CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

Since early 2007, reported sickness and death of pet animals (cats and dogs) was found food contaminated with melamine. It was found that wheat gluten originating from China and used for the production of pet food was at the origin of the animal health problems. The levels of melamine found in wheat gluten and rice protein concentrate were in range of 0.2 to 8.0%. As the protein concentration is measured by analysis of the nitrogen, the fraudulent addition of melamine, a chemical substance rich in nitrogen, aims at enhancing the apparent protein content of protein sources. Some swine, fish, and poultry feeds were reported to be contaminated with 30-120 mg of melamine/kg of diet. Milk products or fresh eggs contaminated with melamine as high as 2500 and 4.7 mg/kg. Therefore, melamine contaminate animal feeds were found worldwide. Although, it is not approved for use as a feed or food additive in any country. melamine and formaldehyde or urea-formaldehyde (UF) are known human health threats and melamine-formaldehyde releases monomers of both. UF are the more important type of pellet binders in both animal and aqua feeds.

This study focuses on modified rapid methods for detection melamine and UF by spectrophotometry followed the method of Hirt et al (1955) in soybean protein products (soybean meal; SBM, fullfat soybean; FFSB, and their equal mixture) and also both broiler and layer feeds. Two experiments were conducted to investigate the effects of dietary graded levels of melamine or UF or their equal mixture on performance and carcass or egg quality, melamine residues, microscopic and histopathological changes in broilers and laying hens tissues.

In first experiment, melamine and its analogues (ammeline, ammelide, and cyanuric acid) can be determined by gas chromatography (GC) after trimethylsilylation (Stoks and Schwarts, 1979) or by high performance liquid chromatography (HPLC) (Beilstein et al., 1981; Sugita et al., 1990). For the determination of melamine as a chemical contaminant in food such as lard, potato

proteins, food-stimulants and beverages, only a few methods have been reported, such as spectrophotometry (Hirt et al., 1955), liquid chromatography (Bisaz and Kummer, 1983; Inoue et al., 1985; Ishiwata et al., 1987) and gas chromatography (Ishiwata et al., 1986). Kim et al. (2008) used HPLC/MS to determine the melamine in pet food by enzyme immunoassay. GC-MS (gas chromatography-mass spectrometry) has also been used after trimethylsilylation for the determination of melamine and its analogues in wheat gluten and pet food matrices. This method has been recommended by the European Commission to analyze consignments of wheat gluten, corn gluten, corn meal, soy protein, rice bran and rice protein concentrate originating from developing countries, in particular from China. This experiment exhibited the ultraviolet and visible spectrophotometric methods for the determination of micro amounts of melamine and UF in aqueous media, soybean protein products and also chicken feeds (broiler and layer) have been modified in order to obtain the rapid desired detectability in soybean protein products and chicken diets. The results of these studies demonstrate that both melamine and UF can be detected by these fast and cheap cost instrument for detection and characterization of melamine and UF compounds in soybean protein products and chicken feeds.

In two experiments (broiler and laying hens), there were thirteen treatment diets were formulated to contain four graded levels (0.25, 0.50, 0.75 and 1.00%) of melamine or UF or their equal mixtures and no added for negative controls. There was no difference ($P>0.05$) in feed intake (FI) of both broilers and laying hens. Chicks fed with melamine showed linear decrease effect on BWG as the levels of melamine in the diets increased with the greatest decreased in BWG observed in chicks fed 1.00% melamine. Feed efficiency or FCR showed similar results as BWG with less efficient linearly FCR as the levels of melamine increased in the diets and also showed the greatest decreased efficiency when fed 1.00% melamine (Experiment II). Hens that are fed on the three products were decreased egg production (EP), egg weight (EW), egg mass (EM) and number of egg ($P>0.05$), with the greatest decrease in hen fed 1.00% melamine (Experiment II). EP, EW, EM and number of egg decreased linearly ($P>0.05$) with increasing levels of dietary melamine or UF or their equal mixtures. Hens that are fed on three products were less efficient ($P>0.05$) in feed conversion ratio compared with the controls. Egg quality were significant difference decreased

(P>0.05) in egg yolk weight and HU for hens (19-34 wk) fed three products level ≥ 0.75% in the diets compared with controls. At 34 to 52 weeks of age, significant difference in specific gravity, egg yolk weight and HU. Hens fed melamine 1.00% showed the greatest decreased egg quality when compared among treatments. The results agree with a early studies by Ledoux et al. (2009) fed graded levels of melamine (0.50-3.00%) in young broilers from hatch to 14 days showed no effect in FI among controls and chicks fed 0.50 or 1.00% melamine, and BWG decreased when fed ≥ 1.00% melamine with the greatest increase in BWG observed in birds fed ≥ 2.00% melamine. Similar previous study also reported by Brand et al. (2009) in young turkey poults fed with graded levels of melamine (0.50-3.00%) from hatch to 21 days indicated that FI was reduced in poults fed diets containing 1.50% melamine, whereas BWG was reduced in birds fed ≥ 1.00% melamine when compared with controls. Feeding with melamine showed graded levels decreased survival percentage with the greatest decreased when birds fed with 1.00% melamine. These results is in agreement with Ledoux et al. (2009) reported that mortality was observed in chicks fed with 2.50 and 3.00% melamine as early as d5 of study, and by d14, mortality was 12, 20 and 30% respectively, in chicks fed 2.00, 2.50 and 3.00% melamine. Similar results also reported by Brand et al. (2009) in young turkey poults. Significant mortality was observed in turkey fed 1.50, 2.00 and 3.00% melamine with 27, 63 and 93% mortality. However, Bai et al. (2010) reported in laying hens fed with 8.6-140.9 mg. melamine/kg of BW for 34 days showed no effects on the survival, BWG, EP and also no pathological changes were observed in the kidney of hens. In addition, Gao et al. (2010) fed six dietary of melamine (0-100 mg of melamine /kg of diet) in laying ducks for 21 days showed no visible signs of illness or mortality were observed and no adverse effects on EW, EP, FI and FCR (P>0.05). The residue levels of melamine in breast meat and liver at d42 can be detected when the birds fed diets contained ≥ 0.50% melamine when added melamine in the diet alone or their equal mixture of both melamine and UF (Experiment II). The distribution of residual melamine in the liver tissues was higher than in the breast meat and after a 7-d withdrawal period, residual melamine was not detected in both breast meat and liver. Similar finding have been reported by Lu et al. (2009). They reported that residue levels of melamine in broiler tissues at d28 and 42 were below the detection limit when the diets contained ≤

50 mg of melamine /kg of diet, and melamine was detected in breast meat and liver only in chicks fed diets containing 500 and 1000 mg of melamine /kg of diet. The distribution of melamine varied in different tissues, with the lowest concentration in breast meat and the highest in the kidney and the melamine residue levels in the tissues were lower on d42 compared with d28. Also, could be explained by an increased melamine clearance with increasing age, but it may also reflect a decreased exposure to melamine on a mg/kg BW per day basis following the feeding behavior of the growing animals. In addition some of the differences between day 28 and 42 might be due to variability in time of sampling. Chen et al. (2010) fed diets spiked with 0 to 100 mg of melamine per kg of feed showed average melamine concentrations in eggs were below limit of detection. In addition, Bai et al. (2010) reported that melamine at 8.6 to 140.9 mg/kg of bw per day for 34 days showed crystals in one of three kidneys of hens treated with melamine at either 62.6 or 140.9 mg/kg. These results show the distribution of melamine in hen tissues and that the highest melamine residue was in the kidney, followed by liver, muscle, ovary, uterus and duodenum. Mast et al. (1983) have reported that the maximum amount of melamine residue was in bladder, followed by the ureter, liver and kidney when rats were administrated with a single oral dose of melamine. There was no significant difference of melamine concentrations in liver, duodenum and kidney between all treatment groups, which may result from the fact that melamine can be quickly excreted from the body.

Microscopic examination of crystal with "spoke wheel" appearance in the tissues of liver, kidney and spleen of broilers fed with diets containing 0.50% melamine having the appearance that were either microscopic in various size or were large white diamond visible to the naked eye. Similar finding have been reported by Brand et al. (2009) they observed that turkey poults fed with 2.00-3.00% melamine that diet in these treatment groups contained crystals that were also either microscopic in size or were large white crystal visible to the naked eye. Lam et al. (2009) and Ledoux et al. (2009) also reported crystal formation in bile and histopathological lesions in kidneys which revealed crystals similar to those observed within the renal tubules of cats with melamine associated renal failure. Furthermore, Bai et al. (2010) evaluation kidney sample from laying hens administrated with melamine at 8.60-140.90 mg/kg of BW/d for 34 days. The crystals were found in one of three kidneys of

hen treated with melamine at either 62.60 or 140.90 mg/kg. In one recent study, Reimschuessel et al. (2008) described that chickens fed only melamine could develop spherulite crystals containing uric acid, a normal excretion product of chickens. Unlike mammals, whose urine contains urea primary, birds excrete uric acid as the primary nitrogen metabolite. Uric acid is synthesized in the liver and is excreted via the urine. It comprises 60 to 80% of the total urinary nitrogen. Birds can, however, produce urea to a limited extent as an end product of purine metabolism and the catabolism of arginine; but the level of urea found in the urine is significant as compared to the uric acid content (William, 2005). Previous studies have also shown that pigs, fish, cats, and rat fed melamine or cyanuric acid in a 1:1 ratio develop renal crystals composed of melamine-cyanurate (Puschner et al., 2007; Dobson et al., 2008; Reimschuessel et al., 2008). The relationship between the melamine concentration in diets fed to chicks and the melamine crystals appearance on amount and size in liver, kidney and spleen of broilers from this present study was best described by a series of linear functions.

Based on this study, melamine concentration $\geq$ 0.75% depressed both growth performance, and survival percentage, and also decreased PI or EPEF. Residue levels of melamine in breast meat and liver were depleted after a 7-d withdrawal period. On the basis of the above results, assuming that most of the melamine within the animal body existed in a free form, which allowed for rapid depletion at the beginning of the withdrawal period (Gao et al., 2010). Higher concentrations of melamine may be widely distributed in different tissues, requiring more time to be cleared. In this study provided some information about melamine residues in the breast meat and liver tissues and depletion or elimination time in the breast meat and liver of broilers, as well as demonstrated the risks to human health posed by melamine adulterants used to increase the nitrogen content in feed.

The two experiments (II and III) in this study were not found crystals in the liver, spleen and egg of broilers and hens. Kidneys tissues found melamine crystal deposited in the interstitial tissue surrounding the renal tubules, with fed 1.00% melamine. Mast et al. (1983) reported that melamine distributes in body water. The relatively high levels in bladder were suggested to be probably due to either back diffusion from urine or to a contamination of the bladder tissue with urine. The

crystals of the pet cases and the experimental cats, pigs, rats and fish are golden brown, are generally arranged in a radial pattern and are birefringent when viewed with polarized light. The appearance of these crystals is similar to that of uric acid crystal "spherulites" or "spherically symmetrical radiating crystal aggregates" that can occur in humans with gout (Fiechtner and Simkin, 1980, 1981). Likewise, the crystals are similar to spherulites of uric acid found in nephrocytes of the ascidian Corella inflate (Lambert et al., 1998). As uric acid crystals are soluble in formalin, several investigators (Dobson et al., 2008; Reimschuessel et al., 2008) examined wet tissue sections at necropsy prior to preserving kidney in formalin for routine histopathology. Reimschuessel et al. (2008) showed that melamine-cyanurate crystals dissolve in formalin over time. This may explain why crystals have not been found in the long-term studies evaluating melamine and cyanuric acid, where animals formed calculi and crystalluria was observed grossly, but no crystals were seen by histopathology. There are no literature data on histopathological changes in organs of broilers and hens induced with UF. The relatively nontoxic nature of melamine and UF noted in this study is consistent with the limited toxicologic information that has been published for these compounds.

Results from the current experiment have implications for the broiler industry because, until now, any melamine found in raw material and broiler feeds was attributed only to the deliberate external addition of melamine to these products. Our results may provide some information for future work on human health risk assessment for melamine and urea-formaldehyde toxicology.