL in eiers was hoër na die albumien as na die dooier. Tydens onttrekking het die MEL konsentrasie vlakke in hierdie weefsels in so ‘n mate afgeneem dat dit onbespeurbaar was binne sewe dae. Geen MEL kon in vleis- en eiermonsters gemeet word vir CYR4 nie. Die verspreidings doeltreffendheid (DE_f) van MEL na vleis en eiers was nie dosis afhanklik nie. Vir vleis het die DE_f gevarieër tussen 1.2 en 2.7% en vir eiers tussen 0.7 en 0.8%.

ACKNOWLEDGEMENTS

On the completion of this work, I would like to express my sincerest appreciation and gratitude to the following people, without whom this work would have been impossible:

The National Research Foundation (NRF) for financing part of the study;

The Post Graduate Supporting Fund for funding part of my studies;

Prof. C.W. Cruywagen, my supervisor, for his dedication, guidance and endless patience during the study;

Dr. E. Pieterse, co-supervisor for her support during my studies;

Mr. S. Barends (Mariendahl Experimental Farm), my fellow students and friends for their assistance in obtaining data for this study;

Ms. B. Ellis and the technical staff of the Department of Animal Sciences, Stellenbosch University, for their assistance during this work;

Ms. M.A. Stander and Ms. M. Adonis of the Central Analytical Facility, Stellenbosch University, for determining the melamine concentrations in the meat and egg samples and their technical support;

Prof. M. Kidd (Stellenbosch University) for his assistance with the statistical analysis;

My mother for her endless love and support and allowing me the opportunity to study;

To God, my heavenly father and centre of my life, who enabled me to accomplish this study by giving me strength and teaching me patience.

Notes

The language and style used in this thesis are in accordance with the requirements of the *South African Journal of Animal Science*. This thesis 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

Melamine ($C_3H_6N_6$) is an industrial chemical utilised in a variety of industrial applications such as plastics, coatings, leather, paints, laminates, flame-retarding agent and table-tops among others (China Chemical Reporter, 2006). In the feed industry the crude protein value for feedstuffs can be obtained by determining the nitrogen (N) value of the ingredient multiplied by 6.25 (Van der Merwe *et al.*, 1991). The N level of pure melamine (MEL) is 667g/kg (Merck, 2001) which is equal to a crude protein level of 4168.75 g/kg. This value is substantially higher than that of pure urea (2917 g/kg) which is renowned for its exceptional high N content. Melamine production increased considerably during the early 2000's in China and in 2006 MEL production was reported to be in excessive surplus (China Chemical Reporter, 2006), with an estimated worldwide production of 1.2 million tons for 2007 (Bizzari *et al.*, 2008). Generally, MEL and its derivates are not present in human foods or animal feeds; however, due to the situation described above, the inclusion of MEL as a fraudulent protein in feed seemed to be an alluring substitute for the more costly protein supplements and at the same time would result in decreasing the severe surplus.

The first serious concerns regarding the inclusion of MEL in food became evident in the 2006/2007 pet food scandal. More than 1000 dogs and cats died in various countries due to renal failure caused by accumulated kidney stones (WHO, 2008). This resulted in 1154 recalls of assorted pet foods, while the main contaminant was promptly identified as MEL tainted corn gluten 60. The second appalling MEL scandal emerged in 2008 after it became known that six babies died and 294 000 became ill after drinking MEL-tainted infant formula (WHO, 2008). More than 50 000 of the infants were reported to be hospitalized with dysfunctional urinary symptoms caused by renal tube blockages and kidney stones (WHO, 2008). More than 22 Chinese companies have sold MEL contaminated milk. These practices were restricted by the Chinese government in an attempt to reduce MEL adulteration.

Various different food sources and certain environmental practices expose humans to MEL and its analogues. The occurrence of MEL exposure may possibly also be due to the use of triazine based

pesticides (for instance cyromazine) used to control fly populations in cattle and poultry manure (Sancho *et al.*, 2005). When cyromazine (CYR) is dealkylized, one of the degradated products is MEL (Sancho *et al.*, 2005), motivating the relevance of investigating the detection of MEL metabolized from CYR in poultry meat and eggs which is reported in this thesis. Cyromazine is also used on field crops or sprayed onto fruits and vegetables. Patakioutas *et al.* (2007) detected MEL residues of less than 1 mg/kg on the edible parts of crops (tomato, lettuce and celery), after applying CYR. Melamine is also used as N source for slow release urea-based fertilizers and may be a major MEL contaminant in food and water (Hilts *et al.*, 2008). The work by Mosdell *et al.* (1987) supports the latter by showing that plants had taken up N in significant amounts, following the application of a MEL-urea combination. Further reports (OECD, 1998) stated that MEL was detected in fish and river water in Japan; however, the data were considered insufficient to determine MEL pollution in general in Japanese river fish populations and drinking-water. Other examples of human exposure to MEL exist, for instance migration of MEL through food packaging material into food (Lu *et al.*, 2009).

In the poultry feed industry of South Africa, MEL was present as an adulterant in maize gluten 60, a raw material commonly used in the industry. Chinese soya products imported to Europe and France during 2008 also tested positive for MEL and were included in poultry rations. Contaminated fishmeal was also exported to Canada (CBC News, 2007) and tainted wheat gluten and rice protein to the US (Weise *et al.*, 2007). All the identified contaminated feeds have been recalled. Cyromazine, the active ingredient of Larvadex® which is included in laying-hen diets to control flies, may be metabolized to MEL in the animal, resulting in the possibility of MEL being detected in the meat or eggs. A residual maximum of 50 ng/g for CYR in the edible parts of eggs and poultry meat are allowed as stipulated by the US Code of Federal Regulations (1987); however, residual levels in consumer products and CYR inclusion levels in animal diets differ between countries depending on their application as a veterinary drug.

There were approximately 94.7 million tons of poultry meat (U.S. Department of Agriculture, 2009) and 60.678 million tons of eggs produced worldwide during 2009 (FAOSTAT, 2010). From these facts it is evident that massive economical losses could be experienced when tainted poultry products have to be destroyed due to products containing MEL residues that exceed legal baseline levels. The general position of animal nutritionists and veterinarians are to not rely on so-called safe inclusion

levels, but that feed should rather contain undetectable levels of MEL. It has been accepted, however, that the presence of MEL in the environment is inevitable and may contribute to a background level of contamination in feeds (WHO, 2008). Therefore, restrictions on maximum MEL inclusion levels in various feedstuffs have been set as an alternative resort. The World Health Organisation (WHO) has set the tolerable daily intake (TDI) levels for humans at 0.2 mg/kg body weight (Setiogi, 2008), and the maximum allowable MEL concentration of human foods at 2.5 mg/kg, while the maximum allowable level for infant formula was set at 1 mg/kg. For animal feeds, the industry has also accepted 2.5 mg/kg MEL as the maximum allowable MEL level. However, products containing excessive MEL residues could still surface in countries with no restriction policies. To justify the current study, the latter could be prevented by implementing scientific recommendations based on experimental findings revealing the maximum inclusion of MEL in feed that would result in MEL contaminated animal products that are within the 2.5 mg/kg restriction limit. From a different perspective, if accidental feeding of MEL contaminated feed occurred, a minimum withdrawal time can be applied as guideline before animal products are considered as safe to harvest for human consumption. However, little scientific data exists that indicate what this minimum withdrawal period should be.

Therefore the current study had the following aims:

1. To determine the distribution rate of three different levels of MEL in poultry meat and eggs.
2. To determine the withdrawal rate of MEL from meat and eggs after terminating MEL exposure in the diet.
3. To determine the efficiency rate at which MEL could be distributed into meat and eggs.
4. To determine the possibility of MEL distribution to meat and eggs after feeding CYR.

References

Bizzari, S. and Yokose, K., 2008. Melamine. IN *Chemical economics handbook.* Menlo Park. CA. SRI Consulting Inc. Available: http://www.sriconsulting.com/CEH/Public/Reports/673.3000/. Accessed: July, 13, 2010.

CBC News, 2007. Tainted fish feed was used in Canada: B.C. minster. Available: http://www.cbc.ca/canada/story/2007/05/09/fish-feed.html. Accessed: July, 14, 2010.

China Chemical Reporter, 2006. Melamine capacity is serious surplus. Available: http://goliath. ecnext.com/coms2/gi_0199-5152838/Melamine-capacity-is-in-serious-surplus.html#abstract. Accessed July, 13, 2010.

FAOSTAT, 2010. Food and agriculture organization of the United Nations. Available: http://faostat.fao.org/site/569/DesktopDefault.aspx?PageID=569#ancor. Accessed: July, 14, 2010.

Hilts, C. and Pelletier, L., 2008. Background paper on occurrence of melamine in foods and feed. Prepared for the WHO expert meeting 1-4 Dec 2008. Available: http://www.who.int/foodsafety/fs_management/Melamine_3.pdf. Accessed: July, 14, 2010.

Lu, J., Xiao, J., Yang, D.J., Wang, Z.T., Jiang, D.G., Fang, C.R. and Yang, J., 2009. Study on migration of melamine from food packaging materials on markets. Bio. Environ Sci. 22: 104-108.

Merck, 2001. Entry 5834. IN The Merck Index 13th ed. Merck Research Laboratories. Whitehouse Station. New York. P. 1038.

Mosdell, D.K., Daniel, W.H. and Freeborg, R.P., 1987. Melamine and ammeline as nitrogen sources for turf grasses. Fert. Res. 11: 79-86.

OECD, 1998. Screening information dataset for melamine. CAS No. #108-78-1. Paris. Organisation for economic Co-op and development. Available: http://www.chem.unep.ch/irptc/sids/OECDSIDS/108781.pdf. Accessed: July, 14, 2010.

Patakioutas, G., Savvas, D., Matakoulis, C., Sakellarides, T. And Albanis, T., 2007. Application and fate of cyromazine in a closed-cycle hydroponic cultivation of bean (*Phaseolus vugaris L*). J. Agric. Fd. Chem. 55: 9928-9935.

Sancho, J.V., Ibáñez, M., Grimalt, S., Pozo, Ó.J. and Hernández, F., 2005. Residue determination of cyromazine and its metabolite melamine in chard samples by ion-pair liquid chromatography coupled to electrospray tandem mass spectrometry. Anal. Chem. Acta. 530: 237-243.

Setiogi, S., 2008. Experts set tolerable level for melamine intake. World Health Organization. Geneva. Available: http://www.who.int/mediacentre/news/releases/2008/pr48/en/print.html.

U.S.,Code of Federal Regulations, 1987. Tolerance for residues of new drugs in food. Part 556.67. Title 21. US.

U.S. Department of Agriculture, 2009. International egg and poultry review. ISSN : 1522-5100. Available:http://search.ams.usda.gov/MNDMS/2009/06/PY20090623WIntlPoultryandEgg.pdf. Accessed: July, 15, 2010.

Van der Merwe, F.J. and Smith, W.A., 1991. Die chemise samestelling van voere. IN *Dierevoeding.* Ed. F.J. Van der Merwe & W.A. Smith. Pinelands. Anim Sci Pty Ltd. P. 5.

Weise, E. and Schmit, J., 2007. FDA: Feed no human threat. USA Today, May, 2, 2007. ISSN: 0734 7356. Available: http://search.ebscohost.com.ez.sun.ac.za/login.aspx?direct=true&db=aph&AN= J0E122276131707&site=ehost-live. Accessed: July, 22, 2010.

World Health Organization, 2008. Expert meeting to review toxicological aspects of melamine and cyanuric acid. Available: http://www.who.int/foodsafety/fs_management/Exec_Summary_melamine.pdf. Accessed: July, 24, 2010.

CHAPTER 2

Literature review

2.1. Introduction

The international agricultural sector is under constant pressure to satisfy the world's infinite demand for dietary resources. Firstly, arable agricultural land is decreasing annually due to the increase in residential areas to support the fast growing world population. New agricultural land can be cultivated, but at a considerable cost and feasible expansions are limited. Secondly, rapidly increasing input costs during the past few years have added increased pressure on raw material prices. In South Africa, the price of fertilizer has more than doubled during the past three years (FSSA-MVSA, 2010), straining marginal profits severely, forcing higher purchase prices for the feed sector, not even mentioning the impact of the fluctuating oil prices (Digital Look, 2010) or the effect of the past recession. These difficulties leave the feed industry with no other choice than to explore cheaper and more effective alternatives for high dietary protein products.

It is a well-known fact in the agricultural industry that feedstuffs containing high protein levels are classified as one of the most expensive raw material groups (McDonald *et al.*, 2002). One of these categorical ingredients is fish meal which is considered to be an excellent protein source for monogastric feeding; however, the protein contents and its digestibility may vary in quality due to different processing practises and the type of fish products that have been used as fish meal ingredients (McDonald *et al.*, 2002). This varying quality is directly linked to the price and it is therefore tricky to formulate least cost rations containing fish meal. Urea contains 466g/kg N (McDonald *et al.*, 2002); however, it is mainly used in ruminant rations and was therefore not applicable for the current research study. Therefore, when comparing melamine (MEL) to fishmeal, it is evident that fishmeal is per weight more expensive to add as a high N feedstuff to the diet than MEL, concluding the more appealing use for MEL.

Melamine is not an effective source of non-protein N for ruminants due to the slow and incomplete hydrolysis of MEL (Newton & Utley, 1978). Furthermore, in a rat study by Mast *et al.* (1983) it has been shown that more than 90% of ingested MEL was excreted within 24 hours, which further accentuated the insignificant nutritional contribution of MEL. Despite the theoretical desirable qualities that MEL displays, the discovery of alternative feed additives should rather be explored on other biological fields, such as entomology. Melamine has not been permitted to be used as a direct feed additive; however, traces may be detected in feed due to crops fertilized with MEL related products, or as a breakdown product from cyromazine (CYR) that has been included as a veterinary drug. Gossner *et al.* (2009) reported that the levels of MEL in contaminated animal feeds, ranged from 3.3 mg/kg to 21 000 mg/kg during 2008 and 2009. These practices are currently considered as unethical and should not be tolerated by authorities while the public should be properly informed of the current situation and possible ill symptoms.

The following review and in depth discussion will reveal the chemical nature of MEL and CYR and their application. Also, the relationship between MEL and cyanuric acid will be mentioned since these two substances are linked to each other throughout the literature (Brown, 2007; Caldas, 2007; Hilts *et al.*, 2008; Reimschuessel *et al.*, 2008; WHO, 2008; Hau *et al.*, 2009; Sebastian, 2009; Kobayashi *et al.*, 2010 and WHO, 2010). The metabolism and toxicological effects of MEL and CYR will be discussed, followed by the distribution characteristics of these two substances in poultry meat and eggs, as well as in products of other species.

2.2. Chemistry and application of melamine

Melamine (1,3,5-triazine-2,4,6-triamine) with chemical formula $C_3H_6N_6$, is an organic compound and a trimer of cyanamide. Melamine is commonly found as a white solid and has a molar mass of 126.12 g/mL, a small molecule with high nitrogen content (Osborne *et al.*, 2008). It is used in the manufacture and processing of a variety products ranging from plastics, coatings, leather, paints, laminates, flame-retarding agent and table-tops and in recent years it has been added to certain food products. Some bacteria have the ability to metabolize MEL; however, the same is apparently not true for mammals and therefore it holds no nutritional value for animals (Osborne *et al.*, 2008).

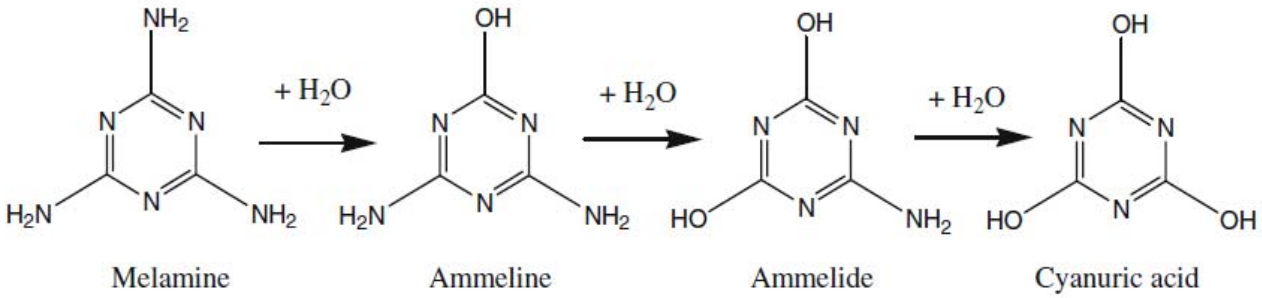


Figure 1 The chemical process in the formation of MEL related compounds (Tyan *et al.*, 2009).

Cook *et al.* (1981) stated that MEL is biodegradable as illustrated in Figure 1 and Jutzi *et al.* (1982) confirmed the derivative pathway of MEL with four different methods. Melamine is commercially synthesized from urea (Hau *et al.*, 2009) and the by-products ammeline, ammelide and cyanuric acid originate during hydrolysis (Figure 1). The melting point of MEL is 345°C, which explain why some plastic ware melts after high heat exposure (Tyan *et al.*, 2009). Melamine is not very soluble in water (3 240 mg/L at 20°C) and according to Chapman *et al.* (1943) less than 1% MEL is required to saturate an aqueous solution after being diluted in water at 20°C. This feature as discussed later in this study is the single most important aspect attributing to renal complications.

2.3. Chemistry and application of cyromazine

Cyromazine (*N*-Cyclopropyl-1,3,5-triazine-2,4,6-triamine) is a triazine with a crystalline appearance. The molecular formula of this substance is $C_6H_{10}N_6$ and it has a molar mass of 116.19 g/mol (Merck, 2001). Cyromazine is a cyclopropyl that is a MEL derivate (Figure 2) and alternative names include Larvadex®, Trigard®, Vetrazin® and CGA-72662 (Merck, 2001).

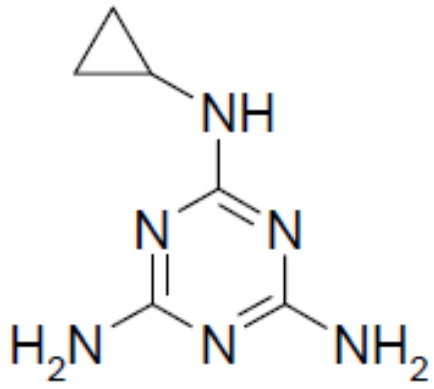


Figure 2 The chemical structure of Cyromazine (Merck, 2001).

The distinctive utility of CYR is to alter the formation of the chitin layer of fly larvae that would hatch in poultry manure. It has been postulated that CYR interferes with the hormone ecdysone, which regulates ecdysis and cuticle deposition in insects (Pfeifer, 1993). Typically it is added to layer feed (marketed under the Larvadex label), when flies start to negatively affect bird comfort over time. The ingested CYR will then pass the digestive system into the faeces, exposing larvae to the product without any additional labour. Conventional methods involved a technique where manure was sprayed with a similar product by hand, which is considered to be more labour intensive than the addition of CYR as feed additive. Reasons for concern in the application of CYR as feed additive arose after it was hypothesized that the ingested CYR could be metabolized by the birds to MEL, which might be deposited in eggs and body tissue intended for human consumption. This hypothesis will be tested in the current study and compared to findings of other authors.

2.4. The relationship between melamine and cyanuric acid

The application of the aims as stipulated under the general introduction is to provide the poultry industry with information concerning the distribution rate of MEL and CYR in meat and eggs. Even though the toxic effects of cyanuric acid (CYAN) will not be evaluated in the current study, it is still inevitable to mention the interaction of this compound with MEL and how it is related to nephrotoxicity in combination with MEL. Cyanuric acid (s-triazine-2,4,6-triol) is a white solid that is

structurally related to MEL (Figure 1) and is generally used as a water stabilizer to minimize hypochlorous acid decomposition in swimming pools after exposure to sunlight (Downes *et al.*, 1984). According to Puschner *et al.* (2007), intake of MEL and CYAN alone seems to be less of a problem than ingesting a combination of the two chemicals (MEL-cyanurate) which has repeatedly been reported to induce kidney disorders. Similar to MEL, CYAN has a low solubility in water (2 000 mg/L at 25°C) and the simultaneous addition of the two substances result in an isomeric interaction, creating an insoluble (2 mg/L) crystalline structure (Plate 1) in the distal nephrons (Hau *et al.*, 2009). The motive for the inclusion of CYAN in animal feed remains uncertain. It is speculated that CYAN has been added as an adulterant similar to MEL in feed due to its high nitrogen content. Another theory is that bacterial MEL metabolism could have occurred in the toxic pet food, creating CYAN as a by-product (Osborne *et al.*, 2008).

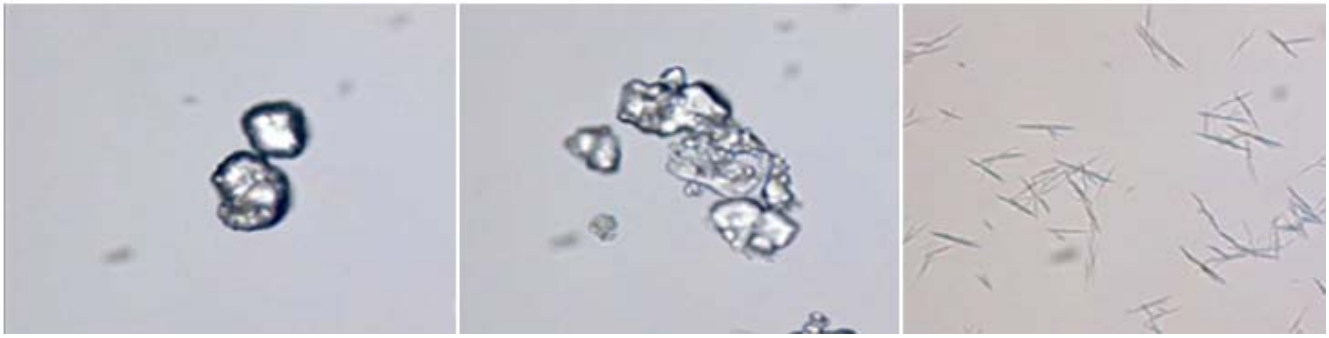


Plate 1 MEL crystals on the left, CYAN crystals in the middle and the pin like crystals of MEL-cyanurate on the right (Kobayashi *et al.*, 2010).

2.5. The metabolism and distribution of melamine and cyanuric acid

Very little is presently known about the exact mechanism of MEL metabolism and nephrotoxicity; however, it appears as if MEL is quickly metabolized by the body. Allen *et al.* (1982) reported that humans excreted 98% of ingested CYAN within 24 hours in urine. Approximately 90% of all ingested MEL in a rat study was excreted in the urine within 24 hours (Mast *et al.*, 1983). Melamine primarily causes severe damage to the kidneys which ultimately may result in death if the animal receives excess

levels of MEL. These levels vary between different species and will be discussed in more detail later. It is said that the mechanism of kidney failure observed in animals due to MEL ingestion can be compared to uric acid nephropathy that occur in humans because both induce mechanical obstructions which is similar to humans enduring gout (Reimshuessel *et al.*, 2008). Therefore, renal failure due to MEL toxicity is the result of intrarenal crystal obstruction causing increased renal pressure which reduces renal blood flow (Reimshuessel *et al.*, 2008).

Approximately 22-25% of the blood flow generated by cardiac output in a human body enters the two kidneys (Sebastian, 2009). Toxins are removed by the kidney via glomerular filtration, tubular excretion through passive diffusion and active tubular secretion (Sebastian, 2009). Sebastian (2009) explains that the most common site for toxin related injury is in the proximal convoluted tubule. Reasons for this is that some toxins are activated by enzymes (cytochrome P450 and cysteine conjugate β-lyase) localized in the epithelial cells of the proximal tubular. Also the epithelium cells of the proximal tubules are more loosely arranged than the distal tubular cells, making toxin intrusion effortless. The proximal convoluted tubular epithelial cells seem to be most susceptible to injuries related to decreased blood flow due to obstruction of the tubule. When feeding high levels of MEL or CYAN alone, stone formation occurs mainly in the proximal tubules (Kobayashi *et al.*, 2010). Interestingly, histopathological evaluations showed complications in the distal tubule as well as in the collection ducts caused by MEL-cyanurate crystals (Dobson *et al.*, 2008; Kobayashi *et al.* 2010). A theory which is currently globally accepted has been developed to explain these findings. It states that the glomerular filtrate has a pH of 7.4 after which it decreases to approximately 6.8 in the proximal tubules due to H^+ secretion and further H^+ secretion in the collecting ducts via luminal proton ATPases can decrease the urinary fluid pH to under 6 (Barac-Nieto, 2004). Bhalla *et al.* (2009) reported that MEL-cyanurate stone formation occurs at pH 5.8 which may be the reason for stone formation in the distal tubules and therefore low urinary pH may be encouraging crystal formation and ultimately causing acute renal failure.

Infants and very small children seem to be more severely affected by MEL than adults due to several reasons including that infants have to take in more feed according to their body weight compared to adults; more frequent feedings; differences in intestinal absorption and immature kidney functioning. However, the main reason why infants are more susceptible is that their blood serum and urine contain

considerably higher levels of uric acid compared to adults and older children, increasing the possibility for uric acid-MEL precipitation in the renal tubules. Also it is said that infants have lower solutes (citrate and phosphate) to compete against MEL for binding sites, making susceptibility even more severe (WHO, 2010). In theory these findings might also apply to the avian species due to their higher uric acid blood serum levels compared to mammals. Layer chickens have lower uric acid levels than non-layer birds and uric acid serum levels decrease with age in most species while low protein diets are also thought to initiate low uric acid levels (Bowes *et al.*, 1989).

The lowest daily intake of MEL that would initiate bladder stone development was reported by Melnick *et al.* (1984) to be 750 mg/kg for rodents after ingesting MEL for 13 weeks. Renal stones collected from patients who ingested MEL alone are primarily composed of MEL and uric acid. These two substances are bonded at a 1:1 ratio in rats and for humans more or less 1:2 due to the higher uric acid levels in humans (Reimshuessel *et al.*, 2008). To identify the origin of the crystals, they are stained with Oil Red O. Crystals composed of calcium phosphate and calcium oxalate did not stain with this reagent; however, MEL-containing crystals will stain with Oil Red O and therefore they are different from the typical calcium stones (Sebastian, 2009). Unlike calcium oxalate crystals, MEL-cyanurate crystals cause renal failure due to stone obstruction in the renal tubules (Kobayashi *et al.*, 2010). Calcium oxalate crystals on the other hand will begin to stick to tubular epithelial cells and will grow by expressing stone matrix proteins (Tawada *et al.*, 1999).

Dobson *et al.* (2008) reported increased kidney weights in rats receiving MEL-cyanurate (3.61 g) compared to the control group (2.05 g). A triazine mixture containing MEL (400 mg/kg), ammeline, ammelide and cyanuric acid (40 mg/kg each) also increased kidney weight (2.62 g). Furthermore urea N levels in blood were substantially higher than normal blood and creatinine clearance was also lower with low urine pH recordings for both MEL mixtures. These findings are all an indication of renal dysfunction. Since it has been established that the binding sites of the MEL-cyanurate molecules are dependent on hydrogen bonding of corresponding amino and hydroxyl group of triazines, Dobson *et al.* (2008) explains that ammelide might substitute CYAN in the lattice if an excess of MEL is present which might explain the increased kidney weight of the mixed triazine group. The author predicted that ammeline could substitute MEL if CYAN is in surplus.

An operating model was subsequently developed to illustrate the toxicity of MEL-cyanurate: MEL-cyanurate was confirmed to remain stable in gluten feed and during the manufacturing of processed pet food after examining samples by infrared spectroscopy. After ingestion the complex was dissociated in the gastric lumen due to the low pH content. From this stage on Dobson *et al.* (2008) suspected that due to the different pKa values of MEL (5) and CYAN (6.9) the acid is absorbed by the stomach and the base in the small intestine or else the two compounds would re-establish the crystal structure. Both compounds were found in the renal filtrate allowed the opportunity to restructure an insoluble complex creating a physical blockage. The author could not explain why the substances did not restructure until reaching the tubules; however, two theories were proposed: The two substances only recombined after reaching a certain concentration point, which might occur as the compounds progress through the osmotic gradient of the kidney. Alternatively, it might be that MEL and CYAN interfere with the metabolism of uric acid and possibly precipitate in the tubules. The presence of ammelide, ammeline and CYAN increases circulating uric acid by inhibiting hepatic uric acid oxidase (Dobson *et al.*, 2008). They also postulated that the compounds compete for a renal acid transporter; however, this remains to be proved. No cellular damage to the tubular epithelia cells were noted before the crystals were formed.

It is worth mentioning the fascinating work conducted by Buur *et al.* (2008) on developing a physiologically based pharmacokinetic model to predict the residual MEL levels in kidney and liver tissues of rats and pigs. The pig model was further applied to estimate withdrawal times after they were accidentally fed with contaminated feed. A similar study has been reported by Baynes *et al.* (2010) in goats. Due to different retention times, glomerular filtration rate and other pharmacokinetical differences between ruminants and monogastric animals, further research is required to refine the accuracy of these predictions to complete these studies.

2.6. The metabolism and distribution of cyromazine

As mentioned, CYR is a derivate of MEL and therefore it was thought that CYR is metabolized similarly to MEL in the body. Similar to MEL, CYR is also rapidly excreted by rats, mainly in urine within 24 hours and 95% of all ingested CYR is excreted within 72 hours (Simoneaux *et al.*, 1978).

Only approximately 3% of the excreted CYR was presented in the faeces. Pfeifer (1993) showed that CYR could not be hydrolyzed *in vitro* at pH 5, 7 or 9 at different temperatures (50°C and 70°C) unless under strong acidic conditions. The main pathway of degradation appeared to be by means of photolysis; however, other researchers' reported contradictory results. When CYR was diluted in an aqueous solution, photo-degradation to MEL only occurred after the addition of acetone. Therefore, the conclusion could be made that very little CYR could be metabolized to MEL in the body and that any complications reported due to dietary CYR inclusions are supposedly due to CYR and not to MEL metabolized from CYR.

Cyromazine is almost completely absorbed after ingestion and distributed to all organs and body tissues (Caldas, 2007). Brake *et al.* (1984) showed evidence of liver abnormalities in broiler breeders fed 1000 mg/kg, thereby implying damage to the metabolic system. Wilson *et al.* (1983) recovered fatty livers from their treatment necropsy tests; however, it could not be associated with any of the treatments. Renal injury has been suggested by Brake *et al.* (1989) after wet litter were recorded for turkeys receiving CYR levels of 1000 mg/kg and more. It was proposed that the kidneys eliminate CYR and related substances from the body and have a metabolizable threshold after which the renal system will not be able to manage toxin elimination sufficiently. From the study by Capps (1990) on rodents, the primary emission route is through renal excretion where 52-78% of the ingested CYR were excreted within 24 hours, which is contradictory to the 97% reported by Caldas (2007). The reports of the two researchers, however, agreed that the excreted components were 72% unchanged CYR, 9% hydroxyl-CYR, 7% MEL and 2% 1-methyl-CYR. According to Keiding (1999) 2-14% of ingested CYR was metabolized to MEL. In the study reported by Caldas (2007), laying hens received a diet containing 5 mg/kg CYR for seven days. Almost all of the residuals were recovered (99.8%) of which the excreta contained the highest residual level (99.1%) followed by egg albumin (0.4%), yolk (0.2%), tissue (0.1%) and expired CO_2 (0.1%). The birds were euthanized and average CYR distribution levels measured for body tissue were the highest for liver (0.31%) whilst renal tissue and breast muscle were 0.019% and 0.010% respectively. Supposing that 7% of the deposited CYR is metabolized to MEL (Caldas, 2007), the calculation could be based on the findings above that 0.00133 mg/kg MEL was potentially deposited in the renal tissue and 0.0007 mg/kg in breast muscle tissue for the study of Caldas (2007) due to CYR ingestion.

2.7. Toxicity and production impact of melamine and melamine-cyanurate

Melamine and MEL-cyanurate most commonly cause renal complications and the degree and prolonged presence of toxicity can be classified as acute or chronic. One of the most evident MEL toxicity studies was performed by Clark *et al.* (1966) on sheep long before the Chinese scandal surfaced. Sheep fed MEL contaminated feed containing more than 25 g a day all died of acute toxicity. Loss of appetite and crystals in the kidney tubules accompanied by nephrosis and haemorrhagic cystitis were observed. High blood urea nitrogen levels were recorded *pre mortem*. Broiler mortalities showed enlarged pale kidneys after being exposed to 1500-3000 mg/kg MEL and opaque bile was observed in the gallbladders (Ledoux *et al.*, 2009). Crystals and lesions were found in the renal tubules and were similar to those found in cats, identifying MEL as the major cause for renal failure (Ledoux *et al.*, 2009).

After chronic exposure to MEL, increased urinary output were noted in dogs with aggressive stone formation (Tusing, 1953). The latter appears to be the only concurrent sign for chronic toxicity. Melnick *et al.* (1984) observed that chronic kidney failure occurred after feeding rats 150 mg/kg MEL for 90 days. Urinary bladder stones were formed and later this probably led to bladder tumours. Bhalla *et al*. (2009) reported inflammation and necrosis of the distal tubular cells and larger stones in the tubules and papilla after chronic exposure for only MEL. Hau *et al.* (2009) stated that the toxicity of MEL alone is low and that the 50% lethal dose (LD_{50}) is 3161 mg/kg body weight for rats. However the researcher also mentioned that high levels of MEL will cause the formation of urinary stones and acute renal failure. No adverse effect on renal function was found after feeding rats 240 mg/kg MEL for 14 days (Kobayashi *et al.*, 2010). Additional reports mentioned that MEL affected the dogs' digestive tract after intake while nausea, vomiting and diarrhoea were some of the observed symptoms (Jeong *et al.*, 2006). Stone formation was more severe in smaller animals and animals with low fluid intake and higher MEL intake. Males were more affected than females due to their increased risk for stone formation (Hau *et al.*, 2009). No explanation for increased stone formation in males was provided by the author; however, it was reported that male serum has higher uric acid levels than females and as explain previously MEL interacts with uric acid and precipitates in the renal tubules

(Bowes, *et al.,* 1989). Therefore animals with higher uric acid levels tend to be more susceptible for MEL-uric acid stone formation.

Some bladder epithelial changes (due to bladder calculi) were reported by Hammond *et al.* (1986) after feeding levels of up to 2200 mg/kg sodium cyanurate to rats with no other occurring defects. Low toxicity of CYAN (LD_{50} of 7700 mg/kg body weight for rats) was reported by the World Health Organization (2008). Cyanuric acid crystals developed in the renal tubules and were reported to cause renal tissue damage such as tubular dilatation necrosis and fibrosis. No adverse effects were observed in rats at levels below 150 mg/kg.

Studies on the simultaneous ingestion of MEL (0.2%) and CYAN (0.2%) in cats resulted in acute renal failure within 48 hours after feeding (Osborne *et al.*, 2008). Severe renal interstitial oedema and haemorrhage were reported and crystals were found in the distal nephrons accompanied by tubular epithelial necrosis. One of the first adverse effects to occur in the nephron during acute renal failure is a drop in the glomerular filtration rate, causing excess urea and non-protein nitrogen substances to accumulate in the blood, termed azotaemia. Azotaemia and hyperphosphatemia were also reported in acute cases by Sebastian (2009). The insoluble crystals had a green colour and were composed of 70% CYAN and 30% MEL. In the study of Kobayashi *et al.* (2010) an administered MEL-cyanurate dose of only 12 mg/kg/day resulted in crystal formation in the renal tubules. Chronic symptoms on the other hand showed inflammation and fibrosis with larger crystals in the distal nephrons. Similar observations were made in fish and pigs (Reimschuessel *et al.*, 2008).

Increased fluid intake seemed to reduce the incidence of stone formation and higher salt inclusion levels in the diet stimulated water intake which also helps to reduce the formation of stones (Ogasawara *et al.*, 1995). The preliminary treatment procedures according to the Chinese Ministry of Health (2008) are to increase daily water intake and to add urinary alkalizes as a food supplement. Pain management and surgical treatment could be required if all else fails.

Many adverse production parameters were observed when feeding MEL diets of 1500 mg/kg and more to broilers over a 14 day period (Ledoux *et al.*, 2009). Feed intake decreased after exceeding 1000 mg/kg and the average daily gain decreased for all groups receiving more than 1500 mg/kg which

became more prominent as the dose increased, especially beyond 2000 mg/kg. Interestingly enough the FCR also decreased significantly ($P< 0.001$) for the 2500 and 3000 mg/kg groups compared to all the other treatments, which indicated that the decrease in weight gain was not affected as severely as the feed intake. The first mortalities were already recorded from day five and mortality rates of more than 12% were observed after 2000 mg/kg and increased up to 30% for the 3000 mg/kg group. Bai *et al.* (2010) provided MEL feed (0, 100, 250, 500, 1000 and 2000 mg/kg) to layers for 34 day and observed no difference between treatment levels regarding egg production, mortality and body weight. After feeding layer ducks different MEL inclusion diets (0, 1, 5, 25, 50 and 100 mg/kg), no differences in egg weight, egg production, feed intake or feed conversion was reported (Yuchang *et al.,* 2010); however, these results could be expected due to the low inclusion levels

2.8. Toxicity and production impact of cyromazine

After following a detailed search, very little relevant literature reported clinical CYR toxicity symptoms. The LD_{50} for rats after ingesting CYR was reported to be between 3387-5033 mg/kg (Bathe *et al.*, 1978; Sabol, 1987; Pheifer, 1993). The overwhelming adverse effect of CYR when chronically administering an oral dose of 1000 mg/kg and more was weight loss due to decreased feed intake and recovery was quickly established after withdrawal of CYR in rats (Bathe *et al.*, 1978; Goldenthal *et al.*, 1979; Sabol, 1987; Pheifer, 1993) and dogs receiving more than 3000 mg/kg (Jessup *et al.*, 1979). Decreased liver weights were reported for rats receiving more than 1000 mg/kg CYR (Goldenthal *et al.*, 1979) and some reproductive complications became evident in rats receiving the same dose (Blair *et al.*, 1981). Pheifer (1993) mentioned that CYR can be considered to have a low acute toxicity level. Even though prominent weight loss were reported for many species receiving more than 1000 mg/kg the NOAEL (no-observed-adverse-effect level) was established at 300 mg/kg.

From the available literature it appears as if broiler breeders are more sensitive to CYR than layers and turkeys (Brake *et al.*, 1984; Brake *et al.*, 1985; Brake *et al.*, 1989). In the study of Brake *et al.* (1983) layers at 27 weeks of age received 3000 mg/kg CYR, but it had to be reduced to 1000 mg/kg due to an elevated mortality rate. These findings were similar to the response observed by Brake *et al.* (1984) where initial doses of 3000 mg/kg fed to broiler breeders were reduced to 2000 mg/kg and further

reduction to 1000 mg/kg was implemented after prolonged high mortality. The authors unfortunately did not report a mortality rate or an indication of the LD_{50}. Levels above 1000 mg/kg reduced reproductive and progeny performance with high mortalities, body weight loss and poor feed conversion ratios (FCR). Decreased fertility, hatchability and egg production were also reported. Cyromazine was reported to be non-toxic to young layers when ingesting 10 000 mg/kg/day CYR for four weeks and 1000 mg/kg for 20 weeks (Brake *et al.*, 1985); however, severe weight loss was recorded for both dosage levels. According to the findings of Brake *et al.* (1989) administered levels of 2000 mg/kg CYR or less are non-toxic to turkeys; however, a marked decrease in feed intake and growth were observed in turkeys receiving levels between 500-2000 mg/kg CYR. Despite the occurrence of suppressed body weights and poor reproductive performance, these production traits were reversible after CYR was withdrawn from the diets of broiler breeders (Brake *et al.*, 1984), layers (Brake *et al.*, 1985) and turkeys (Brake *et al.*, 1989).

It is interesting to note that CYR inclusion of 300 mg/kg in layer diets resulted in increased egg production compared to 0 mg/kg and 30 mg/kg over a period of 32 weeks; however, a decline in egg weight was reported for week 32 where shell weight was significantly decreased compared to the 0 mg/kg treatment (Brake *et al.*, 1983). For turkeys however no significant difference in egg production ($P > 0.05$) were found after feeding different levels of up to 2000 mg/kg, emphasising the hardiness of turkeys compared to broiler breeders against CYR. Inclusions of 1000 mg/kg in layer and broiler breeder diets had adverse effects on egg production and egg weight, but broiler breeders revealed an excellent feed conversion ratio (FCR) compared to the 0 mg/kg group. The birds reduced their intake in an attempt to decrease CYR intake and Brake *et al.* (1985) concluded that a diet containing CYR reduced food wastage or possibly improved nutrient utilization and therefore the enhanced FCR. Decreased shell weights after chronic exposure to 1000 mg/kg CYR were measured; however, no significant ($P > 0.05$) fertility or hatchability difficulties were observed. Inclusion levels of 300 mg/kg or less had no deleterious effects on reproductive and progeny performance (Brake *et al.*, 1984; Brake *et al.*, 1985; Buhr *et al.*, 1983; Cecil *et al.*, 1981). Some residual effects were visible in the progeny and lower body weights were reported for the 1000 mg/kg treatments. The poorer performance for the 1000 mg/kg group could be explained by the decrease in egg weight; however, the 300 mg/kg treatment also had decreased egg weights but chick weight was not negatively affected compared to the 0 mg/kg and 30 mg/kg group with the highest egg weights. According to Brake *et al.* (1984),

metabolic damage is caused by CYR ingestion and therefore nutrient deposition could be retarded in the egg, explaining the decrease in chick body weight without inhibiting liveability.

Contradictory results were reported by Wilson *et al.* (1983) where layer breeder hens who received 1000 mg/kg CYR had the highest egg production rate compared to 0, 50, 100 and 500 mg/kg with no suppressed feed intake and zero mortality. Egg production was however reduced significantly ($P < 0.05$) at 2000 mg/kg which corresponds to similar research (Brake *et al.*, 1983 and Brake *et al.*, 1985). However, in a second experiment for the same study the treatment that received 1000 mg/kg CYR did not differ from the other treatments regarding egg production and was indeed the lowest of all the treatments with the highest mortality rate. Even though no toxic effects can be identified after ingesting CYR, the US EPA (1999) prescribes that the inclusion of CYR may not exceed 5 mg/kg in poultry feed and that a 72 hours withdrawal period must be allowed before slaughter.

2.9. Melamine distribution and withdrawal in animal products

2.9.1. Poultry meat and eggs

According to Thompson *et al.* (2008), MEL and its compounds are rapidly eliminated by the kidneys and do not accumulate in the body. A comparison between the findings of different researchers regarding MEL distribution and withdrawal rates in eggs is displayed in Table 1 in an attempt to obtain an objective idea of the current study field. The values reported by MAFF (2010) as stipulated in Table 1 revealed a plateau on day 12.

Table 1 Comparison between maximum melamine distribution (mg/kg) and melamine residuals in eggs after withdrawal from the feed.

		Inclusion level (mg/kg)									
[1]Day	Author	2	5	30	50	60	100	250	500	1000	2000
	Distribution										
	Bai *et al*. (2010)	-	-	-	-	-	1.6	3.0	6.7	11.7	28.7
	Chen *et al.* (2010)	-	0.3	-	1.0	-	1.9	-	-	-	-
	MAFF (2010)	-	-	0.7	-	1.6	-	-	-	-	-
	Yuchang *et al.* (2010)	0.1	-	-	-	-	2.4	-	-	-	-
	Withdrawal										
10	Bai *et al*. (2010)	-	-	-	-	-	ND	ND	ND	ND	ND
7	MAFF (2010)	-	-	ND	-	0.04	-	-	-	-	-

[1]Days after MEL exposure has been discontinued.

From Table 1 it is evident that there exists an upward trend for MEL distribution as the administered dose increases and that a negligible amount of MEL is present after a seven day withdrawal period. Bai *et al.* (2010) provided MEL feed (0, 100, 250, 500, 1000 and 2000 mg/kg) to layers for 34 days. In eggs MEL levels peaked at a range of 1.6-28.7 mg/kg at day four for all treatments and remained at a plateau for as long as the birds had access to the MEL feed. Similar results were reported by Chen *et al.* (2010) where distribution levels did not differ in eggs from day 1-15, confirming the statement by Thompson *et al.* (2008) that MEL accumulation did not occur. The distribution levels increased (Bai *et al.*, 2010) as the MEL content of the different feeds increased and a similar distribution trend were reported for all treatments.

After the withdrawal period was initiated on day 34, MEL levels rapidly decreased in eggs and no MEL was detected for treatments receiving 0, 100, 250 and 1000 mg/kg MEL already on day three, followed by the treatments receiving 500 and 2000 mg/kg MEL on day four (Bai *et al.*, 2010). Chen *et al.* (2010) established a linear equation ($Y = 0.08491 + 0.01473X$) to calculate the MEL distribution rate in eggs after receiving a certain level of contaminated feed. It states that if a feed containing more than 164 mg/kg MEL are fed to layers, it is predicted that the residual levels in eggs will exceed the maximum permitted level of 2.5 mg/kg (Setiogi, 2008). Note that 2.5 mg/kg is the maximum limit of any veterinary drug that may be traced as a residual in animal tissue or products (Zhu *et al.*, 2009).

Lu *et al.* (2009b) showed that there is a difference in the distribution and metabolism of MEL in eggs and other body tissues. Residues were first detected in egg albumin and the kidney, shortly followed by plasma, liver, drumstick and lastly the breast muscle and egg yolk. Melamine was rapidly metabolized and eliminated within the 14 day withdrawal period from the plasma, liver, kidney and muscle compared to the albumin and yolk. On day 21 no samples revealed MEL levels above 0.30 mg/kg. A few studies have been conducted to determine the rate of MEL distribution and withdrawal from body tissues (kidney, liver, muscle) and the available data has been combined in Table 2 and Table 3 to compare the reported results.

Table 2 Comparison between residue results for melamine distribution in poultry meat.

			Inclusion level (mg/kg)						
[1]Day	Species	Sample type and author	2	100	200	250	500	1000	2000
		Kidney							
42	Broiler	Lü *et al.* (2009)	-	1.7	3.2	-	4.1	9.2	-
34	Layers	Bai *et al*. (2010)	-	1.3	-	1.6	2.7	8.0	21.7
40	Broiler	Yuchang *et al.* (2010)	0.1	4.5	-	-	-	-	-
		Liver							
42	Broiler	Lü *et al.* (2009)	-	ND	ND	-	1.3	2.7	-
34	Layers	Bai *et al*. (2010)	-	0.5	-	0.5	1.5	2.8	6.9
40	Broiler	Yuchang *et al.* (2010)	-	-	-	-	-	-	-
		Muscle							
42	Broiler	Lü *et al.* (2009)	-	ND	ND	-	1.7	3.7	-
34	Layers	Bai *et al*. (2010)	-	0.4	-	0.8	1.6	3.7	9.3
40	Broiler	Yuchang *et al.* (2010)	0.1	1.7	-	-	-	-	-

[1]Day refer to animal age when sample were collected by each author.

Table 3 Comparisons regarding the withdrawal rate of melamine from poultry meat.

			Inclusion level (mg/kg)						
[1]Day	Species	Sample type and author	2	100	200	250	500	1000	2000
		Kidney							
7	Broiler	Lü *et al.* (2009)	-	ND	ND	-	ND	ND	-
10	Layers	Bai *et al*. (2010)	-	ND	-	ND	ND	ND	ND
		Liver							
7	Broiler	Lü *et al.* (2009)	-	ND	ND	-	ND	ND	-
10	Layers	Bai *et al*. (2010)	-	ND	-	ND	ND	0.06	0.40
		Muscle							
7	Broiler	Lü *et al.* (2009)	-	ND	ND	-	ND	ND	-
10	Layers	Bai *et al*. (2010)	-	ND	-	ND	ND	ND	ND

[1]Day refer to animal age when sample were collected by each author.

Lü *et al.* (2009) noted that MEL were detected on day 28 in breast samples above 200 mg/kg and liver samples above 100 mg/kg; however, on day 42 no MEL could be detected in the 200 mg/kg treatments and MEL levels were lower for the 500 and 1000 mg/kg group on day 42 compared to day 28 proving that MEL do not accumulate in the body and that some sort of mechanism might trigger more rapid elimination in the bird. No illness symptoms or production defects were observed for all treatment birds, proving that MEL levels up to 1000 mg/kg are tolerated by birds without affecting production. No MEL was detected for treatment groups receiving less than 100 mg/kg.

Various tissue samples were also analyzed for the study of Bai *et al.* (2010) on day 34 where renal tissue displayed the highest concentrations (1.3-21.7 mg/kg) and MEL was also detected in the liver, muscle and reproductive organs. Liver tissue had higher distribution rates compared to muscle for the low dose groups; however, more MEL was gradually deposited in muscle than the liver as the dose level increased. After a ten day withdrawal time no MEL was detected in the kidneys and muscle with low levels of 0.06 and 0.4 mg/kg in the liver for the treatments receiving 500 mg/kg and 2000 mg/kg MEL respectively. No MEL was detected for any tissue on withdrawal day 20. Similar findings were reported by Yu *et al.* (2009).

2.9.2. Other species and products

After feeding layer ducks different MEL inclusion diets (0, 1, 5, 25, 50 and 100 mg/kg), an increased trend in liver and kidney weights were observed as the MEL dosage increased (Yuchang *et al.*, 2010). Melamine levels peaked around day two for all treatments and the MEL concentration in eggs were higher for the groups that received 25 mg/kg and more compared to the 0-5 mg/kg groups. The half life time of MEL in eggs after withdrawal was found to be 18 hours. Depleted levels were detected four days after withdrawal for all inclusion levels. The 25, 50 and 100 mg/kg treatments reached the 0.05 mg/kg detection level after 3, 4 and 8 days respectively. For the study of Yuchang *et al.* (2010) higher MEL levels were found in poultry eggs compared to kidney and muscle tissue.

In a fish study by Andersen *et al.* (2008) catfish, trout, tilapia and salmon received 400 mg/kg body weight a day in three trials of which MEL or CYAN or MEL-cyanurate were fed for three days. Melamine levels of 0.04-0.12 mg/kg were detected in the trout and salmon control fish, due to contaminated feed that was fed to the fish six months prior to the initiation of this study. For trial one, fish only received MEL and residual levels were detected in the meat for catfish (210 mg/kg), tilapia (177 mg/kg), salmon (94 mg/kg) and trout (80 mg/kg). After a six day withdrawal period, tilapia had considerably lower residual levels (0.02 mg/kg) compared to catfish (81 mg/kg), salmon (58 mg/kg) and trout (34 mg/kg). It could be that tilapia has the ability to excrete MEL more efficient than the other fish species. Or it was likely due to the fact that tilapia emitted some of the MEL containing gel food which resulted in decreased ingestion; however, high levels were comparable to catfish. Trial two was similar to trial one except that MEL was substituted with CYAN in the diets fed. It was interesting to note that even though no MEL was detected in the catfish feed, MEL was found in the muscle tissue (0.006-0.012 mg/kg). Salmon also revealed small muscle concentration levels (0.019-0.083 mg/kg); however, their feed had been found to be contaminated with 6.7 mg MEL /kg. No MEL traces were found in trout and tilapia. In trial three all fish received the MEL-cyanurate contaminated diet for three days after which a 14 day withdrawal period followed. In all fish species MEL-cyanurate crystals were observed in the kidneys, however the level of crystal formation was considerably different from and between species. Melamine was also detected in muscle tissue of individuals from different species

and could be attributed to the level of crystallization where more crystals made less MEL available to be deposited in muscle.

It was reported that during 2007 contaminated pet food were used as a supplement in some swine feed and contained 30-120 mg/kg MEL (US FDA, 2007). Even though these levels were declared to cause no harm to humans, the public remained cautious of consuming pork. The half-life excretion rate of MEL was reported to be 2.7 hours for rats (Mast *et al.*, 1983) and in pigs 4.04 hours (Baynes *et al.*, 2008) with a renal clearance of more or less 27 mL/min for pigs, giving a renal clearance difference of about 1.5 between the two species. This is due to the greater renal clearance mechanism of rats which is about five times more efficient. The observed renal clearance findings of pigs as found by Baynes *et al.* (2008) suggests that the distribution of MEL mainly occurs in the extracellular fluid rather than being deposited or metabolized by organs, except for the kidney where MEL clearance is made. They also reported that the elimination tempo of MEL from pigs were 1.5 times longer than for rats due to different glomerular filtration rates. The bottom line is that 99% of the MEL occurring in pig blood is excreted within 28 hours which is expected to be below the safe level of 50 µL/mL and due to this quick excretion rate, distribution of MEL in the muscle and organ tissue (except for the kidneys) is expected to be low and should be considered to be safe for consumption.

In goat milk MEL could still be detected after 84 hours of 40 mg/kg ingestion (Baynes *et al.*, 2010) and a long plasma half-life (11.12 hours) were reported for small ruminants like goats. Although the half-life of MEL in milk was established to be a quick 9.44 hours, detectable levels were still above the 1 mg/kg safe level for three days. Therefore MEL elimination in milk could be considered to be of higher importance than tissue residues. In a Holstein cattle study conducted by Cruywagen *et al.* (2009) dairy cattle receiving MEL contaminated feed containing 1142 mg/kg for eight days revealed MEL levels of 10-15.7 mg/kg in milk for as long as exposure consisted. Excretion in milk represented 2% of the total dose; however, for the goat study by Baynes *et al.* (2010) only 0.3% of the oral dose was recovered in the goat milk, which could be likely due to the lower milk production relative to body weight of dairy goats compared to dairy cows. This statement has been verified by the higher percentage of ingested MEL in the milk of high producing goats compared to the low production goats. For both studies MEL could be detected in milk for 4-7 days after the last time of exposure. In another dairy study cows received feed containing 50 mg/kg and 100 mg/kg MEL respectively for 28 days

(MAFF, 2010). Milk MEL levels peaked at 0.9 mg/kg and 1.6 mg/kg for the low and high groups respectively within two days and rapidly decreased to below 0.01 mg/kg within seven days after withdrawal (MAFF, 2010). Tissue samples were also collected on day 28 for muscle (0.46-0.69 mg/kg), fat (0.25-0.63 mg/kg), liver (0.58-1.0 mg/kg) and kidneys (2.3-3.4 mg/kg).

After feeding lambs 2-100 mg/kg MEL, maximum levels were reported for muscle (0.435 mg/kg) and liver (0.469 mg/kg) samples on day 49 (Lv *et al.*, 2010). Kidney maximum levels (1067 mg/kg) were observed on day 53 which differs from other species. Treatment groups receiving 2 mg/kg had tissue distribution levels below the so called safe level of 0.05 mg/kg. When MEL intake was discontinued, detectable levels below 0.020 mg/kg were reported for all tissue samples within 4.5 days after withdrawal. Except for fish, MEL residues in tissue samples of cattle, swine, chicken, sheep and duck exposed to 100 mg/kg a day could not be detected after a four day withdrawal period. For fish, five days for muscle and 14 days for renal tissue were necessary for complete depletion. Yuchang *et al.* (2010) reported higher residual levels in chicken muscle (1.86 mg/kg) compared to pig (1.36 mg/kg), sheep (0.53 mg/kg) and cattle (0.47 mg/kg) after feeding 100 mg/kg MEL per day. Also eggs had more MEL residuals (2.366 mg/kg) compared to milk (0.487 mg/kg); however it was interesting to note that chicken, duck and layer muscle tissue samples had the fastest depletion rate of 24 hours compared to pigs (48 hours), cattle (65 hours) and sheep (98 hours).

2.10. Cyromazine distribution and withdrawal in animal products

2.10.1. Poultry meat and eggs

There has been some concern that CYR could be metabolized in poultry breeds to MEL which could be deposited in meat and eggs, since it has been reported that 10% of the ingested CYR will convert metabolically to MEL (USEPA, 2007). Chou *et al.* (2003) tested chicken, egg, beef, mutton and pork samples for any CYR and MEL residues. None of the samples revealed detectable MEL levels (> 0.02 mg/kg) and only one beef sample contained 0.04 mg/kg CYR.

Cyromazine were detected (100 mg/kg) in layer eggs after feeding 2.5 mg CYR /kg feed/day for five weeks (Miller & Corley, 1980) and no traces were found in liver or muscle tissue. It was concluded that there is a positive correlation between induced dose levels and residues in eggs. Cyromazine was also detected in the liver, muscle and faeces after an increase in inclusion level. In a study by Anderson *et al.* (1987), CYR (0.00044 mg/kg added to diet) traces were found in only three out of 32 eggs with detection levels of <0.01, 0.11 and 0.22 mg/kg respectively and no traces were found in the thigh and breast portions and the liver, gizzards and fat. These findings might not be considered accurate since tissue samples were harvested from only two birds; however, these results have the same trend as the previous mentioned trial of Miller and Corley (1980).

Cecil *et al.* (1981) determined the amount of CYR distribution in the liver, fat and muscle of layers after feeding a CYR derivate known by the industry as CGA-19255 at four different levels (2.5, 12.5, 25 and 125 mg/kg). CGA-19255 is metabolized in the body to CYR. Cyromazine were not traced in the first group and increasing levels were found in the liver and muscle as a result of increased administration levels. Cyromazine deposition in fat was only detected after 125 mg/kg ingestion of CGA-19255. No CYR were detected after only a week of withdrawal in all tissues for all treatment levels and the authors concluded that these inclusion levels did not suppress any production performance qualities. No MEL was detected in any egg or meat samples in the study of Boone *et al.* (1985) after feeding layers 0-5 mg/kg CYR. The CYR maximum levels were already reached on day three in eggs and day 14 in meat samples. Withdrawal times were rapid and within one and two days no CYR were detected in meat and eggs samples respectively.

Even though CYR is rapidly eliminated from the body as mentioned under section 2.6, undeniable evidence published by Brake *et al.* (1991) shows that contaminated faeces were passed several weeks after birds were withdrawn from a diet containing CYR. The higher the administered dose, the longer the residual toxicity duration persisted. Brake *et al.* (1991) reported that when feeding layers 25 mg/kg/day CYR for the first 20 weeks of age, residuals could be found in the droppings 13 weeks after CYR withdrawal and it was suggested that residues were deposited in body tissues. The recycling of CYR might have occurred since the birds were reared on the floor and according to the later studies of Caldas (2007) 97% of ingested CYR (5 mg/kg) are excreted within 24 hours. Although the inclusion levels in the study of Caldas (2007) were only 5 mg/kg which might have relieved some renal pressure

to manage CYR excretion compared to the 25 mg/kg applied by Brake *et al.* (1991), the delay in CYR excretion reported by Brake *et al.* (1991) is questionable since contamination could repeatedly occur by ingesting faeces containing CYR.

2.10.2. Other species and products

In studies on other species, findings similar to the poultry trials were reported. In a lactating goat study by Simoneaux *et al.* (1984), a low (4.8 mg/kg) and high (48 mg/kg) CYR concentration were administered to two groups. Approximately 90.4% and 82.2% of the recovered residues were excreted via urine and 7.5% and 5.7% in the faeces respectively for the low and high doses. Less than 2% of the administered dose was recovered in body tissue. Again the liver had the highest residue levels (0.791 mg/kg and 1.522 mg/kg) followed by kidney tissue (0.043 mg/kg and 0.437 mg/kg) and loin cuts (0.009 mg/kg and 0.104 mg/kg). Cyromazine detected in the milk accounted for 0.2% of the total recovery with an average of 0.017 mg/kg for the low dose and 0.35 mg/kg for the high CYR dose. In a similar study by Tortora (1991), 150 mg/kg were administered to dairy goats which is higher than the dose provided by the previous researcher. Also Tortora (1991) had a lower recovery rate of 73.9% compared to Simoneaux *et al.* (1984) (102% and 90.4% for the low and high dosed animals respectively). The highest recoveries were in the kidneys (3.78mg/kg) followed by the liver (0.93 mg/kg) and loin cut (0.79 mg/kg) which is contradictory to the higher liver recovery rates of Simoneaux *et al.* (1984). Milk contained 0.76 % of the total recovery (0.656 mg/kg). These studies added to the confirmation that higher administered CYR doses will result in higher residual traces as mentioned before. Melamine was also detected in the kidney (1.24 mg/kg), liver (0.16 mg/kg) and loin cut (0.03 mg/kg) as a result of CYR metabolism *in vivo*. No MEL was detected in fat and CYR levels were 0.11 mg/kg.

In a small study on sheep, 5 mg/kg CYR were administered for nine days on only one sheep (Simoneaux *et al.*, 1981). The results were similar to the goat studies. The liver yielded the highest CYR recovery (0.174 mg/kg) followed by the kidneys (0.048 mg/kg) and leg muscle (0.013 mg/kg). It was interesting to note that a higher level of MEL was detected in the liver (0.645 mg/kg) compared to

CYR, which might be explained by the relatively low recovery rate of 67% that could have influenced accurate detection.

No detectable CYR residues were found in rat tissue after feeding 0.5 mg/kg CYR (Simoneaux *et al.*, 1978), except for the liver (0.007 mg/kg); however, the author discarded this value due to a lack of accuracy. Caldas (2007) proposed a possible pathway for CYR metabolism in different animal species. In the rat, goat, hen and sheep CYR can be metabolized to MEL; however, very little data on these reports are available. Only in rats and goats the residual methyl-CYR were observed and goats were the only animal investigated to have hydroxyl-CYR present as a CYR metabolite.

2.11. Melamine and cyromazine contamination due to processing and food package material

In cattle MEL levels of 0.010-0.170 mg/kg were reported due to the ingestion of CYR. Processing and curing of beef containing CYR seemed to help reduce the CYR content by 35% (Epstein *et al.*, 1988). The author also reported that beef processing at 68°C increased the CYR content of the product due to water loss. A MEL trace of 1 mg/kg were found in canned beef that were treated with and without CYR and the possible explanation was that it could be contaminated by the MEL-formaldehyde resin originating from the can lining. The US FDA (2007) allows MEL-formaldehyde coatings as long as the chloroform soluble extractives yield does not exceed a food contact surface of 0.5 mg/inc^2. Zhu *et al.* (2009) could however not trace any MEL back to their milk containers and therefore the contaminated milk was not due to packaging materials. Lu *et al*. (2009a) also reported no MEL levels in milk migrated from containers, polypropylene and polycarbonate materials. Low levels were detected in milk samples packed in MEL resin containers and it was reported that these results met the European Unit's requirements.

2.12. Conclusion

It has been reported that MEL and CYR are rapidly eliminated by the body via urine. Small doses of MEL are tolerable in most animals; however it has been widely reported that levels above 1000 mg/kg can cause severe kidney damage and other adverse production effects. The kidneys appeared to be damaged more severly by MEL-cyanurate followed by MEL and CYR. Altough no clinical defects were reported after ingesting high levels of CYR, some production parameters were adversly affected, indicating that CYR should be used with caution as a feed additive in layer diets.

A definite trend exists for the distribution of MEL in meat and other animal products. It appears as if MEL are rapidly noticed (within a day or two) in products such as eggs and milk followed by muscle tissue. There is also a link between the level of MEL exposure and the concentration deposited. The higher the administered MEL levels, the more MEL is deposited in the eggs, milk and meat. Also it has been found that MEL residues increased rapidly in animal products up to a certain point where after it reaches a plateau where no further increases are noted. These finding led to the conclusion that MEL does not accumulate in the body and that some sort of mechanism exists to help the animals to manage the ingested MEL.

After MEL administration is terminated, MEL is rapidly removed from meat (within four days) and eggs (within 7-10 days) and therefore chickens reared on MEL contaminated feed could still be marketed if a sufficient withdawal time was allowed prior to slaughter. From the literature CYR is probably not metabolized and deposited as MEL in animal tissues or eggs; however, in theory a small proportion could be deposited as MEL. The literature covering this specific field was extremely limited regarding the latter and the possibility of CYR being metabolized to MEL in animal products remains uncertain.

The toxicity sensitivity level between animal species varied greatly and residuals remained longer in animal products such as milk and eggs rather than muscle tissue after withdrawal. Rats metabolize MEL the fastest followed by chickens and pigs and small ruminants such as goats and sheep have the longest half life for MEL excretion compared to all the other investigated animal species.

Many studies reported on the rate of MEL distribution and many similar rates were comparable while a few contraditions exist; however, all results only sketch a vague picture accompanied with many assumptions. Prediction models have also been formulated for rats and pigs to estimate MEL distribution and withdrawal rates; however, further investigation is required to refine this field. Although alot has been learned on the distribution and withdrawal times in many species as well as the characteristic toxicity symtoms, the exact mechanism of MEL and CYR metabolism still needs to be confirmed. More accurate distribution and withdrawal rates should also be established in order to guide the industry on MEL and CYR safety levels.

2.13. References

Allen, L.M., Briggle, T.V. and Pfaffenberger, C.D., 1982. Absorption and excretion of cyanuric acid in longdistance swimmers. Drug. Met. Rev. 13: 499-516.

Andersen, W.C., Turnipseed, S.B., Karbiwny, C.M., Clark, S.B., Madson, M.R., Gieseker, C.M., Miller, R.A., Rummel, N.G. and Reimschuessel, R., 2008. Determination and confirmation of melamine residues in catfish, trout, tilapia, salmon and shrimp by liquid chromatography with tandem mass spectrometry. J. Agric. F. Chem. 56: 4340-4347.

Anderson, J.F., Spandorf, A., Magnarelli, L.A. and Glowa, W., 1987. Control of house flies in commercial poultry houses in Connecticut. J. Poult. Sci. 65: 837-844.

Bai, X., Bai, F., Zhang, K., Lv, X., Qin, Y., Li, Y., Bai, S. and Lin, S., 2010. Tissue deposition and residue depletion in laying hens exposed to melamine-contaminated diets. J. Agric. F. Chem. 58: 5414-5420.

Barac-Nieto, M., 2004. Outline of regulation of bicarbonate. Faculty of Medicine. Department of Physiology, Kuwait University. Available: http://www.acbrown.com/renal/OutBicarb.htm. Accessed: Aug, 7, 2010.

Bathe, R. And Sachsse, K., 1978. Acute oral LD_{50} in the rat of technical CGA 72662. Unpublished report no. Siss 6446. Ciba-Geigy Ltd. Switzerland.

Baynes, R.E., Smith, G., Mason, S.E., Barrett, E., Barlow, B.M. and Riviere, J.E., 2008. Pharmacokinetics of melamine in pigs following intravenous administration. F. Chem. Tox. 46: 1196-1200.

Baynes, R.E., Barlow, B., Mason, S.E., Riviere, J.E., 2010. Disposition of melamine residues in blood and milk from dairy goats exposed to an oral bolus of melamine. F. Chem. Tox. doi: 10.1016/j.fct.2010.04.040.

Bhalla, V., Grimm, P.C., Glen, M.C. and Pao, A.C., 2009. Melamine nephrotoxicity: an emerging epidemic in an era of globalization. Kid. Inter. 75: 774-779.

Blair, M., Slezak, S.E. and Schardein, J.L., 1981. Two generation reproduction study with CGA-72662 in albino rats. Unpublished study. Project no. 382-086. International Research and Development Corp. Mattawan. USA.

Boone, T. and Cheung, M.W., 1985. Residues of cyromazine and melamine in chicken tissues and eggs resulting from the feeding of cyromazine in the diet. Unpublished Report. Ciba-Geigy Corp. Greensboro. United States. Report No. ABR-84082.

Bowes, V.A., Julian, R.J. and Stirtzinger, T., 1989. Comparison of serum biochemical profiles of male broilers with female broilers and white leghorn chickens. Can. J. Vet. Res.53: 7-11.

Brake, J., Ort, J.F. and Carter, T.A., 1983. Effect of Larvadex on broiler breeder performance. J. Poult. Sci. 62: 1388.

Brake, J., Ort, J.F. and Carter, T.A., 1984. Effect of the insect growth regulator CGA-72662 (Larvadex®) on broiler breeder production, hatchability, and subsequent chick performance. J. Poult. Sci. 63: 910-916.

Brake, J., Berry, W.D., Peebles, E.D. and Ort, J.F., 1985. Effect of feeding CGA-72662 (Larvadex®) during growth on body weight, feed consumption, livability, production, and egg quality of single comb white leghorn layers. J. Poult. Sci. 64: 247-252.

Brake, J. and Ort, J.F., 1989. Effects of CGA-72662 (Larvadex®) in turkeys during rearing and reproduction. J. Poult. Sci. 68: 731-738.

Brake, J. And Axtell, R.C., 1991. Retention of Larvicidal activity after feeding cyromazine (Larvadex®) for the initial 20 weeks of life of single comb whith leghorn layers. J. Poult. Sci. 70: 1873-1875.

Brown, C.A., Jeong, K.S., Poppenga, R.H., Puschner, B., Miller, D.M., Ellis, A.E., Kang, K.I., Sum, S., Cistola, A.M. and Brown, S.A., 2007. Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. J. Vet. Diagn. Invest. 5: 525-531.

Buhr, R.J. and Abbott, U.K., 1983. Fertility and hatchability of breeding chickens fed Larvadex. J. Poult. Sci. 62: 1393-1394.

Buur, J., Baynes, R.E. and Riviere, J.E., 2008. Estimating meat withdrawal times in pigs exposed to melamine contaminated feed using a physiologically based pharmacokinetic model. Reg. Tox. Pharm. 51: 324-331.

Caldas, E.D., 2007. Cyromazine (169). First draft. University of Brasilia. Brazil. P. 256-261.

Capps, T.M., 1990. Characterization and identification of ^{14}C-cyromazine and metabolites in rats. DPR Registration Document No. 414082.

Cecil, H.C., Miller, R.W. and Corley, C., 1981. Feeding three insect growth regulators to white leghorn hens: Residues in eggs and tissues and effects on production and reproduction. J. Poult. Sci. *60*: 2017-2027.

Chapman, R.P., Averell, P.R. and Harris, R.R., 1943. Solubility of melamine in water. Indus. Eng. Chem. 35: 137-138.

Chen, Y., Yang, W., Wang, Z., Peng, Y., Li, B., Zhang, L. and Gong, L., 2010. Deposition of melamine in eggs from laying hens exposed to melamine contaminated feed. J. Agric. F. Chem. 58: 3512-3516.

Chinese Ministry of Health, 2008. Melamine-contamiation event. Available: http://www.who.int/foodsafety/fs_management/infosan_events/en/index.html. Accessed: Aug, 1, 2010.

Chou, S.S., Hwang, D.F. and Lee, H.F., 2003. High performance liquid chromatographic determination of cyromazine and its derivative melamine in poultry meats and eggs. J. Food. Drug. Anal. 11: 290-295.

Clark, R., 1966. Melamine crystalluria in sheep. J.S. Afr. Vet. Med. Assoc. 37: 349-351.

Cook, A.M. and Hütter, R., 1981. S-Triazines as nitrogen sources for bacteria. J. Agric. Food. Chem. 29: 1135-1143.

Cruywagen, C.W., Stander, M.A., Adonis, M. and Calitz, T., 2009. Hot topic: Pathway confirmed for the transmission of melamine from feed to cow's milk. J. Dairy. Sci. 92: 2046.

Digital Look, 2010. This is money financial website of the year. Available: http://investing.thisismoney.co.uk/companyresearch/100815/Brent_Crude/company_charts.htm. Accessed: July, 23, 2010.

Dobson, R.L., Motlagh, S., Quijano, M., Cambron, R.T., Baker, T.R. and Pollen, A.M., 2008. Identification and characterization of toxicity of contamination in pet food leading to an outbreak of renal toxicity in cat and dogs. Tox. Sci. 106: 252-262.

Downes, C.J., Mitchell, J.W., Viotto, E.S. and Eggers, N.J., 1984. Determination of cyanuric acid levels in swimming pool waters by U.V. absorbance, HPLC and melamine cyanurate precipitation. Water. Res. 18: 277-280.

Epstein, R. L., Randecker, V., Corrao, P., Keeton, J.T. and Cross, H.R., 1988. Influence of heat and cure preservatives on residues of sulfamethazine, chloramphenicol and cyromazine in muscle tissue. J. Agric. Food. Chem. 35: 1009-1012.

FSSA-MVSA, 2010. Fertilizer Society of South Africa. Available: http://www.fssa.org.za/pebble.asp?relid=64.

Goldenthal, E.L. and Hughes, R., 1979. 90 day subacute toxicity study with CGA-72662 in albino rats. Unpublished report. Project no. 382-052. International Research and Development Corp. Mattawan, USA.

Gossner, C.M., Schlundt, J., Ben Embarek, P., Hird, S., Lo-Fo-Wong, D. And Beltran, J.J., 2009. The melamine incident: implications for international food and feed safety. Environ. Health Perspect. 117: 1803-1808.

Hau, A.K., Kwan, T.H. and Li, P.K., 2009. Melamine toxicity and the kidney. J. Am. Soc. Nephr. 20: 245-250.

Hammond, B.G., Barbee, S.J. and Inoue, T., 1986. A review of toxicology studies on cyanurate and its chlorinated derivates. Environ. Health. Perspect. 69: 287-292.

Hilts, C. and Pelletier, L., 2008. Background paper on occurrence of melamine in foods and feed. Prepared for the WHO expert meeting 1-4 Dec 2008. Available:

http://www.who.int/foodsafety/fs_management/Melamine_3.pdf. Accessed: July, 14, 2010. P. 3-5.

Jeong, W., Do, S.H. and Jeong, D., 2006. Canine renal failure syndrome in three dogs. J. Vet. Sci. 7: 299-301.

Jessup, D.C. and Hughes, R., 1979. 90 day subacute oral toxicity study with CGA-72662 in purebred beagle dogs. Unpublished report no. 382-048. International Research and Development Corp. Mattawan, USA.

Jutzi, K., Cook, A.M. and Hütter, R., 1982. The degradative pathway of the s-triazine melamine. Biochem. J. 208: 679-684.

Keiding, J., 1999. Review of the global status and recent development of insecticide resistance in field populations of the housefly, *Musca domestica* (Diptera: Muscidae). Bull. Entomol. Res. 89: 9-67.

Kobayashi, T., Okada, A., Fujii, Y., Kazuhiro, N., Hamamoto, S., Yasui, T., Tozawa, K. and Kohri, K., 2010. The mechanism of renal stone formation and renal failure induced by administration of melamine and cyanuric acid. Urol. Res. 38: 117-125.

Ledoux, D.R., Zavarize, K.C., Murarolli, R.A., Kutz, R.E., Rottinghaus, G.E., Bermudez, A.J. and Lin, M., 2009. Effects of graded levels of melamine in young broiler chicks. University of Mussouri. Columbia.

Lu, J., Xiao, J., Yang, D.J., Wang. Z.T., Jiang, D.G., Fang, C.R. and Yang, J., 2009a. Study on migration of melamine from food packaging materials on markets. Biomed. Environ. Sci. 22: 104-108.

Lu, Y., Liu, L., Wu, Y., Jiang, J., Feng, N., Xie, W. and Zhou, X., 2009b. Study on the distribution and metabolism of melamine in laying-hens and eggs. College of Veterinary Medicine. China Agriculture University. Beijing.

Lü, M.B., Yan, L., Guo, J.Y., Li, Y., Li, G.P. and Ravindran, V., 2009. Melamine residues in tissues of broilers fed diets containing graded levels of melamine. J.Poult. Sci. 88: 2167-2170.

Lv, X., Wang, J., Wu, L., Qiu, J., Li, J., Wu., Z. and Qin, Y., 2010. Tissue deposition and residue depletion in lambs exposed to melamine and cyanuric acid-contaminated diets. J. Agric. Food. Chem. 58: 943-948.

MAFF, 2010. Summary of studies on the transfer of melamine from feeds to tissues of lactating cows and chickens. Japanese Ministry of Agriculture. Forestry and Fisheries. Unpublished report.

Mast, R.W., Jeffcoat, A.R. and Sadler, B.M., 1983. Metabolism, disposition and excretion of [^{14}C]melamine in male Fisher 344 rats. Food. Chem. Tox. 21: 807-810.

McDonald, P., Edwards, R.A., Greenhalgh, J.F.D. and Morgan, C.A., 2002. Non-protein nitrogen compounds as protein sources. IN *Animal Nutrion 6*th Ed. Pearson Eduacation Limited. Edinburgh Gate. Harlow. P. 602, 603, 610.

Melnick, R.L., Boorman, G.A., Haseman, J.K., Montali, R.J. and Huff, J., 1984. Urolithiasis and bladder carcinogenicity of melamine in rodents. Tox. Appl. Pharm. 72: 292-303.

Merck, 2001. Entry 5834. IN *The Merck Index 13*th*Ed*. Merck Research Laboratories. Whitehouse Station. New York. P. 1038.

Miller, R.W. and Corley, C., 1980. Feed-through efficacy of CGA-19255 and CGA-72662 against manure-breeding flies and other arthropods and residues in feces, eggs, and tissues of laying hens. Southwest. Entomol. 5: 144-148.

Newton, G.L. and Utley, P.R., 1978. Melamine as a dietary nitrogen source for ruminants. J. Anim. Sci. 47: 1338-1344.

Ogasawara, H., Imaida, K. and Ishiwata, H., 1995. Urinary bldder carcinogenesis induced by melamine in F344 male rats: Correlation between carcinogenicity and urolith formation. Carcinogenesis. 16: 2773-2777.

Osborne, C.A., Lulich, J.P., Ulrich, L.K., Koehler, L.A., Albasan, H., Sauer, L. and Schubert, G., 2008. Melamine and cyanuric acid-Induced crystalluria, uroliths, and nephrotoxicity in dogs and cats. Vet. Clin. Sci. Small. Anim. 39: 1-14.

Pfeifer, K., 1993. Cyromazine (Larvadex) risk characterization document. Medical toxicology and worker health and safety branches. Department of pesticide regulation, California environmental protection agency. Available: Http://www.cdpr.ca.gov/docs/risk/rcd/cyromazine.pdf. Accessed: August, 6, 2010.

Puschner, B., Poppenga, R.H., Lowenstine, L.J., Filigenzi, M.S. and Pesavento, P.A., 2007. Assessment of melamine and cyanuric acid toxicity in cats. J. Vet. Diagn. Invest. 19: 616-624.

Reimschuessel, R., Hattan, D.G. and Gu, Y., 2008. Background paper on toxicology of melamine and its analogues. Prepared for the WHO Expert Meeting on toxicological and health aspects of melamine and cyanuric acid.

Sabol, R.J., 1987. Acute oral toxicity study in rats – EPA guidelines no. 81-1. Unpublished report no. 5020-87. Stillmeadow Inc. Houston. USA.

Sebastian, M., 2009. Renal Toxicity. IN *Handbook of toxicology of chemical warfare agents.* Ed. C.G. Ramesh. Academic Press.

Setiogi, S., 2008. Experts set tolerable level for melamine intake. World Health Organization. Geneva. Available: http://www.who.int/mediacentre/news/releases/2008/pr48/en/print.html. Accessed: Aug, 14. 2010.

Simoneaux, B, and Cassidy, J.E., 1978. Metabolism and balance study of ^{14}C-CGA 72662 in the rat. Unpublished report no. ABR-78072. Ciba-Geigy Corp. Greensboro, United States.

Simoneaux, B. and Cassidy, J.E., 1981. Metabolism and balance study of ^14C-CGA-72662 in a mature sheep. Report no. ABR-80055. Ciba-Geigy Corp. Greensboro. United States.

Simoneaux, B. and Marco, G., 1984. Balance and metabolism of ^{14}C-Cyromazine in lactating goats. Report No. ABR-84067. Ciba-Geigy Corp. Greensboro. United States.

Tawada, T., Fujita, K., Sakakura, T., Shubutani, T., Nagata, T., Iguchi, M. and Kohri, K., 1999. Distrubution of osteopontin and calprotectin as matrix protein in calcium-containing stone. Urol. Res. 4: 238-242.

Thompson, M.E., Lewin-Smith, M.R., Kalasinsky, V.F., Pizzolato, K.M., Fleetwood, M.L. and McElhaney, M.R., 2008. Characterization of melamine-containing and calcium oxalate crystals in three dogs with suspected pet food-induced nephrotoxicosis. Vet. Pathol. 45: 417-426.

Tortora, N.J., 1991. Metabolism of [triazine-14C]-cyromazine in lactating goats. Unpublished Report. Ciba-Geigy Corp. Greensboro. United StatesEnviron. Health Center. Farmington. United States. Report No. F-00105. GLP.

Tusing, T.W., 1953. Chronic feeding: Dogs. Available: http//www.cdpr.ca.gov/docs/risk/toxsums/pdfs/1023.pdf. Accessed: July, 28, 2010.

Tyan, Y.C., Yang, M.H., Jong, S.B., Wang, C.K. and Shiea, J., 2009. Melamine contamination. Bionanal. Chem. 395: 729-735.

United States Environmental Protection Agency (US EPA), 1999. Cyromazine: Pesticide Tolerance. IN *Federal Register*. *64*: 50043-50050. Available: http//www.epa.gov/EPA-PEST/1999/September/Day-15/p24047.htm. Accessed: Aug, 1, 2010.

United States Environmental Protection Agency (USEPA), 2007. Cyromazine: Registration review summary document. Washington. D.C. United States Environmental Protection Agency. Available: http://www.regulations.gov/fdmspublic/component/main?main=DocumentDetai&d=EPA-HQOPP-2006-0108-0003. Accessed: July, 21, 2010.

United States Food and Drug Administration, 2007. Interim melamine and analogues safety/risk assessment. Available: http://www.fda.gov/OHRMS/DOCKETS/98fr/07n-0208-ra00001.pdf. Accessed: Aug, 3, 2010.

Wilson, H.R., Harms, R.H., Simpson, C.F. and Campbell, W.R., 1983. Effect of high levels of Larvadex® on reproduction in Leghorn breeders. Journal of Poultry Science. *62*: 1731-1733.

World Health Organization, 2008. Melamine and cyanuric acid: Toxicity, preliminary risk assessment and guidance on levels in food.

World Health Organization, 2010. Proposed draft maximum levels for melamine in food and feed (N13-2009). Joint FAO/WHO food standards programme. Codex committee on contaminants in foods, 4th Session. Izmir. Turkey. April, 26-30, 2010.

Yu, X., Sun, Z., Lu, Y., Yang, T. and Fang, W., 2009. Determination of residues and metabolism of melamine in chicken using GC/MS. Acta. Agric. Zhejiangensis. April 2009.

Yuchang, Q., Xiaowen, L., Jun, L., Guanghai, Q., Qiyu, D., Guohua, L., Min, X., Jiaqi, W., Jianming, T., Liying, Z. and Keying, Z., 2010. Assessment of melamine contamination in crop, soil and water in China and risks of melamine accumulation in animal tissues and products. Environ. Internat. 36: 446-452.

Zhu, X, Wang, S., Liu, Q., Xu, Q., Xu, S. and Chen, H., 2009. Determination of residues of cyromazine and its metabolite, melamine, in animal-derived food by gas chromatography-mass spectrometry with derivatization. J. Agric. Food. Chem. 57: 11075-11080.

CHAPTER 3

Absorption of dietary melamine and cyromazine and subsequent distribution to in poultry meat

3.1. Introduction

Melamine ($C_3H_6N_6$) is an organic industrial chemical with many applications, but it is mainly used to manufacture paints, laminates, table tops and other plastic ware. Melamine has an exceptionally high N content of 667g/kg on a molecular weight basis (Merck, 2001), which relates to a crude protein (CP) content of 4169 g/kg (N x 6.25). This is approximately 45% higher than the CP content of urea. Therefore, the opportunity emerged to add this substance to high protein feed products such as infant formula (WHO, 2008), wheat gluten, corn gluten, rice protein (Weise *et al.*, 2007), soya and fishmeal (CBC News, 2007). These products were mainly used for animal feed production.

In South Africa, maize gluten meal (also known as Gluten 60 or Prime Gluten) is the only known imported feed ingredient where MEL contamination could be confirmed. Approximately 50 000 tons of maize gluten is annually used for animal feed manufacturingin South Africa (Briedenhann, 2010). It is not exactly clear how much of the adulterated gluten 60 was imported during 2008, but the Department of Agriculture has confiscated some 300 tonnes. Despite the misuse of MEL as feed adulterant, concern has been expressed that MEL might end up in animal tissues as a result of cyromazine (CYR) ingestion, as MEL is an intermediate compound of CYR metabolism in the animal. Cyromazine is the active ingredient of some pesticides such as Larvadex® which is commonly added to poultry layer feed to inhibit fly larvae from hatching in the manure. Furthermore, MEL can be present in pelleted feed as binding agent or in minute quantities as a result of migration from the environment (WHO, 2008). Groundbreaking work confirmed the distribution of MEL from feed to broiler meat (Bai *et al.*, 2010; Chen *et al.*, 2010; Lü*et al.*, 2009a and Yuchang *et al.*, 2010). In another study, Boone *et al.* (1985) determined MEL residues in meat which might have been the result of the metabolization of ingested CYR.

The popularity of broiler meat consumption increases annually in South Africa with a total production of 931 443 000 broilers during 2009 (Vauqulin, 2010) and new processed chicken products are consistently demanded. This seems to be proof that the impact of MEL included in broiler feed is far beyond the impact scope that manufactures could have ever imagined and this is only one division of many that are contributing to the food chain. It would be of interest to feed manufacturers to asses the inclusion of MEL and CYR in broiler feeds and to determine the distribution pathways of these two substances in meat to be able to implement the necessary safety measures.

Therefore, the objectives of this study were:

1. to determine the distribution efficiency of MEL from feed to chicken meat and the effect of MEL on production responses when MEL was added to broiler diets at different levels, ranging from 0 to 500 mg/kg.
2. to determine if MEL would be cleared from the meat after contaminated feed had been withdrawn.
3. to compare the distribution of MEL in different tissues (liver, kidneys and muscle).
4. to explore the possibility of MEL detection in muscle tissue as a result of CYR metabolism by including Larvadex® in one of the experimental diets.

3.2. Materials and methods

All feeding, husbandry and harvesting methods were practised strictly according to the ethical requirements that have been approved by Subcommittee B of the University of Stellenbosch (Reference # 2008B03003). The Larvadex® product contained 99% inert products and only 1% CYR (Drugs.com, 2010). Since it was recommended by the suppliers (Profile Feeds, Paarl, South Africa) that Larvadex® should be included at 400 mg/kg, the CYR treatment therefore effectively received 4 mg/kg CYR, which is within the maximum legal level of 5 mg/kg (Drugs.com, 2010).

3.2.1. Birds, housing and management

A total number of 480 day old Cobb 500 chickens were obtained from a local hatchery (Vredebest Farms, Stellenbosch). On arrival, the birds were vaccinated for Infectious Bronchitis and placed promptly to minimize further travelling stress. Environmental temperature and lighting regimes were applied according to the Cobb 500 guidelines of 2010. All birds received a balanced pre-starter diet (Table 4) until Day 9. The diet was offered *ad libitum* and it contained no detectable levels of MEL or CYR. Water was freely available at all times via 250 mL cups and after Day 18 via nipple drinkers. On Day 9, four chicks were randomly allocated to each of 120 cages after which the cages, which were regarded as experimental units, were randomly assigned to five treatments.

3.2.2. Treatments and experimental diets

The five experimental treatments were as follows:

1. Treatment 1 (CON) received a diet containing no detectable levels of MEL or CYR.
2. Treatment 2 (MEL50) received a diet containing 50 mg/kg MEL and no CYR.
3. Treatment 3 (MEL100) received a diet containing 100 mg/kg MEL and no CYR.
4. Treatment 4 (MEL500) received a diet containing 500 mg/kg MEL and no CYR.
5. Treatment 5 (CYR4) received a diet containing 4 mg/kg CYR and no MEL.

Diets were mixed on the Mariendahl Experimental Farm of the Stellenbosch University. Maize gluten meal imported from China found to be an adulterated product (Cruywagen & Reyers, 2009), was used as one of the MEL sources in the diets. The MEL content of the product was 15 117 mg/kg. It also contained urea, wheat bran, maize bran, gluten 20 and actually very little gluten 60. Because of the nature of the product it was thus decided to limit the inclusion level thereof and to balance the required MEL content of the diets with pure MEL (Sigma-Aldrich M2659, Supplied by Sigma, Cape Town). The minor ingredients were carefully weighed out and mixed in an industrial blender with a 10 kg capacity. The mixture was then transferred to a vertical industrial mixer (1 tonne capacity) and the major ingredients were added. First, the CON diet was mixed followed by the CYR4, MEL50,

MEL100 and lastly the MEL500 diet to avoid contamination between diets. All feeds were presented as a meal and stored in a cool dry area in 50 kg bags.

The pre-starter diet was offered *ad libitum* to all the chickens until Day 9 when the different experimental diets were introduced. Chickens received the respective starter diets from Day 9 until Day 18, the grower diets from Day 19 to 28 and the finisher diets from Day 29 until Day 36 – all these diets being offered *ad libitum*.

Chicken mortalities and morbidities were recorded throughout the trial and feed intake was determined by measuring the total amount of starter, grower and finisher feed that each treatment had consumed for the duration of the trial. On Day 19 birds were moved to a similar house with larger pens. Intake was not influenced after this move and it is accepted that the stress of moving did not have an adverse effect.

Table 4 The composition and calculated analysis (g/100g "as fed") of broiler diets in the melamine trial.

	Ration Name															
	Pre-starter	Starter diet treatments[1]					Grower diet treatments					Finisher diet treatments				
Item	All	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Ingredient																
Maize Gluten 60[2]	-	-	0.02	0.02	0.02	-	-	0.33	0.67	2.50	-	-	0.33	0.67	3.00	-
Melamine[3]	-	-	-	0.01	0.05	-	-	-	-	0.01	-	-	-	-	0.01	-
Larvadex	-	-	-	-	-	0.04	-	-	-	-	0.04	-	-	-		0.04
Vit+Mineral Premix[4]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	1.24
Maize	43.4	49.9	49.9	49.9	49.8	49.9	50.4	50.0	49.7	47.7	50.4	58.8	58.4	58.1	55.7	58.8
Soybean Full Fat	50.8	42.1	42.1	42.1	42.2	42.1	29.0	29.0	29.0	29.0	29.0	1.24	1.26	1.28	9.24	1.24
Soybean 46	1.02	3.36	3.35	3.34	3.26	3.34	16.4	16.5	16.5	16.7	16.4	35.8	35.8	35.8	27.9	35.8
L-lysine HCl	0.41	0.37	0.37	0.37	0.37	0.37	0.12	0.11	0.11	0.08	0.12	0.07	0.07	0.06	0.09	0.07
DL-methionine	0.26	0.23	0.23	0.23	0.23	0.23	0.15	0.14	0.14	0.10	0.15	0.10	0.09	0.08	0.06	0.10
L-threonine	0.15	0.13	0.13	0.13	0.13	0.13	-	-	-	-	-	-	-	-	-	-
Limestone	1.62	1.66	1.66	1.66	1.66	1.66	1.66	1.66	1.66	1.66	1.66	1.71	1.71	1.71	1.71	1.71
Salt	0.15	0.21	0.21	0.21	0.21	0.21	0.25	0.25	0.25	0.25	0.25	0.23	0.23	0.23	0.24	0.23
Monocalcium Phosphate	1.63	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.63	1.64	1.65	1.65	1.65	1.64	1.65
Sodium Bicarbonate	0.30	0.21	0.21	0.21	0.21	0.21	0.16	0.16	0.16	0.15	0.16	0.18	0.18	0.18	0.17	0.18
Calculated composition																
AMEn (MJ/kg)[5]	13.0	12.8	12.80	12.8	12.8	12.8	12.0	12.0	12.0	12.0	12.0	11.3	11.3	11.3	11.8	11.3
Crude Protein	23.6	22.0	22.0	21.97	22.0	22.0	22.8	23.0	23.2	24.2	22.8	22.1	22.3	22.4	23.0	22.1
Calcium	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Total phosphorus	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82
Available phosphorus	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Digestible lysine	1.51	1.37	1.37	1.37	1.37	1.37	1.24	1.24	1.24	1.24	1.24	1.14	1.14	1.14	1.14	1.14
Digestible methionine	0.55	0.51	0.51	0.51	0.51	0.51	0.45	0.45	0.43	0.45	0.40	0.40	0.40	0.40	0.39	0.40
Melamine	0.00	0.00	0.005	0.01	0.05	0.00	0.00	0.005	0.01	0.05	0.00	0.00	0.005	0.01	0.05	0.00

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[2]The maize gluten 60 contained melamine at a level of 15117 mg/kg.
[3]Melamine powder (Sigma-Aldrich M2659, Supplied by Sigma, Cape Town).
[4]Vitamin+mineral premix provided (per kg of diet): 8160 IU vitamin A, 1700 IU vitamin D3, 30.6 IU vitamin E, 2.7 mg vitamin K3, 2.05 mg vitamin B1, 2.05 mg vitamin B2, 27.2 mg niacin, 10.2 mg calcium pantothenate, 0.02 mg vitamin B12, 4.1 mg vitamin B6, 1.7 mg folic acid, 0.068 mg biotin, 120 mg ronozyme p500, 350 mg choline, 0.08 mg I, 0.34 mg Co, 0.2 mg Se, 70 mg Mn, 70 mg Zn, 6 mg C and 50 mg Fe.
[5]AMEn = Apparent metabolizable energy.

Samples (100 g) were collected before the start of the trial from all the experimental diets and milled through a 1 mm screen after which the diets were analyzed to confirm MEL inclusion levels using the LC-MS/MS method (described later). The obtained values are displayed in Table 5.

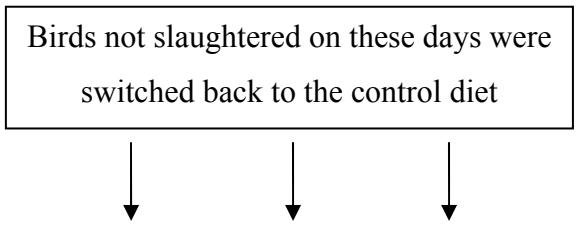
Table 5 The expected and analyzed melamine values (mg/kg) of the diets for trial purposes.

Treatment[1]	Expected value	Analyzed value			
		Pre-Starter	Starter	Grower	Finisher
1	0	ND	ND	ND	ND
2	50		36.24	60.12	82.16
3	100		118.2	116.2	124.9
4	500		356.5	543.2	656.3
5	0		ND	ND	ND

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.

3.2.3. Measurements and sampling

To determine the rate at which MEL could be disturbed to body tissues, six birds were euthanized from each treatment (except for CYR4) on Days 11, 13, 15, 18, 22, 29 and 36 (Figure 3).



	Day 11	Day 13	Day 15	Day 18	Day 22	Day 29	Day 36
CON	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds
MEL50	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds
MEL100	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds
MEL500	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds	6 birds
CYR4							6 birds
[1]C18 (MEL50, MEL100, MEL500)							18 birds
C22 (MEL50, MEL100, MEL500)							18 birds
C29 (MEL50, MEL100, MEL500)							18 birds

Figure 3 Diagrammatical illustration of the slaughtering process of broiler birds in the melamine trial. [1]C18; C22; C29 = Birds slaughtered on day 36 received the control diet from Day 18, Day 22 or Day 29, respectively.

The rate at which MEL could be cleared from the respective tissues was investigated by replacing the MEL diet with the control diet for the remaining chickens from the cages that were selected for slaughtering on Days 18, 22 and 29. These birds were then slaughtered on Day 36 to establish a MEL withdrawal period of 18, 14 and 7 days, respectively. Regarding CYR4, it should be mentioned that only the possibility of MEL being detected as a metabolite (derived from cyromazine) in the liver, kidneys and the breast muscles were of interest, rather than the rate at which it would be distributed to the various tissues. Therefore, birds from CYR4 were only slaughtered on Day 36 and these results compared to the CON treatment.

Birds were weighed before slaughter and were euthanized by means of the cervical dislocation technique. The *pectoralis major* and *minor* muscles, liver (without the gallbladder), heart, spleen and the kidneys were removed and weighed. Only the breast, liver and kidney samples were analyzed for

MEL residues and these samples were promptly frozen at -18°C. The same sampling technique and procedure were applied for dissected CYR4 birds on Day 36.

3.2.4. Laboratory analyses

The frozen samples were thawed overnight and then cut into pieces to increase evaporation area. The correct technique would have been to homogenize the samples; however, most of the samples were too small for effective homogenization and nonetheless the dried samples were milled as described later. The samples were weighed and dried in an oven (Memmert, 220V, 2000W, 0-280°C, West-Germany) at 50-59°C in small aluminium foil dishes. Temperature readings from the top and bottom sections of the oven revealed the inevitable variation as stated. The nitrogen compounds of the tissue samples may become volatile above 60°C; therefore, the differences in temperature reported were assumed not to have affected the results. All the samples were dried to constant weight. The livers required more than 48 hours to dry, due to their high fat content. The kidneys required only 24 hours drying time, while for the breast samples, 33 hours were sufficient. The reason for using the oven drying method instead of freeze-drying was due to limited freeze-drying capacity and that oven drying seemed to be as efficient as freeze-drying.

The dried samples were ground with a mill (Russel Hobbs Model no. 10934 obtained from a local retail store) until a powdery texture was obtained. These samples weighed between 1.92 and 91.50 g. The average percentage moisture loss for the breast and kidney samples were similar (73.80% and 73.84% respectively) and for the livers only 65.22%, emphasising the high fat contents of the latter once more. All the samples were frozen again in plastic bags at -18°C for further analyses.

For MEL analysis, the method described by Shai *et al.* (2008) was used with some modifications. Cruywagen *et al.* (2009) described the method as follows:

Chemicals and Reagents:

- 1 g ground sample
- 50% Acetonitrile
- 10 mL0.1% formic acid
- Methanol (12 mL)
- Water (6 mL)
- 100 µL of a 0.5 mg/L stable isotope labelled MEL ($^{13}C_3H_6{}^{15}N_3$) internal standard solution (Cambridge Isotope Laboratories, Inc., Andover, MA)
- 0.1N HCl (6 mL)
- 6 mL ammonium hydroxide:methanol:dichloromethane (1:5:5)
- 1 mL acetonitrile (50%).
- Nitrogen

Other Consumables and Equipment:

6. Ultrasonic bath
7. Standard 24 –port vacuum manifold (Vac Elut SPS 24)
8. Cation exchange solid phase extraction (SPE) cartridges (Phenonenex Strata SCX, 55 µm, 70 A, 500 mg/3 mL, supplied by Separations, Randburg, South Africa)
9. Tubes (15 mL glass)
10. Waters API Quattro Micro triple quadruple mass spectrometer
11. Waters 2690 HPLC (Waters Corporation, Milford, MA)

Method:

- The grounded samples (1 g) were extracted with 50% acetonitrile (10 mL) and 0.1% formic acid (10 mL) by sonication in an ultrasonic bath for an hour.
- The cation exchange SPE cartridges were then conditioned with 6 mL methanol followed by 6 mL water.

- The cartridges were washed with 6 mL of 0.1 *N* HCL and 6 mL methanol respectively while aspirating under vacuum for one minute.
- The MEL were then eluted with 6 mL ammonium hydroxid:methanol:dichloromethane (1:5:5) into a clean tube.
- The extracts were dried for 48 hours in a fume cupboard and resuspended in 1 mL of 50% acetonitrile.
- The samples were analyzed with liquid chromatography–tandem mass spectrometry (LC-MS/MS) on the Waters API Quattro Micro triple quadruple mass spectrometer, coupled to a Water 2690 high performance liquid chromatographer (HPLC).
- For tissue samples, the detection limit of this method was 0.05 mg/kg.
- The internal standard was used to correct dissimilarities in recovery rates.

Several other methods (i.e. Capillary electrophoresis; gas chromatography and gas chromatography–mass spectrometry) have been suggested to determine the presence of MEL in various media (Xia *et al.,* 2009). However, the LC–MS/MS method is the most accurate one for determining MEL in muscle (Smoker & Krynitsky., 2008) and kidney (Filigenzi *et al.*, 2008).

3.2.5. Statistical analyses

All the data were analyzed using the Statistica Version 9 (2010) package. Production data, as well as MEL concentration in meat data, were subjected to a two-way ANOVA (where treatment and day were the main effects) using the general linear models (GLM) procedure. Since the MEL residues observed in slaughtered birds (experimental unit) were independent of each other on the different slaughter days, the repeated measures analysis method was considdered as inappropriate. Comparisons between the liver, kidney and muscle tissues were performed by implementing the repeated measures of analysis method. Due to the fact that no MEL could be detected in samples from birds subjected to withdrawal, CON and CYR4, no statistical analyses were performed on these data.

3.3. Results

3.3.1. Production parameters

The following production parameters were recorded: feed intake, weight gain, feed conversion ratio (FCR), mortality, protein efficiency and European production efficiency (EFEF). No visible differences occurred between treatments. Because significant treatment x day interactions was observed, the main effects cannot be interpreted for feed intake. It appeared, however, that CYR4 chickens had a greater appetite, which is confirmed by the fact that on Days 13, Day 18 and Day 22 the CYR4 chickens had a significantly ($P < 0.01$) higher feed intake than those in any of the other treatments. Some vomiting was noted from a few birds that ingested the MEL diets and similar observations in dogs and cats were reported by Brown *et al.* (2007) and Hau *et al.* (2009).

Table 6 Average daily feed intake (g/d) of broilers in the melamine trial.

	Day						
[1]Treatment	11	13	15	18	22	29	36
1	27.7^{a}	34.8^{d}	48.7^{f}	72.2^{h}	92.5^{j}	136.7^{lm}	169.3^{n}
2	28.0^{ab}	31.5^{abcd}	47.2^{f}	70.8^{h}	91.8^{j}	135.3^{l}	167.0^{n}
3	29.7^{abc}	33.8^{d}	48.3^{f}	71.0^{h}	89.7^{j}	136.8^{lm}	168.8^{n}
4	27.7^{a}	31.8^{bcd}	53.0^{g}	71.5^{h}	92.8^{j}	134.8^{l}	167.3^{n}
5	33.0^{cd}	41.0^{e}	56.0^{g}	83.3^{i}	106.7^{k}	140.0^{m}	168.0^{n}

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[a-n]Means within rows and columns with different superscripts differed significantly ($P < 0.01$).

The cumulative feed intake was recorded and average daily feed intakes were calculated, which are shown in Figure 4.

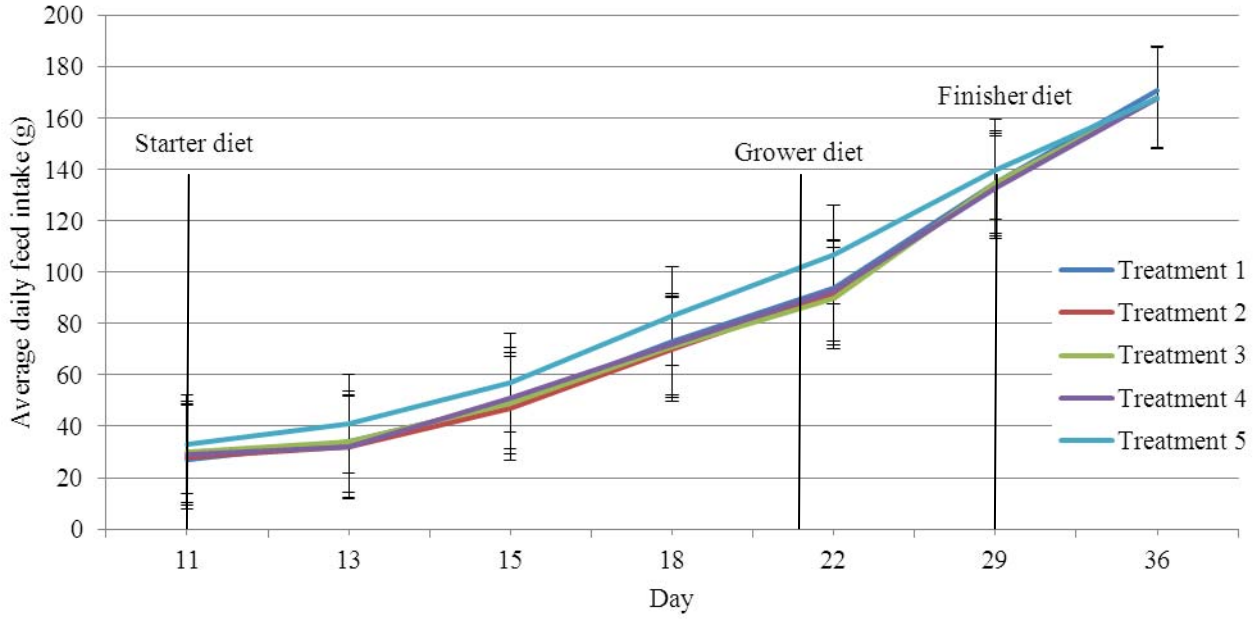


Figure 4 Average daily feed intake (g/d) of broilers in the melamine trial. Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.

No differences between treatments were recorded for live weight (Table 7). As expected, significant differences ($P < 0.01$) were obtained between days, except for Days 11 and 13.

Table 7 The cumulative weight gain (g/d) of broilers in the melamine trial.

	Day							
Treatment	11	13	15	18	22	29	36	*P*
1	239.7[a]	310.0[a]	396.8[b]	553.0[c]	666.7[d]	1309.3[e]	1756.0[f]	<0.01
2	290.5[a]	287.8[a]	389.7[b]	595.0[c]	719.2[d]	1189.5[e]	1750.5[f]	<0.01
3	297.7[a]	309.0[a]	374.7[b]	588.2[c]	703.7[d]	1235.7[e]	1822.2[f]	<0.01
4	241.3[a]	296.7[a]	400.8[b]	549.0[c]	760.2[d]	1171.2[e]	1728.1[f]	<0.01

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[a-f]Means within rows and columns with different superscripts differed significantly ($P < 0.01$).

The feed conversion ratio was calculated by dividing the cumulative feed intake (g) by the total live weight gain (g). No significant differences were found between treatments for FCR at termination - Day 36 (Table 8). Regarding mortality, only four birds died during the trial and two had to be culled due to leg abnormalities.

Table 8 The production results of feed conversion ratio (FCR), protein efficiency rate and European production efficiency rate (EPEF).

	Treatment					
Parameter	1	2	3	4	5	*P*
FCR	1.61	1.34	1.49	1.53	1.47	0.26
Protein efficiency (%)	2.97[a]	2.86[a]	2.77[a]	2.82[a]	3.40[b]	0.049
EPEF	296	319	335	310	334	0.90

[a-f]Means within rows with different superscripts differed significantly ($P < 0.05$).

The protein efficiency ratio (grams body weight gain / grams protein ingested, expressed as a percentage) was also calculated as an additional measurement of the production performance (Table 8). The crude protein level of the finisher feed was 19.26 %. The results indicated that CYR4 differed ($P < 0.05$) from all the other treatments with the best protein efficiency.

Finally, production efficiency was calculated by means of the European Production Efficiency Factor (EPEF) in order to establish a broader broiler performance rate (Perić *et al.*, 2009).

EPEF = (end weight – start weight) x (100 – mortality %) / (days in production x FCR x 10) x 1000

The EPEF for all treatments were satisfactory and none of the treatments differed from each other. Therefore it seems as if MEL and CYR inclusion in the diet did not have a marked effect on production overall.

3.3.2. Melamine concentration in muscle tissue

The MEL concentrations detected in breast meat are displayed in Table 9. No MEL could be detected in the withdrawal groups on day 36 or in the CYR4 treatment on day 36. These results were therefore not included in the statistical analyses.

Table 9 The concentration of melamine (mg/kg dry weight) in breast meat after feeding diets containing graded levels of melamine over time.

	Day							
Treatment[1]	11	13	15	18	22	29	36	*P*
1	ND[2]	ND	ND	ND	ND	ND	ND	
2	4.4 ± 1.7[3]	3.7 ± 1.3	4.6 ± 1.0	4.3 ± 0.8	7.1 ± 1.7	4.1 ± 1.9	2.7 ± 0.6	0.018
3	7.6 ± 2.5	6.6 ± 2.8	7.9 ± 2.2	7.4 ± 1.5	11.9 ± 3.4	9.4 ± 3.8	5.0 ± 1.0	0.018
4	38.2 ± 17.5	45.3 ± 13.0	22.8 ± 16.1	34.9 ± 10.8	52.5 ± 6.7	33.1 ± 15.7	27.1 ± 9.1	<0.001
5	-	-	-	-	-	-	ND	
P	0.2	0.3	0.2	0.3	0.04	0.3	0.2	

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[2]ND = Not detected. The detection limit of the method was 0.05 mg/kg.
[3]Data are displayed as means ± SD (n = 6).

The MEL distribution levels in breast meat increased as the feed MEL concentration increased (Figure 5). An interaction ($P < 0.05$) was observed between treatments and days.

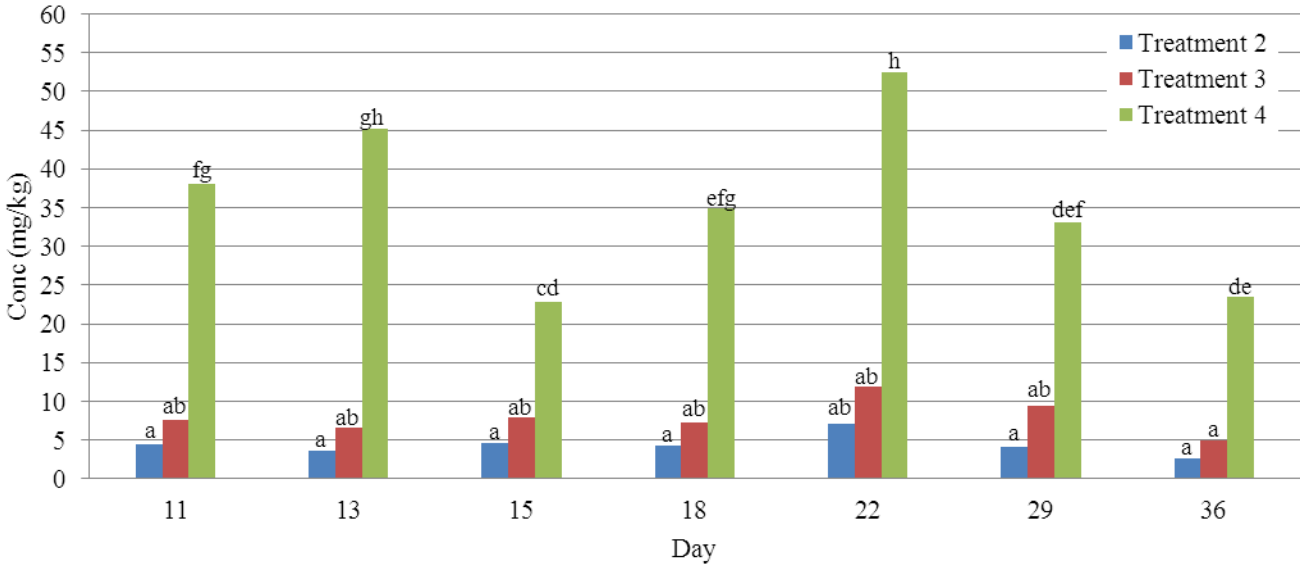


Figure 5 An illustration of the mean distribution values (mg/kg) of melamine in muscle after ingesting diets containing graded levels of melamine over time (day). [a-h]Means with different superscripts differed significantly ($P < 0.05$). Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500mg/kg; 5 = dietary cyromazine 4 mg/kg.

For each day tested, MEL500 differed ($P < 0.05$) from MEL50 and MEL100 whilst the latter two did not differ from each other. Within MEL500, differences occurred as indicated by Figure 5. Within MEL500, Day 22 differed ($P < 0.01$) from all the other days except for Day 13. Also Day 15 differed ($P < 0.05$) from Days 11, 13, 18 and 22. During slaughter on Day 15 it was noted that the gallbladders of all the birds of MEL500 were enlarged compared to MEL50 and MEL100. Day 13 differed from Days 28 ($P < 0.05$) and 36 ($P < 0.01$) and Day 11 also differed ($P < 0.05$) from Day 36. It was interesting to note that on Day 22 a peak concentration level for all treatments were achieved after which a definite decline can be observed until Day 36.

3.3.3. Melamine concentrations in liver and kidneys

Melamine concentrations in the liver and kidneys were only tested on Day 36 (Table 10). Since it was assumed that no MEL would be detected in the CON treatment samples, these samples were not tested for MEL residues.

Table 10 The concentration melamine (mg/kg dry weight) in breast meat, liver and kidneys of broilers that received diets with graded levels of melamine.

	Tissue		
Treatment[1]	Muscle	Liver	Kidney
2	[2]2.72 [a] ± 0.59	1.57 [a] ± 0.57	10.28 [b] ± 2.10
3	4.99 [ab] ± 0.97	2.88 [a] ± 0.96	16.7 [c] ± 3.49
4	23.5 [d] ± 9.12	16.9 [c] ± 6.21	73.0 [e] ± 9.55
P	0.29	0.29	< 0.01

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[2]Data are displayed as means ± SD (n = 6). The detection limit of the current study was 0.05 mg/kg.
[a-h]Means within rows and columns with different superscripts differed significantly ($P < 0.05$).

From Table 10 it is evident that the kidneys had significantly higher ($P < 0.05$) MEL levels than both the liver and muscle tissues for all treatments. Also all the treatments within the kidney group differed significantly ($P < 0.01$). The results for MEL50 and MEL100 in the liver and muscle did not differ from each other, but a difference ($P < 0.05$) was observed between muscle and liver tissue for MEL500. The MEL distribution level increased in all tissues for each treatment as the concentration increased (Figure 6).

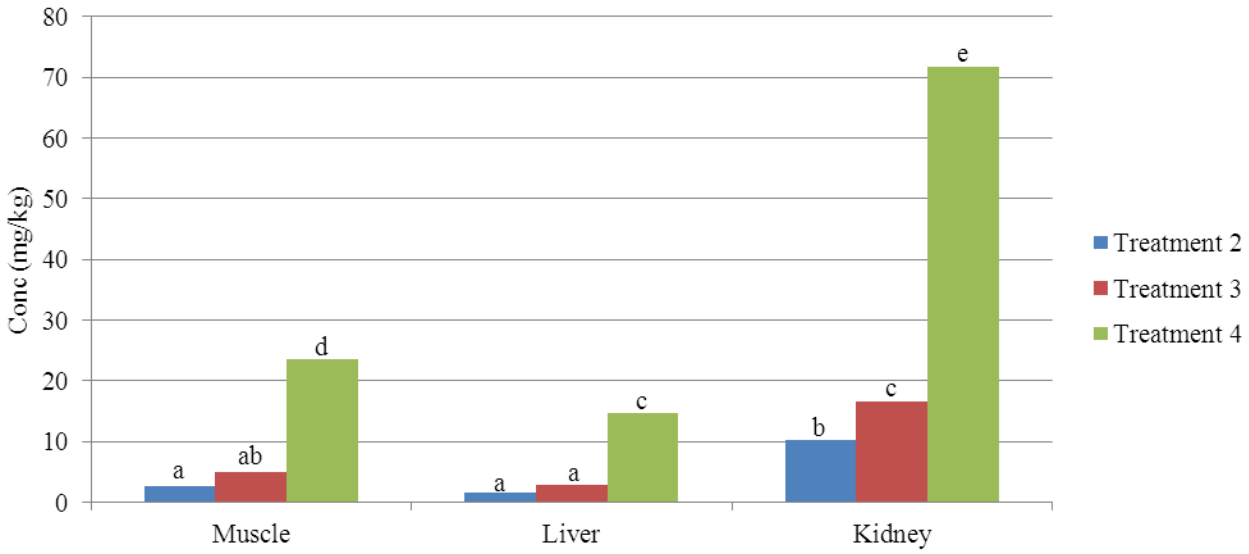


Figure 6 An illustration of the relationship between the distribution of melamine (mg/kg) in muscle, liver and kidney tissue on Day 36. [a-e]Means with different superscripts differed significantly ($P < 0.05$). Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500mg/kg; 5 = dietary cyromazine 4 mg/kg.

3.3.4. Distribution efficiency rate

The distribution efficiency (DE_f) of MEL to muscle tissue was calculated as follows:

DE_f (%) = MEL residues (mg) in total muscle mass tissue / MEL intake (mg) per day

The chemical analyses were done on dried tissue samples and the results were converted back to a wet basis. The average moisture content of the lean muscle samples was 738 g/kg. For total muscle mass determination, it was assumed that the total average lean muscle content of the whole bird was 62 % (Lin *et al.*, 2001). Because only the *Pectoralis major* and *minor* muscle groups were sampled in the current trial, it was further assumed that MEL was distributed equally to all the muscle groups. Calculations were also based on the assumption of a seven day depletion rate of MEL from muscle tissue (Lü *et al.*, 2009a and Bai *et al.*, 2010). Results are presented in Table 11 and Figure 7.

Table 11 The distribution efficiency (%) of melamine to muscle tissue in broilers receiving different levels of dietary melamine.

	Day			
Treatment[1]	18	22	29	36
2	1.749^{ab,x}	2.659^{c,x}	1.727^{b,xy}	1.294^{a,y}
3	1.447^{ab,xy}	2.223^{c,xy}	2.028^{b,x}	1.238^{a,y}
4	1.263^{ab,y}	2.048^{c,y}	1.370^{b,y}	1.274^{a,y}
Pooled SEM[2]	0.093	0.141	0.176	0.081

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[2]Pooled standard error means.
[a-c]Means within rows with different superscripts differed significantly ($P < 0.05$).
[x-y]Means within columns with different superscripts differed significantly ($P < 0.05$).

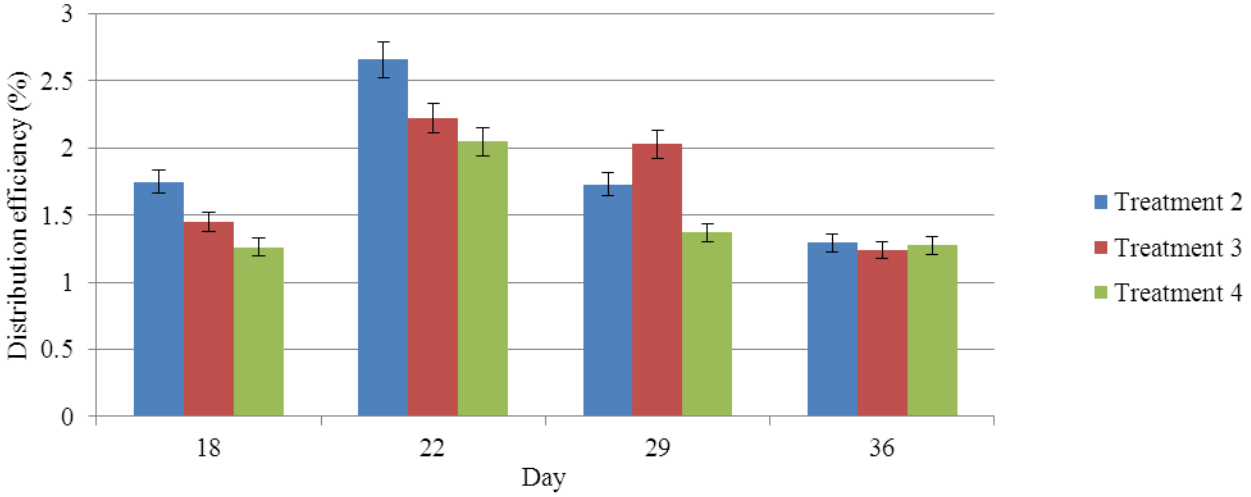


Figure 7 An illustration of the mean distribution efficiency (%) values of the total melamine deposited in the whole bird. Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500mg/kg; 5 = dietary cyromazine 4 mg/kg.

It was apparent that there was a definite pattern for DE_f to increase as the concentration MEL decreases. A significant difference ($P < 0.01$) was found between Day 22 and the other days. Day 29 also differed significantly ($P < 0.05$) from Days 22 and 36.

3.4. Discussion

3.4.1. Production parameters

In this study, no differences in feed intake, body weight gain and FCR were found between treatment groups that received graded levels of MEL and the control group (CYR4 will be discussed in the next paragraph). According to Ledoux *et al.* (2009) the effect of MEL ingestion on feed intake, body weight gain and FCR was mainly observed after ingested MEL levels of 1000 mg/kg was provided, after which a decrease in these production parameters could be observed. A further decrease can be expected if the MEL content of feed increased to levels of 1000 mg/kg and above. For broilers (Lü *et al.*, 2009a) and ducks (Lü *et al.*, 2009b) it was reported that feed intake, body weight gain and FCR was not suppressed in birds receiving feed containing 1000 mg/kg MEL and mortalities only increased after 1500 mg/kg. Therefore, it seems as if broilers can tolerate MEL levels up to 1000 mg/kg without adversely affecting feed intake, body weight, FCR and mortality. Since the highest inclusion level in the current study was only 500 mg/kg, these results are in accordance with Ledoux *et al.* (2009) and Lü *et al.* (2009a).

Even though no differences in feed intake, body weight gain and FCR between MEL50, MEL100 and MEL500 are being reported, CYR4 differed significantly ($P < 0.01$) from the other treatments (Figure 4) with superior feed intake and FCR ($P < 0.05$). Related literature reported no differences in feed intake after including 1000 mg/kg Larvadex® in the diet (Cecil *et al.*, 1981; Wilson *et al.*, 1983; Brake *et al.*, 1984; Brake *et al.*, 1985 and Brake *et al.*, 1989) with a rapid decrease after 1000 mg/kg which contradicts these results, where 400 mg/kg resulted in a significant increase ($P < 0.01$) in feed intake. The reason for the increased feed intake due to CYR ingestion is unknown. Other researchers did report an improved FCR, but it was based on the fact that a decrease intake improved feed utilization. Body weight was not affected by CYR since CYR4 did not differ from the other treatments. These findings are similar to the authors mentioned in this paragraph who did not find any changes in body weight for birds receiving Larvadex® up to 1000 mg/kg.

The study of Kidd *et al.* (1996) has shown that feed protein was better utilized in low protein diets and that higher protein efficiency percentages indicate more efficient utilization. Kidd *et al.* (1996) fed a diet containing a crude protein level of (16.8%) to broiler chicks and reported protein efficiency values of 2.46-2.86%. In the current study, diets containing 19.26% crude protein were fed during the finisher phase and it was found that MEL50, MEL100 and MEL500 had similar values (2.859%, 2.768% and 2.817% respectively) as presented by Kidd *et al.* (1996), which further supports the findings of Kidd *et al.* (1996) that the increased dietary crude protein had lower utilization compared to diets containing less crude protein. However, the MEL included in the test diets could have contributed to the poorer performance. In fact, the protein efficiency values of CYR4 (3.403%) and CON (2.968%) contradicted the findings of Kidd *et al.* (1996). An obvious explanation would be that improved genetic selection for production parameters over the past 15 year has been achieved and therefore the difference between Kidd *et al*. (1996) and the present study.

Considering the EPEF (Table 8), no difference occurred between treatments and all values were above 310. In the industry a value above 300 could be considered as a fine achievement and therefore it seems as if the inclusion of MEL up to 500 mg/kg did not influence overall performance and that a CYR inclusion also had a tendency to improve performance.

3.4.2. Melamine concentration in muscle tissue

Following the inclusion of graded levels of MEL in three broiler diets on Day 9, detectable MEL levels were already observed on Day 11 in the breast muscle for MEL50, MEL100 and MEL500. No differences were observed between MEL50 and MEL100 throughout the trial, but MEL500 resulted in higher concentration of MEL residues in muscle. This is in accordance with results reported by Lü *et al*. (2009a), Bai, *et al.* (2010) and Yuchang *et al.* (2010), who found that MEL increased. The muscle MEL concentrations of MEL500 on Days 15 and Day 18 obscured the general pattern, but it would appear that MEL residue levels reached a maximum value on Day 22 and then decreased towards Day 36 of the trial. It could be mentioned that the birds of MEL500 that were executed on Day 15 had enlarged gallbladders which were almost twice the size of the other treatment groups. This might indicate some disturbance in digestion that, by speculation, could have caused reduced MEL

absorption and resulted in the lower MEL values that were observed on Days 15 and 18. The decrease in MEL concentration that had been observed after Day 22 might indicate a possible metabolic response that develops over time and which enabled the birds to clear MEL residues from the muscle more efficiently. A similar observation was reported by Lü *et al*. (2009a) and they speculated that broilers may develop an advanced capacity to clear MEL from the body with increasing age.

Lü *et al.* (2009a) reported that MEL was undetectable in meat samples within seven days after withdrawal of MEL from the feed, and Bai *et al.* (2010) detected a very low level of 0.0025 mg/kg MEL in meat 10 days after withdrawal. Yuchang *et al.* (2010) reported that a single feeding of feed that contained 100 mg/kg of MEL was eliminated within 24 hours in chicken and duck meat and up to 96 hours in other animal species, except for fish (up to 14 days). In the current study, no MEL residues could be detected in muscle tissue after a seven day withdrawal period. It might have been more useful to determine the MEL residues in meat within a shorter withdrawal period (i.e. 1 or 2 days after withdrawal) instead of 7, 14 and 18 days. Nevertheless, the results were comparable to that of Lü *et al.* (2009a) and therefore supporting the fact that MEL concentrations of < 0.05 mg/kg (if any) might be present in poultry meat after a withdrawal time of 7 days, but that such low levels are of no concern for human consumption.

No MEL could be detected in the meat samples of CYR4 and similar results were reported by Boon *et al.* (1985). Many reports discussing the detection of CYR in meat samples after ingesting a diet containing CYR are available; however, very few reports are available that report on the distribution of MEL in meat due to the inclusion of CYR in the diet. It has been reported that 10% of ingested CYR is metabolizable to MEL (US EPA, 2007). In the current study 4 mg/kg CYR was applied to CYR4 and therefore, theoretically 0.4 mg/kg MEL could have been metabolized to MEL. Based on the results of MEL50, which resulted in a muscle MEL concentration of 7.13 mg/kg (Table 9), and assuming a similar distribution efficiency, it was calculated that 0.4 mg/kg MEL originating from the metabolization of 4 mg/kg CYR, would theoretically yield a residue concentration of 0.057 mg/kg. If the current tolerable daily intake for humans is 0.2 mg/kg body weight (WHO, 2008), any possible MEL residues in broiler muscles resulting from CYR metabolization are no reason for concern. Therefore, if the current recommendations for the application of the CYR product Larvadex® (1% premix) in poultry feed are followed, muscle MEL residues should be well within tolerable limits.

3.4.3. Melamine concentrations in liver and kidneys

From the results, it is evident that the kidneys contained a significantly higher ($P < 0.01$) concentration of MEL, followed by muscle and liver tissues (Figure 6). It was once again evident that an increase in the MEL dietary level resulted in an increased MEL distribution to all tissues. Similar findings were reported by Lü *et al.* (2009a), Bai *et al.* (2010) and Yuchang *et al.* (2010) where the kidney had higher residual levels confirming the findings that there is a difference between the distribution patterns of MEL to different body tissues. Even though it was not statistically significant, muscle tissues were found to contain slightly more MEL than the liver. Lü *et al*. (2009a) and Bai *et al.* (2010), on the other hand, reported definite findings that the MEL concentration was higher in the liver than the muscle tissue. No MEL from CYR could be detected in any of the tissue samples.

3.4.4. Distribution efficiency (DE_f)

Other relevant studies (Lü *et al*., 2009a; Bai, *et al.*, 2010 and Yuchang *et al.*, 2010) reported the distribution of MEL into muscle tissue as a ratio of the concentration MEL in the feed. However, these finding do not take into account the actual amount of MEL ingested that is retained in the muscle. Therefore, the method used by Cruywagen *et al.* (2010) was followed where the MEL values obtained are expressed as a fraction of the MEL ingested. A retention time of 7 days was used as reported by Lü *et al.* (2009). In the current study, the DE_f of MEL to meat was 1.3 to 2.7%. Cruywagen *et al.* (2009) reported an efficiency value of 2.1% in milk and 3.6% in muscle tissue of Merino sheep (Cruywagen *et al.*, 2010).

From Figure 7 it can be seen that the treatment with the lowest MEL content (MEL50) resulted in the highest efficiency rate, suggesting that lower dietary MEL levels would be distributed to meat more efficiently than higher levels. Kidd *et al.* (1996) observed similar tendencies for protein utilization. In the current study a significant increase ($P < 0.05$) in efficiency rates was observed for Day 22 in all treatments, followed by an even decrease in Days 29 and 36. It could probably be ascribed to the rapid muscle growth rate from around Day 20 and thus also explaining the reason for the higher value in MEL100 on Day 29 since a slight increased growth rate was observed for this group.

According to the results, it is calculated that, if a 36 day old broiler is fed a diet containing 100 mg/kg MEL and consumes 168 mg/kg feed a day, the total MEL deposited in the muscle fraction of the whole chicken would contain 1.456 mg MEL. These values are within the detection limit of 2.5 mg/kg (Setiogi, 2008) that are allowed for food. However broiler diets containing 500 mg/kg MEL revealed values of 7.493 mg/kg and therefore MEL inclusions in broiler diets above 100 mg/kg should be avoided to ensure that MEL concentration levels are below the legal limit for food.

3.5. Conclusion

The current trial showed that dietary MEL levels of up to 500 mg/kg did not have an effect on production parameters. It has been confirmed that MEL could be distributed to breast meat within two days after chickens had ingested MEL tainted diets. As the dietary MEL concentration increased from 50 and 100 mg/kg to 500 mg/kg, a significant increase was observed in tissue MEL residue concentrations. A peak MEL concentration was observed at 22 days of age for all treatments, followed by a decrease until day of slaughter, which might indicate that broilers developed an enhanced capacity with advancing age to clear MEL from their system. After a seven day withdrawal time, no more MEL could be detected in meat samples. As expected, the kidneys contained the highest MEL residue levels, followed by the muscle and liver tissues. Furthermore, no MEL could be detected in meat samples of broilers subjected to a diet containing 4 mg/kg CYR. Finally, distribution efficiencies of MEL to meat were determined and these values were comparable to those reported for other species (Merino *L. dorsi*) and products (Holstein milk).

The illegal MEL contamination of food products will surely persist until such products can be banned from every country; however, the realization of this suggestion is unlikely. Even though doctors and veterinarians agree that no MEL should be included in food products for human and animal consumption, tolerable daily intake levels have been implemented in an attempt to control MEL adulteration. Therefore, the current study is considered to be relevant and was intended to provide knowledge on the inclusion of MEL in poultry feed. For future studies it might be useful to harvest meat samples earlier than two days after exposure to determine how fast MEL would occur in meat

samples. However, birds should preferably not be younger than 10 days, since the harvested samples might be too small to analyze. Also, it would be interesting to determine if any detectable MEL residues are still present in meat within a shorter withdrawal period than seven days to set an accurate withdrawal period if commercial birds have accidentally been fed a diet containing MEL contaminated raw materials. This will also be useful to determine a more accurate DE_f.

3.6. References

Bai, X., Bai, F., Zhang, K., Lv, X., Qin, Y., Li, Y., Bai, S. and Lin, S., 2010. Tissue deposition and residue depletion in laying hens exposed to melamine-contaminated diets. J. Agric. Food. Chem. 58: 5414-5420.

Brake, J., Ort, J.F. and Carter, T.A., 1984. Effect of the insect growth regulator CGA-72662 (Larvadex®) on broiler breeder production, hatchability, and subsequent chick performance. J. Poult. Sci. 63: 910-916.

Brake, J., Berry, W.D., Peebles, E.D. and Ort, J.F., 1985. Effect of feeding CGA-72662 (Larvadex®) during growth on body weight, feed consumption, livability, production, and egg quality of single comb white leghorn layers. J. Poult. Sci. 64: 247-252.

Brake, J. and Ort, J.F., 1989. Effects of CGA-72662 (Larvadex®) in turkeys during rearing and reproduction. J. Poult. Sci. 68: 731-738.

Boone, T. and Cheung, M.W., 1985. Residues of cyromazine and melamine in chicken tissues and eggs resulting from the feeding of cyromazine in the diet. Unpublished Report. Ciba-Geigy Corp. Greensboro. United States. Report No. ABR-84082.

Briedenhann, E., 2010. Chairman's report 2009/2010. Animal feed manufacturers association. Available: http://www/afma.co.za/imgs/AFMA%20-%20Chairman's%20Report%202009_10.pdf. Accessed: November, 4, 2010.

Brown, C.A., Jeong, K.S., Poppenga, R.H., Puschner, B., Miller, D.M., Ellis, A.E., Kang, K.I., Sum, S., Cistola, A.M. and Brown, S.A., 2007. Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. J. Vet. Diagn. Invest. 5: 525-531.

CBC News, 2007. Tainted fish feed was used in Canada: B.C. minster. Available: http://www.cbc.ca/canada/story/2007/05/09/fish-feed.html Accessed: July, 14, 2010.

Cecil, H.C., Miller, R.W. and Corley, C., 1981. Feeding three insect growth regulators to white leghorn hens: residues in eggs and tissues and effects on production and reproduction. J. Poult. Sci. 60: 2017-2027.

Chen, Y., Yang, W., Wang, Z., Peng, Y., Li, B., Zhang, L. and Gong, L., 2010. Deposition of melamine in eggs from laying hens exposed to melamine contaminated feed. J. Agric. Food. Chem. 58: 3512-3516.

Cruywagen, C.W. and Reyers, F., 2009. The risk of melamine contaminated ingredients in animal feeds. AFMA Matrix. 18: 4-6.

Cruywagen, C.W., Stander, M.A., Adonis, M. and Calitz, T., 2009. Hot topic: Pathway confirmed for the transmission of melamine from feed to cow's milk. J. Dairy. Sci. 92:2046.

Cruywagen, C.W., van de Vyver, W.F.J. and Stander, M.A., 2010. Quantification of melamine absorption, deposition and excretion by sheep. Unpublished report. Department of Animal Science. Stellenbosch University.

Drugs.com., 2010. Larvadex 1% Premix. Drug information online. Available: http://www.drugs.com/vet/larvadex-1-premix.html. Accessed: Nov, 19, 2010.

Filigenzi, M.S., Puschner, B., Aston, L.A., Poppenga, R.H., 2008. J. Agric. Food. Chem. 56: 7593.

Hau, A.K., Kwan, T.H. and Li, P.K., 2009. Melamine toxicity and the kidney. J. Am. Social. Nephr. 20: 245-250.

Kidd, M.T. and Kerr, B.J., 1996. Growth and carcass characteristics of broilers fed low-protein threonine-supplemented diets. J. App. Poult.Sci. 5: 180-190.

Ledoux, D.R., Zavarize, K.C., Murarolli, R.A., Kutz, R.E., Rottinghaus, G.E., Bermudez, A.J. and Lin, M., 2009. Effects of graded levels of melamine in young broiler chicks. University of Mussouri. Columbia.

Lin, R.S., Chen, L.R., Huang, S.C., Liu, C.Y., 2001. Electromagnetic scanning to estimate carcass lean content of Taiwan native broilers. Meat. Sci. 61: 295-300.

Lü, M.B., Yan, L., Guo, J.Y., Li, Y., Li, G.P. and Ravindran, V., 2009a. Melamine residues in tissues of broilers fed diets containing graded levels of melamine. Poult. Sci. 88: 2167-2170.

Lü, M.B., Uan, L., Guo, J., Sun, Z. and Zhu, S., 2009b. Melamine residues in tissues of ducks fed diets containing graded levels of melamine. Research and development center. Liuhe Feed Co. Ltd. Qingdao. Shandong. China.

Merck, 2001. Entry 5834. IN The Merck Index 13th ed. Merck Research Laboratories. Whitehouse Station. New York. P. 1038.

Perić, L., Milošević, N., Zikić, D., Kanački, Z., Džinić, N., Nollet, L. and Spring, P., 2009. Effect of selenium sources on performance and meat characteristic of broiler chickens. J. Appl. Poult Res. 18: 403-409.

Setiogi, S., 2008. Experts set tolerable level for melamine intake. World Health Organization. Geneva. Available: http://www.who.int/mediacentre/news/releases/2008/pr48/en/print.html. Accessed: Aug, 14. 2010.

Shai, J., Mallet, C., Young, M., Li, J., Meng, Y. and Qi, C., 2008. Application note: Rapid specific analysis of melamine contamination in infant formula and liquid milk by UPLC/MS/MS. Waters Corporation, Milford, MA.

Smoker, M. and Krynitsky, A.J., 2008. Interimmethod for determination of melamine and cyanuric acid residues in foods using LC-MS/MS: Version 1.0. *U.S. Food and drug administration.* Available: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/DrugChemical ResiduesMethdology/ucm071673.htm. Accessed: August, 15, 2010.

Statistica Version 9, 2010. StatSoft Inc. USA.

United States Environmental Protection Agency, 2007. Cyromazine: Registration review summary document. Washington. D.C. United States Environmental Protection Agency. Available: http://www.regulations.gov/fdmspublic/component/main?main=DocumentDetai&d=EPA-HQOPP-2006-0108-0003. Accessed: July, 21, 2010.

Vauqulin, C., 2010. Report of the broiler organisation committee. Available: http://www.sapoultry.co.za/2010%20 DOWNLOADS/AviAfrica%202010/2010%20BROILE %20REPORT.pdf.

Weise, E. and Schmit, J., 2007. FDA: Feed no human threat. USA Today, May, 2, 2007. ISSN: 07347356. Available: http://search.ebscohost.com.ez.sun.ac.za/login.aspx?direct=true& db=aph&AN=J0E1222761 31707&site=ehost-live. Accessed: July, 22, 2010.

Wilson, H.R., Harms, R.H., Simpson, C.F. and Campbell, W.R., 1983. Effect of high levels of Larvadex® on reproduction in leghorn breeders. J. Poult. Sci. *62*: 1731-1733.

World Health Organization, 2008. Expert meeting to review toxicological aspects of melamine and cyanuric acid. Available: http://www.who.int/foodsafety/fs_management/Exec_Summary_ melamine.pdf. Accessed: July, 24, 2010.

Xia, X., Ding, S., Li, X., Gong, X., Zhang, S., Jiang, H., Li, J. and Shen, J., 2009. Validation of a confirmatory method for the determination of melamine in egg by gas chromatography–mass spectrometry and ultra-performance liquid chromatography–tandem mass spectrometry. Anal. Chimica. Acta. 651: 196-200.

Yuchang, Q., Xiaowen, L., Jun, L., Guanghai, Q., Qiyu, D., Guohua, L., Min, X., Jiaqi, W., Jianming, T., Liying, Z. and Keying, Z., 2010. Assessment of melamine contamination in crop, soil and water in China and risks of melamine accumulation in animal tissues and products. Environ. Inter. *36*: 446-452.

CHAPTER 4

Absorption of dietary melamine and cyromazine and subsequent distribution to poultry eggs

4.1. Introduction

In the year 2006, hundreds of pets have fallen ill (WHO, 2008) due to unknown reasons after consuming certain brands of pet food. The problem was soon identified to be caused by the presence of the industrial chemical melamine (MEL). Then in 2008, after it was confirmed once again that MEL was the main contaminant in infant formula, causing renal failure in thousands of infants, global attention was focussed on this substance and its inclusion in consumer food products (WHO, 2008). In the industry MEL ($C_3H_6N_6$) or 1,3,5-triazine-2-4-6 triamine, has various applications and is primarily known for its use in plastic ware (China Chemical Reporter, 2006). Since MEL has a nitrogen content of 670 g/kg on a molecular weight basis (Merck, 2001), it has been included in food products (especially animal feeds) with a deceptive purpose to increase the apparent crude protein level of feed (AOAC, 2000).

The major illness effect of MEL is renal failure due to crystal formation in the proximal tubules, effectively causing blockage (Kobayashi *et al.*, 2010). In the poultry layer industry hens are productive until approximately 72 weeks of age and therefore the effect of renal complications can have a major influence on overall performance efficiency. Melamine is also an intermediate compound of cyromazine (CYR) metabolism in the animal. Cyromazine is the active ingredient of some pesticides, such as Larvadex ®, which is commonly added to poultry layer feed to control flies (Sancho *et al.*, 2005). Larvadex® can conveniently be added to layer diets after which it is passed in the faeces where the fly eggs are laid. Therefore, by including products that contain CYR in layer feed, MEL could potentially occur in eggs even though no MEL has been added to the feed.

Relevant literature on the current study field is limited to only a handful of contributing authors (Lu *et al.*, 2009; Bai *et al.*, 2010; Chen *et al.*, 2010; MAFF, 2010 and Yuchang *et al.*, 2010). Two similar

studies have also been executed on layer ducks (Gao *et al.,* 2010 and Yuchang *et al*., 2010) and definite MEL deposition tendencies exists for the two layer species as will be discussed later. However, further research would be beneficial to enhance the reliability of the reported results to provide more accurate and consistent technical support to the industry regarding this matter.

It has been reported that over 17 million cases of eggs have been produced during 2009 in South Africa (Vauqulin, 2010). Poultry and duck eggs are used in a wide variety of food products which means that the majority of the global population could potentially be exposed to MEL contaminated eggs. As a result, it is important to establish the distribution rate of MEL to eggs after ingesting contaminated feed, emphasising the necessity of the current study. The objectives of the current study were:

1. to determine the distribution efficiency of MEL from feed to eggs when MEL was added to layer diets at different levels, ranging from 0 to 500 mg/kg.
2. to determine if a MEL withdrawal period would result in eggs with non-detectable levels of MEL and how long it would take
3. to determine if CYR in the feed would result in MEL being detected in eggs had been withdrawn
4. to determine if dietary MEL and CYR would have an effect on layer production.

4.2. Materials and methods

All harvesting methods were practised strictly according to the ethical requirements that have been approved by Subcommittee B of the University of Stellenbosch (Reference # 2008B03003). The Larvadex® product contains 99% inert products and only 1% CYR (Drugs.com, 2010). Since it was recommended by the suppliers that Larvadex® should be included at 400 mg/kg, CYR4 therefore effectively received 4 mg/kg CYR which is below the maximum legal level of 5 mg/kg (Drugs.com, 2010).

4.2.1. Birds, housing and management

One hundred and twenty Hyline Silver hens (24 weeks of age), provided by the Mariendahl Poultry unit from the University of Stellenbosch, were relocated for trial purposes from a commercial unit to an experimental house. At the end of the trials, all participating hens were euthanized according to the ethical protocol. The trial was conducted during the summer months and hens were allowed three days to adapt to their new surroundings. The birds were exposed to a similar housing system (open houses and natural ventilation with sprayers on the roof for additional cooling) and received the same diet, lighting regime (16 hours light and eight hours darkness) and climate control than what they were accustomed to. Four hens were allocated per cage and six cages per treatment were used.

4.2.2. Treatments and experimental diets

1. Treatment 1 (CON) received a diet containing no detectable levels of MEL or CYR.
2. Treatment 2 (MEL50) received a diet containing 50 mg/kg MEL and no CYR.
3. Treatment 3 (MEL100) received a diet containing 100 mg/kg MEL and no CYR.
4. Treatment 4 (MEL500) received a diet containing 500 mg/kg MEL and no CYR.
5. Treatment 5 (CYR4) received a diet containing 4 mg/kg CYR and no MEL.

Diets were mixed on the Mariendahl Experimental Farm of the Stellenbosch University. Maize gluten meal imported from China found to be an adulterated product (Cruywagen & Reyers, 2009), was used as one of the MEL sources in the diets. The MEL content of the product was 15 117 mg/kg. It also contained urea, wheat bran, maize bran, gluten 20 and actually very little gluten 60. Because of the nature of the product it was thus decided to limit the inclusion level thereof and to balance the required MEL content of the diets with pure MEL (Sigma-Aldrich M2659, Supplied by Sigma, Cape Town). The minor ingredients were carefully weighed out and mixed in an industrial blender with a 10 kg capacity. The mixture was then transferred to a horizontal ribbon mixer (100 kg capacity) where the major ingredients were then added. First, the CON treatment diet was mixed followed by the CYR4, MEL50, MEL100 and lastly the MEL500 diet to avoid contamination between diets. All feeds were presented as a meal and stored in a cool dry area in 50 kg bags. At the end of Day 3, just

before the lights went out, the five diets were made available to the hens. The experimental diets were offered to the hens until Day 10 after which they all received a control diet. The formulations and calculated nutritive analysis for the five different diets are presented in Table 12.

Table 12 The composition and calculated analysis (g/100g as fed) of layer treatment diets.

	Layer treatment[1] diet				
Item	1	2	3	4	5
Ingredient					
Maize Gluten 60[2]	-	0.33	0.67	3.33	-
Melamine[3]	0.00	0.005	0.01	0.05	0.00
Larvadex	-	-	-	-	0.04
Vit+Mineral Mix[4]	0.15	0.15	0.15	0.15	0.15
Maize	52.3	44.9	52.0	51.2	48.9
Wheat Bran	-	6.84	-	-	-
Soybean Full Fat	25.2	34.8	24.7	22.6	29.2
Soybean 46	9.33	-	9.42	9.79	-
Sunflower	-	-	-	-	8.65
L-lysine HCl	0.06	0.08	0.07	0.08	0.15
DL-methionine	0.19	0.19	0.18	0.15	0.17
Limestone	10.6	10.7	10.6	10.6	10.6
Salt	0.27	0.28	0.27	0.26	0.25
Monocalcium Phosphate	1.68	1.54	1.68	1.68	1.66
Sodium Bicarbonate	0.29	0.27	0.29	0.29	0.29
Calculated Analysis					
AMEn[5] (MJ/kg)	11.50	11.50	11.50	11.50	11.50
Crude Protein	18.28	18.28	18.28	18.28	18.28
Calcium	4.20	4.20	4.20	4.20	4.20
Total phosphorus	0.77	0.77	0.77	0.77	0.77
Available phosphorus	0.50	0.50	0.50	0.50	0.50
Digestible lysine	0.94	0.94	0.94	0.94	0.94
Digestible methionine	0.44	0.44	0.44	0.44	0.44
Digestible threonine	0.60	0.60	0.60	0.60	0.60

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[2]The maize gluten 60 contained melamine at a level of 15117 mg/kg.
[3]Melamine powder (Sigma-Aldrich M2659, Supplied by Sigma, Cape Town).
[4]Vitamin+mineral premix provided (per kg of diet): 8160 IU vitamin A, 1700 IU vitamin D3, 30.6 IU vitamin E, 2.7 mg vitamin K3, 2.05 mg vitamin B1, 2.05 mg vitamin B2, 27.2 mg niacin, 10.2 mg calcium pantothenate, 0.02 mg vitamin B12, 4.1 mg vitamin B6, 1.7 mg folic acid, 0.068 mg biotin, 120 mg ronozyme p500, 350 mg choline, 0.08 mg I, 0.34 mg Co, 0.2 mg Se, 70 mg Mn, 70 mg Zn, 6 mg C and 50 mg Fe.
[5]AMEn = Apparent metabolizable energy.

Samples of all experimental diets were analysed to determine the true MEL inclusion levels by using the LC-MS/MS method (Table 13). Samples (100 g) were milled with a 1 mm screen prior to evaluation.

Table 13 The expected and analyzed values (mg/kg) of the melamine diets for trial purposes.

Treatment[1]	Expected values	Analyzed values
1	0	ND
2	50	56.50
3	100	130.8
4	500	531.7
5	0	1.971

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.

The MEL50, MEL100 and MEL500 birds received the diets as described above from Days 1-10 to determine if dietary MEL would result in MEL residues in the eggs and to determine the distribution efficiency. As from Days 11-20, MEL 50, MEL 100 and MEL 500 groups were switched back to a control diet identical to the diet fed to the CON treatment to determine if a MEL withdrawal period would result in eggs with non-detectable levels of MEL.

Hens in CYR4 received a diet containing Larvadex, as mentioned above, and were compared to the CON treatment. The justification for the CYR4 group was to determine if MEL (derived from the metabolism of CYR, the active ingredient in Larvadex) was distributed to eggs and to determine the efficiency of the distribution. The rate of depletion in the absence of Larvadex was not tested, since previous trials conducted by the University revealed low distribution rates in the first instance. Therefore, CYR4 received the Larvadex diet for Days 1-20.

4.2.3. Measurements and sampling

The trial was conducted during the summer months and temperature readings were recorded daily to explain possible changes for feeding patterns. All birds were weighed prior to the experimental initiation and again on the last day of the experimental period (20 days). Feed intake was measured for each cage separately throughout the duration of the trial. Mortality and morbidity were monitored and reported daily. Eggs were collected once every day at the same time; weighed individually and then marked with a pencil to indicate the date, weight, cage and treatment number. One cage's eggs represented one experimental unit to account for birds who would skip a day. The collected eggs were stored below 25°C for 28 days until relocation to a cold room (4°C) for 1-9 days prior to the beginning of laboratory analysis.

4.2.4. Laboratory analyses

All the eggs were individually weighed again and the egg contents were cracked into a polypropylene jar, previously tared on a scale and the weight of each egg was recorded in this manner. The reason why the eggs were weighed again prior to breakage was to determine moisture loss from the fresh eggs until sample processing for consumer purposes. The egg contents were individually homogenized with a Polytron (Patent–Lizenz Prof. P. Willems, Luzern Kinematisches Hochfrequenz – Gerät, Type: PT45/50 OD no. 4860, Volt 220, Amp 5, Hz 50) and poured into marked plastic bags. One egg from each cage of MEL500 (collected on Day 10) was separated to estimate MEL distribution ratio to the albumin and yolk and were treated the same as the whole egg samples. The bagged samples were stored at -18°C until further laboratory analyses commenced.

Eggs collected on Days 1, 2, 3, 4, 5, 7, 10, 11, 12, 14 and 16 were selected to be analyzed for MEL detection. The eggs collected for each cage were pooled together to represent an experimental unit and were homogenized once again to establish an even mixture. The following chemicals, equipment and sample preparations (as described by Xia *et al.*, 2009) were necessary before the LC-MS/MS analysis:

Chemicals and Reagents:

- Egg sample (2 g)
- 5% Trichloroacetic acid
- Methanol (12 mL)
- Water (6 mL)
- 100 µL of a 0.5 mg/L stable isotope labelled MEL ($^{13}C_3H_6{}^{15}N_3$) internal standard solution (Cambridge Isotope Laboratories, Inc., Andover, MA).
- 0.1N HCl (6 mL)
- 6 mL ammonium hydroxide:methanol:dichloromethane (1:5:5)
- 1 mL acetonitrile (50%)
- Nitrogen

Other Consumables and Equipment:

- Scale (Mettler AE 160)
- Micro pipette (5 mL)
- 5 mL Pipette tips
- Vortex (Heidolph, Batch no: 10834, 230 V ~ 50 Hz, 30 Watt, U/min 200-2400, Made in West Germany)
- Tubes (15 mL polypropylene and 15 mL glass)
- Centrifuge (Sigma Laborzentrifugen, D-37520 Ost-rode am Harz, Germany, Type Model 2-16K, Fabrik no: 112234, V/Hz 230/50 1 Ph, 720 Watt)
- Ultrasonic bath
- Cation exchange solid phase extraction (SPE) cartridges (Phenonenex Strata SCX, 55 µm, 70 A, 500 mg/3 mL, supplied by Separations, Randburg, South Africa)
- Waters API Quattro Micro triple quadruple mass spectrometer
- Waters 2690 HPLC (Waters Corporation, Milford, MA).

Sample Preparation:

- A representative sample (2 g) was collected from each mixture and transferred to a 20 mL tube.
- 5% Trichloroacetic acid (10 mL) was added and the mixture was vortexed for one minute.
- The mixture was then centrifuged (4°C) at 8603 x g for 10 minutes.
- The supernatant (5 mL) was transferred to clean marked tubes and frozen at -18°C.

Method:

- Condition the cation exchange SPE cartridges with 6 mL methanol followed by 6 mL water.
- Load the supernatant of the extracts (5 mL) and 100µL of a 0.5 mg/L stable isotope–labelled MEL ($^{13}C_3H_6{}^{15}N_3$) internal standard solution onto the SPE cartridges. Therefore, 0.05µg MEL is loaded onto each cartridge.
- Wash the cartridges with 6 mL of 0.1 *N* HCL and subsequently with 6 mL methanol while aspirated under vacuum for one minute.
- Elute the MEL with 6 mL ammonium hydroxid:methanol:dichloromethane (1:5:5) into a clean tube.
- Dry the extracts under a nitrogen stream and resuspend it in 1 mL of 50% acetonitrile.
- Analyse the samples with liquid chromatography–tandem mass spectrometry (LC–MS/MS) on the Waters API Quattro Micro triple quadruple mass spectrometer, coupled to a Water 2690 high performance liquid chromatographer (HPLC).
- For egg samples, the detection limit of this method is 0.01 mg/kg.
- Use the internal standard to correct for possible incomplete extractions.

4.2.5. Statistical analyses

All of the production data were subjected to a two-way analysis of variance (ANOVA) using the general linear models (GLM) procedure and were analysis with the Statistica Version 9 (2010) package. The repeated measure of analysis method was conducted on the MEL concentrations data. Mixed models were used to compare distributed MEL concentrations in the yolk, albumin and whole egg and a one-way ANOVA to calculate differences between the distribution efficiency rates.

4.3. Results

4.3.1. Production parameters

The trial was conducted during the summer months and environmental temperature had a definite effect on body weight and feed intake. Maximum temperatures fluctuated between 27-40°C and night temperatures were 13-22°C while most of the temperature readings above 35°C were recorded during the last five days of the trial. All treatment groups lost between 2-5% of their total body weight during the 20 day study. Results are reported in Table 14.

Table 14 The average body weight (kg) measured[1] for treatments on the first and last day of the trial.

	Day		
Treatment	1	20	*P*
1	7.8[a]	7.4[b]	0.05
2	7.8[a]	7.4[b]	0.05
3	7.6[a]	7.4[b]	0.05
4	7.7[a]	7.5[b]	0.05
5	7.9[a]	7.5[b]	0.05

[1]Mean body weight of six cages.
[a-d]Means with different superscripts differed significantly ($P < 0.05$).

No differences were found between treatments; however, weight on Day 1 differed significantly ($P < 0.05$) from weights on Day 20. The greatest weight loss (5%) was reported for CON and CYR4 while MEL100 and MEL500 only lost 2% of their body weight.

Feed intake for all treatments declined during the last 10 days of the trial and could be contributed to the high ambient temperatures. No interaction between treatments and days were found; however, the treatments ($P < 0.05$) as well as the days ($P < 0.01$) differed significantly from each other (Table 15).

Table 15 The average feed intake (kg) of an experimental unit during the first and second stage of the trial. Layer hens were subjected to graded levels of melamine as well as cyromazine over a period of 20 days.

	Day	
Treatment	1-10	11-20
1	$4.3^{a,x}$	$3.4^{b,x}$
2	$4.5^{a,xy}$	$3.6^{b,xy}$
3	$4.7^{a,y}$	$3.6^{b,y}$
4	$4.2^{a,x}$	$3.5^{b,x}$
5	$4.6^{a,y}$	$3.6^{b,y}$

[a-b]Means in a row with different superscripts differed significantly ($P < 0.01$).
[x-y]Means in a column with different superscripts differed significantly ($P < 0.05$).

It is evident from Table 15 that hens in MEL100 and CYR4 consumed the most feed during the first 10 days of the study. There was a significant difference ($P < 0.01$) between Days 10 and 20 where all the treatments showed a decrease in feed intake over time. From all of these findings it is probable that the high environmental temperatures suppressed feed intake and body weights during the last 10 days of the trial.

Table 16 The total daily egg production for treatments over a 20 day trial.

	Day																				
[1]Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total
1	21	23	23	23	24	24	24	24	24	22	23	23	23	23	23	23	22	23	22	22	459[a]
2	24	23	20	23	23	22	23	23	23	23	23	23	23	23	22	22	21	23	23	23	453[a]
3	24	23	22	22	23	24	24	24	24	24	23	24	24	23	24	24	23	22	22	23	466[a]
4	24	23	22	24	24	24	24	24	24	21	24	23	21	22	20	22	22	21	22	21	452[a]
5	20	22	23	20	22	21	21	21	21	21	21	21	20	21	21	21	20	19	20	19	415[b]
Total	113	114	110	112	116	115	112	116	112	111	114	114	111	112	110	112	108	108	109	108	

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[a-b]Means in a column with different superscripts differed significantly ($P < 0.01$).

Table 17 The average egg weight (g) for each treatment over the 20 day trial period.

	Day				
Treatment	1-5	6-10	11-15	16-20	Average
1	52.8[abc,uv]	52.7[abcde,uv]	53.2[defg,vw]	53.7[efg,wx]	53.1
2	52.9[abc,uv]	53.3[abcde,vw]	53.8[defg,wx]	55.0[efg,z]	53.7
3	51.6[abc,t]	52.8[abcde,uv]	53.7[defg,wx]	53.9[efg,wx]	53.0
4	51.2[abc,t]	52.5[abcde,uv]	53.2[defg,vw]	53.1[efg,vw]	52.5
5	50.5[abc,s]	53.1[abcde,vw]	54.0[defg,xy]	54.8[efg,yz]	53.1

[1]Trt = Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500mg/kg; 5 = dietary cyromazine 4 mg/kg.
[a-g]Means in a row with different superscripts differed significantly ($P < 0.05$).
[s-z]Means in a column with different superscripts differed significantly ($P < 0.05$).

Only two birds died (CON and MEL 500) during the whole study while one hen was morbid (MEL50) and was removed from the trial. Regarding egg production, no differences were found over time. There was, however, a tendency for production to decrease from Day 8 to Day 20 (Table 16). A significant difference ($P < 0.01$) was found between treatments and throughout the trial CYR4 produced the least number of eggs, while egg production did not differ between CON, MEL50, MEL100 and MEL500. Egg weight differed between treatments ($P < 0.05$), as well as between days ($P < 0.05$) (Figure 8). MEL500 had the lowest average egg weight compared to CON, MEL50, MEL100 and CYR4 (Table 17). There seemed to be an increase in egg weight during the trial.

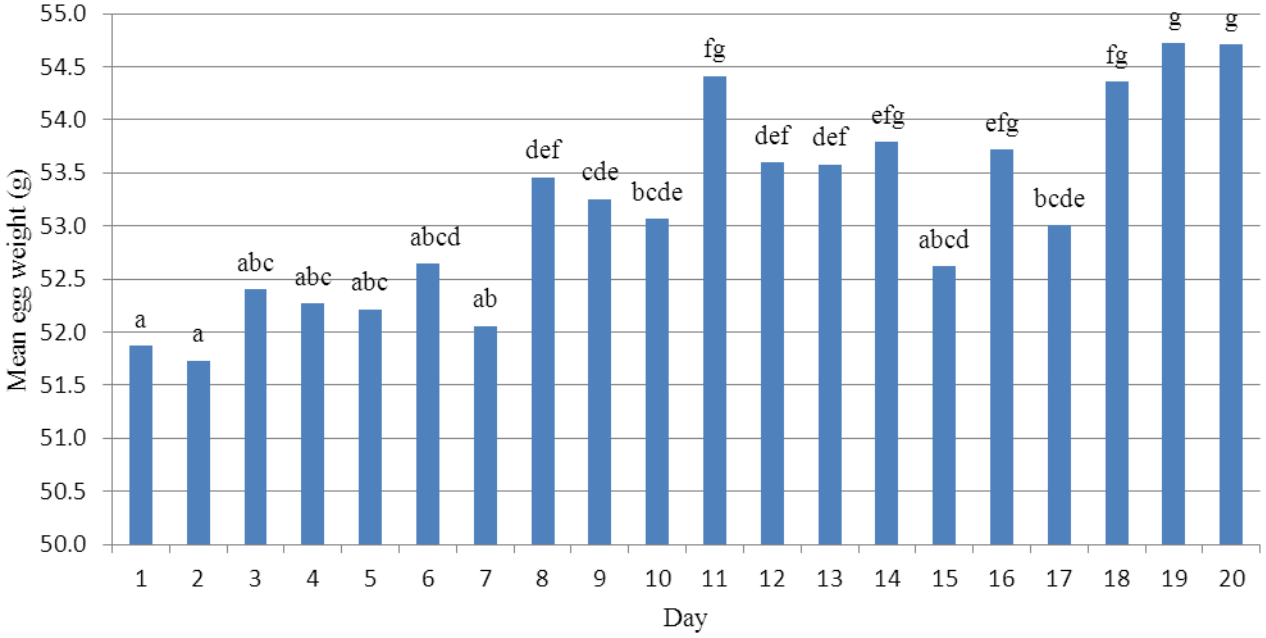


Figure 8 The mean egg weight (g) of each treatment combined to illustrate the effect of time (day) on egg weight. [a-g]Means with different superscripts differed significantly ($P < 0.05$).

As a slight decrease in egg number over time was observed, the tendency for egg weight to increase with time as revealed in Figure 8 seems sensible. Egg weight content was also measured and it attributed 86% of the total egg weight for all treatments.

4.3.2. Melamine concentrations in eggs

The data that were collected for the MEL concentrations in the eggs are displayed in Table 18. Days 1, 2, 3, 4, 5, 7 and 10 were tested to determine the manner of MEL distribution and Days 11, 12, 14 and 16 were evaluated to establish a withdrawal rate after MEL ingestion. From the statistical analyses there appeared to be significant interaction ($P < 0.01$) between the treatments and days. In Figure 9 it is clear that MEL was deposited in eggs within 24 hours after ingestion. There was an increasing trend for MEL distribution in eggs as the dietary MEL content increased.

Table 18 The concentration of melamine (mg/kg dry weight) in layer eggs after feeding diets containing graded levels of melamine over time.

Treatment[1]	Day										
	1	2	3	4	5	7	10	11	12	14	16
1	ND[2]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	4.4 ± 0.5[3]	1.4 ± 0.8	2.7 ±1.0	2.8 ± 1.5	1.8 ± 0.8	1.5 ± 0.2	0.8 ± 0.4	0.9 ± 0.3	0.1 ± 0.02	0.1 ± 0.02	0.04 ± 0.02
3	4.5 ± 0.9	2.4 ± 0.7	2.9 ± 0.6	3.7 ± 0.5	2.2 ± 1.3	3.2 ± 0.5	2.3 ± 1.2	2.6 ± 0.5	0.5 ± 0.08	1.0 ± 0.1	0.02 ± 0.01
4	3.1 ± 1.2	16.3 ± 9.4	14.7 ± 2.3	16.7 ± 2.8	13.1 ± 2.4	12.9 ± 1.4	9.0 ± 2.9	4.3 ± 3.9	0.9 ± 4.1	0.3 ± 0.04	0.04 ± 0.01

[1]Treatments: 1 = no added melamine; 2 = dietary melamine 50 mg/kg; 3 = dietary melamine 100 mg/kg; 4 = dietary melamine 500 mg/kg; 5 = dietary cyromazine 4 mg/kg.
[2]Not detected.
[3]Data are displayed as means ± SD (n = 6).

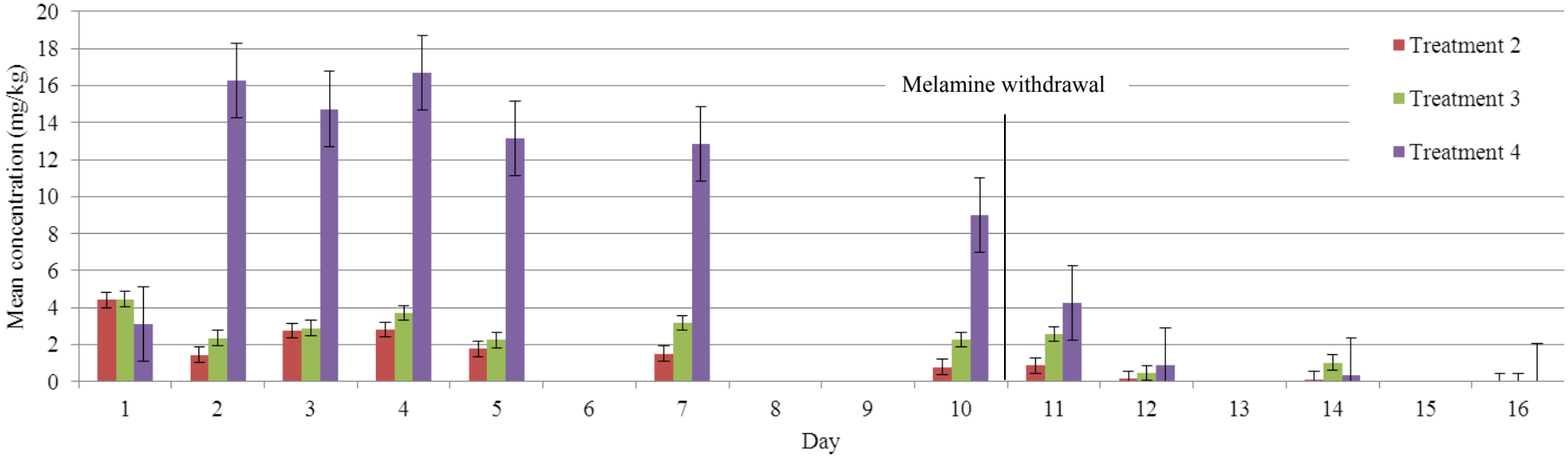


Figure 9 An illustration of the mean concentrations (mg/kg) of melamine in eggs after ingesting diets containing graded levels of melamine over time (day).

From Figure 9, MEL500 differed significantly ($P < 0.05$) from MEL50 and MEL100 except for Days 1, 12, 14 and 16. The major differences between MEL50 and MEL100 occurred on Days 12, 14 and 16. It seems as if MEL levels peaked between Day 1 and Day 4 and then slowly decreased until Day 10. The residual concentration of MEL rapidly decreased after the withdrawal period was initiation at the end of Day 10. On Day 16, MEL levels for all treatments were below 0.05 mg/kg. No MEL could be detected as a result of CYR ingestion and therefore no statistical analysis was performed on the latter.

4.3.3. A comparison of the melamine concentrations detected in albumin, yolk and whole egg

No differences were found between treatments regarding MEL concentration in albumin (9.586 mg/kg), yolk (7.710 mg/kg) and whole egg (8.460 mg/kg).

4.3.4. Distribution efficiency rate (DE_f)

A DE_f of MEL to eggs was established by applying the following calculation:

Distribution efficiency (%) = MEL residues (mg) in total egg / MEL intake (mg) per day

Results were calculated on an as is basis since the MEL concentration results were determined on a similar basis, as well as egg content measurements. To calculate the MEL distributed to the total egg, the weight (kg) of the whole egg (without the shell) were multiplied with the detected MEL concentration (mg/kg). The daily MEL intake was calculated by multiplying the daily feed intake (kg) with the MEL inclusion level (mg/kg). Unlike the DE_f rate that was calculated in Chapter 3 for meat, no assumptions were necessary for the DE_f calculation in eggs, since all the parameters were measured.

No difference in MEL DE_f was found between MEL50, MEL100 and MEL500 on Day 10, although efficiency in MEL50 (0.686%) was slightly lower than MEL100 (0.778%) and MEL500 (0.756%).

4.4. Discussion

4.4.1. Production parameters

Since there was no difference between treatments regarding body weight on Day 1 and on Day 20, it can be concluded that neither MEL nor CYR had an effect on body weight and that the decrease in body weight that had been observed from Day 1 to Day 20, was mainly caused by high ambient temperatures that affected feed intake. Similar conclusions had been made from the feed intake data, where feed intake declined from Day 10-20 due to high environmental temperatures. Gao *et al.* (2010) reported no damaging effects of MEL diets containing less than 100 mg/kg on neither body weight nor feed intake in layer ducks. It was interesting from the results that CYR4 had a superior feed intake but ultimately revealed a decreased body weight.

No adverse effects were observed for egg production over time; however, there did seem to be a slight tendency for egg production to decrease, especially from Day 8 until Day 20, which could have been due to the decreased feed intake observed in all treatments during the same period. It is postulated that this phenomenon may be due to the high ambient temperatures. There was however, a significant difference ($P < 0.01$) in egg production between CYR4 and all the other treatments (Table 16).

The observed increase in egg weight over time for all the treatments (Figure 8) might have been due to the decrease in egg production; however, it appeared as if the level of dietary MEL had some effect on egg weight since MEL500 had the lowest egg weight and differed significantly ($P < 0.05$) from all the other treatments except for MEL100. Gao *et al.* (2010) reported no negative effects on egg weight after feeding diets containing MEL levels of up to 100 mg/kg to layer ducks. From the results, it can be concluded that some detrimental effects in egg weight can already be observed at MEL inclusion levels of 100 mg/kg and that 500 mg/kg MEL had a definite adverse effect on egg weight.

4.4.2. Melamine concentrations in eggs

In the current study, MEL was already detected in eggs within the first day, similarly to Chen *et al*. (2010) who indicated that MEL was rapidly distributed to eggs. Bai *et al.* (2010) did not measure MEL concentrations on Day 1, but they found peak MEL levels already on Day 2. Melamine concentrations peaked very soon after first ingestion and for MEL50 and MEL100, peak values were already observed on Day 1. It happened that concentrations started to decrease after about a week. In the broiler trial (Chapter 3), it was also noted that MEL concentrations in tissues started to decrease over time. Similar observations were reported by Lü *et al*. (2009) in broilers and Cruywagen *et al*. (2010) in lambs, who hypothesized that the animal's ability to clear MEL from tissues might increase over time. The decrease was the most prominent in the MEL500 group and started on Day 7 while the decrease in MEL50 already manifested on Day 4. There was a dose-response relationship between the dietary MEL inclusion levels and MEL concentrations in the eggs, which was confirmed by Bai *et al.* (2010), Chen *et al.* (2010) and Gao *et al.* (2010). The results were proportionally much higher than those reported by Bai *et al.* (2010), Chen *et al.* (2010) and Gao *et al.* (2010) and might be due to different detection methods used. Bai *et al.* (2010) used the high-performance liquid chromatography-ultraviolet method and Chen *et al.* (2010) the gas chromatography-mass spectrometry method, whereas Gao *et al.* (2010) used the LC-MS/MS method. From the results reported by Chen *et al.* (2010), 100 mg/kg dietary MEL did not result in egg samples containing more than 2.5 mg/kg MEL, which is the maximum residue level allowed in food. In the current study, results revealed distribution levels of 2.24 to 4.45 mg/kg for similar dietary levels, suggesting that a diet containing more than 100 mg/kg MEL could violate the permitted 2.5 mg/kg inclusion level.

It was evident that the MEL levels rapidly decreased after the onset of the withdrawal period and on Day 6 all the treatment samples contained <0.05 mg/kg MEL. Similar results were reported (MAFF, 2010) where undetectable levels of MEL (<0.04 mg/kg) were found in eggs within seven days after withdrawing diets that contained 30 mg/kg of MEL. A model constructed by Gao *et al.* (2010) revealed that the half-life of MEL in eggs was <18 hours, emphasising the rapid excretion of MEL from the body. Elevated MEL concentrations needed a longer time period to be withdrawn from eggs, which is in agreement with Bai *et al.* (2010), Chen *et al.* (2010) and Gao *et al.* (2010). The

assumption has been made by Gao *et al.* (2010) that MEL mainly exists in a free form in the body, which attributed to the rapid elimination and that higher dose levels were deposited more extensively in other tissues which is why it may have taken longer to be eliminated. It might also be that the kidneys can only manage a certain load and that excess MEL would therefore need more time to be cleared. No MEL from the CYR4 treatment could be detected in any of the samples in the current study.

4.4.3. The comparison of melamine concentrations in yolk, albumin and whole eggs

The available literature gave the impression that the highest MEL concentrations detected in body tissues are found in tissues containing high protein levels (Anderson *et al.*, 1987; MAFF, 2010), excluding the kidneys and liver which have to eliminate toxins from the body and high MEL levels in these organs are to be expected. The current test results revealed that albumin contained the highest MEL concentration, compared to the whole egg and yolk and Yang *et al.* (2009) reported similar findings. The albumin contains most of the protein fraction of the egg and therefore the reported results appeared to be in order.

4.4.4. Distribution efficiency rate (DE_f)

The main reason for calculating the DE_f rate was to express the MEL concentrations measured in eggs as a fraction of the ingested MEL. The current method can be described as an alternative for reporting the distribution efficiency and differed from other reported methods to express efficiencies (Bai *et al.*, 2010; Chen *et al.*, 2010; Gao *et al.*, 2010; MAFF, 2010 and Yuchang *et al.*, 2010). In the current study, the method accounted for the actual amount of MEL that had been ingested per day and retained in the egg. Other reports only mentioned the concentration at which MEL was deposited in eggs as a ratio of the concentration of MEL in the feed. The calculation method used in the current trial was first reported by Cruywagen *et al.* (2009) for MEL distribution to milk of Holstein cows (2.1%), followed by a reported 3.6% in the *L. dorsi* of Merino sheep (Cruywagen *et al.*, 2010). The DE_f of MEL to eggs was calculated on Day 10 to be 0.69% for MEL50, 0.78% for MEL100 and

0.76% for MEL500. There were no differences between treatments regarding distribution. It was difficult to determine the retention time of MEL in the body and, as most others, a retention time of seven days were assumed. These findings might be subjected to change in the future when daily withdrawal depletion rates have been confirmed.

4.5. Conclusion

Layer hens were fed diets containing 0, 50, 100 and 500 mg/kg of MEL to determine the effect of MEL on production parameters and the possible distribution of MEL to eggs. The current test results suggests that MEL levels up to 500 mg/kg had no marked effect on body weight; however, feed intake and egg weights noticeably decreased in the 500 mg/kg group. Within 24 hours after ingestion, MEL could be detected in egg samples for all treatment groups receiving MEL. A plateau was reached for each treatment and a definite decreasing trend was observed for all treatments from seven until ten days. This would suggest that the birds developed an increased capacity over time to clear MEL from the body. Even though the actual concentration levels differed from other reports, all of the results had similar tendencies as reported by other researchers. The MEL distribution levels were found to be dose-related to the concentration of MEL included in the feed.

A rapid decrease in MEL residual levels were detected after a withdrawal period had been implemented and within six days MEL levels were below 0.05 mg/kg. Despite an increased feed intake and a decrease in egg production, no production parameters were influenced by CYR inclusion and no MEL could be detected in eggs due to the inclusion of 4 mg/kg dietary CYR. Therefore, it could be concluded that the application of the Larvadex® 1% premix in layer feed did not result in any MEL distribution in eggs due to CYR metabolism. Albumin contained the highest MEL residual concentration followed by the whole egg and yolk. A MEL DE_f was estimated by expressing the total amount of MEL deposited in eggs as a fraction of the total MEL ingested per day. These results were comparable to those reported for other species (Merino *L. dorsi*) and products (Holstein milk).

4.6. References

AOAC, 2000. Official Methods of Analysis. 17th ed. Assoc. Off. Anal. Chem. Int., Gaithersburg, MD.

Anderson, J.F., Spandorf, A., Magnarelli, L.A. and Glowa, W., 1987. Control of house flies in commercial poultry houses in Connecticut. J. Poult. Sci. 65: 837-844.

Bai, X., Bai, F., Zhang, K., Lv, X., Qin, Y., Li, Y., Bai, S. and Lin, S., 2010. Tissue deposition and residue depletion in laying hens exposed to melamine-contaminated diets. J. Agric. Food. Chem. 58: 5414-5420.

Chen, Y., Yang, W., Wang, Z., Peng, Y., Li, B., Zhang, L. and Gong, L., 2010. Deposition of melamine in eggs from laying hens exposed to melamine contaminated feed. J. Agric. Food Chem. 58: 3512-3516.

China Chemical Reporter, 2006. Melamine capacity is serious surplus. Available: http://goliath.ecnext.com/coms2/gi_0199-5152838/Melamine-capacity-is-in-serious-surplus.html#abstract. Accessed: July, 13, 2010.

Cruywagen, C.W. and Reyers, F., 2009. The risk of melamine contaminated ingredients in animal feeds. AFMA. Matrix. 18: 4-6.

Cruywagen, C.W., Stander, M.A., Adonis, M. and Calitz, T., 2009. Hot topic: Pathway confirmed for the transmission of melamine from feed to cow's milk. J. Dairy. Sci. 92: 2046.

Cruywagen, C.W., van de Vyver, W.F.J. and Stander, M.A., 2010. Quantification of melamine absorption, deposition and excretion by sheep. Unpublished report. Department of Animal Science. Stellenbosch University.

Drugs.com., 2010. Larvadex 1% Premix. Drug information online. Available: http://www.drugs.com/vet/larvadex-1-premix.html. Accessed: Nov, 19, 2010.

Gao, C.Q., Wu, S.G., Yue, H.Y., Ji, F., Zhang, H.J., Liu, Q.S., Fan, Z.Y., Liu, F.Z. and Qi, G.H., 2010. Toxicity of dietary melamine to laying ducks: Biochemical and histopathological changes and residue in eggs. J. Agric. Food. Chem. 58: 5199-5205.

Kobayashi, T., Okada, A., Fujii, Y., Kazuhiro, N., Hamamoto, S., Yasui, T., Tozawa, K. and Kohri, K., 2010. The mechanism of renal stone formation and renal failure induced by administration of melamine and cyanuric acid. Urol. Res. 38: 117-125.

Lu, Y., Liu, L., Wu, Y., Jiang, J., Feng, N., Xie, W. and Zhou, X., 2009. Study on the distribution and metabolism of melamine in laying-hens and eggs. College of Veterinary Medicine. China Agriculture University. Beijing.

MAFF, 2010. Summary of studies on the transfer of melamine from feeds to tissues of lactating cows and chickens. Japanese Ministry of Agriculture. Forestry and Fisheries. Unpublished report.

Merck, 2001. Entry 5834. IN The Merck Index 13th ed. Merck Research Laboratories. Whitehouse Station. New York. P. 1038.

Sancho, J.V., Ibáñez, M., Grimalt, S., Pozo, Ó.J. and Hernández, F., 2005. Residue determination of cyromazine and its metabolite melamine in chard samples by ion-pair liquid chromatography coupled to electrospray tandem mass spectrometry. Anal. Chimica. Acta. 530: 237-243.

Statistica Version 9, 2010. StatSoft Inc. USA.

Vauqulin, C., 2010. Report of the broiler organisation committee. Available: http://www.sapoultry.co.za/2010%20DOWNLOADS/AviAfrica%202010/2010%20BROILER%20REPORT.pdf.

World Health Organization, 2008. Expert meeting to review toxicological aspects of melamine and cyanuric acid. Available: http://www.who.int/foodsafety/fs_management/Exec_Summary_melamine.pdf. Accessed: July, 24, 2010.

Xia, X., Ding, S., Li, X., Gong, X., Zhang, S., Jiang, H., Li, J. and Shen, J., 2009. Validation of a confirmatory method for the determination of melamine in egg by gas chromatography–mass spectrometry and ultra-performance liquid chromatography–tandem mass spectrometry.Anal. Chimica. Acta. 651: 196-200.

Yang, S. P., Chen, H.W., Yang, Y.L., Hu, B., Zhang, X., Zhou, Y.F., Zhang, L.L. and Gu, H.W., 2009. Imaging melamine in egg samples by surface desorption atmospheric pressure chemical ionization mas spectrometry. Chinese J. Anal. Chem. 37: 315-218.

Yuchang, Q., Xiaowen, L., Jun, L., Guanghai, Q., Qiyu, D., Guohua, L., Min, X., Jiaqi, W., Jianming, T., Liying, Z. and Keying, Z., 2010. Assessment of melamine contamination in crop, soil and water in China and risks of melamine accumulation in animal tissues and products. Environ. Inter. 36: 446-452.

CHAPTER 5

General conclusion

During the past four years, the illegal inclusion of the organic chemical, melamine (MEL), has been detected in infant formula, pet food and certain other food products. Since it is known that MEL has no nutritional value and has been proven to be a causative agent of renal damage, MEL should not be present in any food product. Even though MEL adulteration could be banned from society, environmental MEL could still surface in food products, including poultry meat and eggs. Melamine derived from the metabolism of other compounds, such as cyromazine, also has the potential to end up in foods. Cyromazine (CYR) is the active ingredient of pest control agents, such as Larvadex and Trigard, often included in layer hen diets to control flies. It is thus necessary to determine the distribution behaviour of MEL in food products, even if only for risk assessment purposes.

The current study showed that dietary MEL levels of up to 500 mg/kg did not have any detrimental effect on production parameters for broilers. Apart from a decreased feed intake and lower egg weights observed in layer hens that received diets with 500 mg/kg of MEL, no other production parameters were influenced by MEL. Melamine was distributed to breast meat within two days after broilers had ingested MEL tainted diets and, regarding eggs, MEL was already detectable within 24 hours after ingestion. As the dietary MEL concentration increased from 50 and 100 mg/kg to 500 mg/kg, a significant increase was observed in muscle tissue and egg MEL residue concentrations. For all broiler treatments, a peak MEL concentration was observed at 22 days of age, which decreased until slaughtering at 36 days of age. In layer hens, a MEL distribution plateau in eggs was reached for each MEL treatment between Days 1 and 4, followed by a definite decrease in MEL distribution from Day 7 until Day 10. These findings are indicative of a possible adaption of broilers and layers in their capacity to clear MEL from their system. The kidneys contained the highest MEL residue levels, compared to other organ tissues, such as muscle and liver. In eggs, the distribution of MEL was higher to albumin than to the yolk.

After a seven day withdrawal period, no more MEL could be detected in meat samples. In eggs, a rapid decrease in MEL residual levels were detected after a 10 day withdrawal period and within six

days MEL levels were below 0.05 mg/kg. Furthermore, no MEL could be detected in meat and egg samples subjected to a diet containing 4 mg/kg CYR. Estimated distribution efficiencies of MEL to meat and MEL to eggs were comparable to reports on other species (Merino *L. dorsi*) and products (Holstein milk).

It was concluded that dietary MEL is absorbed by broilers and layers and rapidly distributed to various organs, muscle and eggs. Upon withdrawal, MEL concentration in these tissues can be expected to decline to undetectable levels within seven days.

Thus far, global efforts to completely clear food from MEL remain as failed attempts. Joined forces between governments and all participating food manufactures will ensure safe food. However, differing priorities between governments (food safety) and manufacturers (generating profit) will remain to ruin the perfect concept.