# CHAPTER III
# EXPERIMENT I
# RAPID MODIFIED SPECTROPHOTOMETRIC DETERMINATION OF MELAMINE AND UREA-FORMALDEHYDE IN SOYBEAN PROTEIN PRODUCTS AND CHICKEN FEEDS

## 3.1 Introduction

Melamine is a synthetic chemical (1,3,5-triazine-2,4,6-triamine) used primarily for the production of amino resins, plastics (Anderson, 1995; Subrayan and Rasmussen, 1995; Weil and Choudhary, 1995) and fertilizer because of its high nitrogen content (Lim et al., 1990). Recently, reports indicating that pet food contaminanted with melamine resulted in renal disease and deaths in cats and dogs have placed melamine contamination in the spotlight (Thomas and Kulkarni, 2007; Burns, 2007). In the case of pet food, it has been speculated that melamine was added intentionally in feed for a false high level of crude protein determined by the Kjeldahl method (Lachenmeier et al., 2009). Some swine, fish, and poultry feeds were reported to be contaminated with 30-120 mg of melamine /kg of feed (Burns, 2007; Nestle and Nesheim, 2007; Ingelfinger, 2008). The concentration of melamine in some infant formulas and fresh eggs was determined to be as high as 2500 and 4.7 mg/kg, respectively (Guan et al., 2009; The Government of the Hong Kong Special Administration Region Centre for Food Safety, 2008). Therefore, addition of melamine to feed may contaminate animal products and has raised worldwide concerns about food safety. Both melamine and formaldehyde or urea-formaldehyde (UF) are known human threats and melamine-formaldehyde releases monomers of both (Ishiwata et al., 1986; Bradley et al., 2005). UF (polymer with formaldehyde) are the more important type of pelleting binders in both animal and aquatic feeds. The objectives of the present studies were to modified rapid methods for 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.

## 3.2 Materials and Methods

Ultraviolet and visible automatic recording spectrophotometer with fused quartz cells of various light path lengths were used for the ultraviolet spectrophotometric work and a photomultiplier tube and 10-mm glass cells were used for the visible spectrophotometric work.

Melamine and UF industrial grade were purchased from Tianjn BASF Chemical Company, Tianjn, China. To determine the correct concentrations of melamine and UF for quantitative analysis, their solubilities were initially examined using 0.02 grams of melamine and UF were individually added to 2 L of 0.1 *N* HCl and water respectively and stirred for 4 h at 60°C. The solution were filtered through Whatman filter paper No.4 to remove any insoluble material. From the comparison of the weights of the filter paper after and before the filtration, the solubilities of melamine and UF were approximated and then their stock standard solution were used for the quantitative analyses.

Iso-octane and 12 *N* and 0.1 *N* hydrochloric acid were used. Solutions of phenylhydrazine hydrochloride (1%) and of potassium ferricyanide (5%) in distilled water were prepared fresh daily. Soybean protein products and both basal broiler and layer feeds (Table 3.1) containing no antioxidants or inhibitors, were used in the recovery and storage tests (Commercial antioxidants have ultraviolet absorption which interferes with the detection of melamine). In order to test the efficiency of the extractions and the quantitative recovery of the known amounts of melamine and UF were added to the soybean protein products and both also broiler and layer feeds and the described methods were applied.

### 3.2.1 Ultraviolet spectrophotometric method for melamine

The strong absorption band of melamine at 235 mμ in dilute acid solution has been used for the determination of melamine in stock melamine standard solution, soybean protein products and chicken feeds and the theoretical detectability has been calculated to be 4 μg. The detectability of melamine in feedstuff and animal

feeds may be improved by an extraction procedure which serves to separate the melamine from interfering materials and to concentrate the melamine in a minimum volume corresponding to the volume of the absorption cell to be used. As the resin and the melamine have very nearly the same absorptivity at the analytical wave length 235 mμ, the data may be reported as melamine and/or resin.

**Procedure details**

Approximately 15 grams of soybean protein products or chicken feeds were weighed by difference on an analytical balance, dissolved in 100 ml. of iso-octane, and transferred to a separatory funnel. Ten milliliters of 0.1 *N* hydrochloric acid were added, and the funnel was shaken well and allowed to stand for 20 minutes for separation to take place. The bottom (acid) layer was removed, and the extraction procedure repeated with another 10 ml. acid portion, which was added to the first portion. The sample was transferred to a 20-mm. absorption cell and examined versus a matched cell filled with 0.1 *N* hydrochloric acid, scanning from 270 mμ to the lower wave-length limit of the instrument. Absorbance readings were taken at 235 mμ. both standard and exposed samples were examined in this manner.

The concentration of melamine and/or resin was calculated from

$$Cm = \frac{A235}{(b)(a235)}$$

where *Cm* is the concentration of melamine in grams per 100 ml, *A*235 is the observed absorbances at the subscript wave length of the extracts of the test samples, *b* is the cell light path length in millimeters, *a235* is the absorptivity of melamine at 235 mu (81.0). The concentration of melamine is the extraction test sample is given by

$$Mc = \frac{(Cm)(V)}{W}$$

where *Mc* is the concentration of melamine in test sample, *V* is the volume of the extracting hydrochloric acid in milliliters, and *W* is the weight of the test sample in grams.

### 3.2.2 Visible spectrophotometric method for urea-formaldehyde

A red color is formed when dilute solutions of formaldehyde phenylhydrazone (1%) are treated with potassium ferricyanide (5%) in the presence of anexcess of hydrochloric acid. Formaldehyde phenylhydrazone is formed by the reaction of formaldehyde and phenylhydrazone hydrochloride. Time was found to be an important factor in this reaction and in the color development. Ten minutes were allowed between the addition of the phenylhydrazone hydrochloride and the addition of the remaining reagents; 10 minutes were also allowed for full color development after the addition of the hydrochloric acid. A reagent blank was run with stock standard and each set of samples.

**Procedure details**

A sample of soybean protein products or chicken feeds of approximately 15 grams was weighed by difference on an analytical balance and dissolved in 30 ml. of iso-octane. This solution was extracted four times with 3 ml. of distilled water using a separatory funnel. The first two extracts were combined and the third and fourth extracts were combined prior to the development of the color. These two sets of combined extracts were examined separately. The volumes of the extracting medium were kept to a minimum in order to improve the detectability through concentration, as in the melamine procedure. The water extract (of not more than 6-ml. volume) was treated with 1.0 ml. of 1% phenylhydrazone hydrochloride and allowed to stand for 10 minutes. Then 0.5 ml. of 5% potassium ferricyanide and 2.0 ml. of 12*N* hydrochloric acid were added in rapid succession. The solution was made up to 10-ml. volume, and the color was measured after 10 minutes, in a glass cell of 10-mm. light path length. The analytical wave length was 520 mμ. The concentration of UF was calculated from

$$Cuf = \frac{A520}{(b)(a520)}$$

where *Cuf* is the concentration of UF in grams per 100 ml, *A520* is the observed at 520 mμ of the sample, *b* is the cell light path length in millimeters, and *a520* is the absorptivity of the UF (in the formaldehyde-phenylhydrazone), which is 660. The concentraton of UF in the test sample is given by

$$UFc = \frac{(Cuf)(V)}{W}$$

where *Cuf* is the concentration of UF in the test sample, *V* is the volume of the extracting hydrochloric acid in milliliters, and *W* is the weight of the test sample in grams.

### 3.3 Results and Discussion

The melamine and UF in both standard solution illustrates the relationship between optical density (absorbance) and the amount of melamine or UF obeyed Beer's Law (Table 3.2 and also Figure 3.1 melamine and Figure 3.1). Prediction results by contracting the absorbance value of predicted melamine and UF concentration against the values of actual melamine and UF concentrations. Good prediction results were obtained with $R^2$=0.9857 for standard melamine solution, $R^2$=0.9641 for standard UF. The results of the determinations of melamine and UF in soybean protein products and chicken feeds are also shown in Table 3.2 and Figure 3.2-Figure 3.6. Good linear regression ($R^2$) for melamine and UF also found in SBM, FFSB, mixture (1:1) of SBM and FFSB, broiler and layer feeds. The amount of melamine extracted and recovery from FFSB and the mixture of SBM and FFSB (1:1) showed $R^2$ values lower than 0.9500 with $R^2$=0.9121 for FFSB and $R^2$=0.9226 for the mixture of both products. The presence of high fat content in the test sample showed interferes with the extraction and detection of melamine (Table 3.2, Figure 3.3). It may be seen from examination of Table 3.2 and Figure 3.1– Figure 3.6 that these extraction procedures and spectrophotometric determination can be applied to determination of melamine and UF in other feedstuffs and animal feeds similar to soybean protein products and chicken feeds, with probably only minor modifications

in the techniques. The presence of interfering materials (fat and commercial antioxidants) which would extract along with the melamine or the UF would be the greatest source of difficulty.

**Table 3.1** Composition and nutrient contents in the basal chicken feeds

| Ingredient (%) | Broiler (%) | Layer (%) |
|---|---|---|
| Corn | 47.95 | 54.00 |
| Fullfat soybean | 20.00 | - |
| Soybean meal | 25.70 | 24.00 |
| Fish meal | - | 4.50 |
| Rice bran | - | 5.00 |
| Monocalcium phosphate (P21) | 2.42 | - |
| Dicalcium phosphate (P18) | - | 1.49 |
| Rice bran oil | 1.00 | 2.00 |
| Salt | 0.40 | 0.25 |
| Limestone | 1.70 | 8.00 |
| DL-Methionine | 0.31 | 0.18 |
| L-lysine | 0.17 | 0.08 |
| Choline chloride 60% | 0.10 | - |
| Premix[a] | 0.25 | - |
| Premix[b] | - | 0.50 |
| Calculated chemical analysis: | | |
| ME, kcal/kg | 3,069.00 | 2,810.00 |
| Crude protein (%) | 23.05 | 18.45 |
| Calcium (%) | 1.05 | 3.60 |
| Available phosphorus (%) | 0.48 | 0.43 |

[a]Provided per kilogram of feed: vitamin A, 11,025 IU; vitamin $D_3$, 3,528 IU; vitamin E, 33 IU; $K_3$, 0.91 mg; thiamin, 2 mg; vitamin $B_1$, 18 mg; vitamin $B_2$, 8 mg; nicotinic acid, 55 mg; pantothenic acid, 18 mg; vitamin $B_6$, 5 mg; vitamin $B_{12}$, 0.028 mg; folic acid, 1 mg; biotin, 0.221 mg; manganese, 64 mg; iodine, 2 mg; zinc, 75 mg; iron, 40 mg; copper, 10 mg; selenium, 0.3 mg; choline, 478 mg.

[b]Provided the following per kilogram of feed: vitamin A, 14,440 IU; vitamin $D_3$, 2,220 IU; vitamin E, 22.2 IU; vitamin K, 3.3 mg ; vitamin $B_1$, 2.2 mg; vitamin $B_2$, 6.7 mg ; nicotinic acid, 38.9 mg; pantothenic acid, 15.6 mg; vitamin $B_6$, 6.7 mg; vitamin $B_{12}$, 0.028 mg; folic acid, 1.1 mg; manganese, 50 mg; iodine, 0.333 mg; zinc, 88.9 mg; iron, 66.7 mg; copper, 8.9 mg; selenium, 0.111 mg.

**Table 3.2** Prediction of melamine (M) or UF from standard soybean meal (SBM), fullfat soybean (FFSB), mixture (1:1) of SBM and FFSB, broiler and layer feeds

| Parameter | Prediction equation, y | $R^2$ value |
|---|---|---|
| Standard sample: | | |
| M | y = 0.0260x + 0.0044 | 0.9857 |
| UF | y = 0.0780x + 0.9270 | 0.9641 |
| Soybean meal: | | |
| M | y = 0.0056x + 0.1530 | 0.9640 |
| UF | y = 0.2820x – 0.0995 | 0.9875 |
| FFSB: | | |
| M | y = 0.0330x + 0.1635 | 0.9121 |
| UF | y = 0.0147x + 0.1110 | 0.9780 |
| SBM:FFSB (1:1) | | |
| M | y = 0.0293x + 0.0608 | 0.9226 |
| UF | y = 0.0494x + 0.2077 | 0.9828 |
| Broiler feed: | | |
| M | y = 0.0191x + 0.0350 | 0.9920 |
| UF | y = 0.1081x + 0.1045 | 0.9963 |
| Layer feed: | | |
| M | y = 0.0229x + 0.1120 | 0.9944 |
| UF | y = 0.2010x + 0.1520 | 09986 |

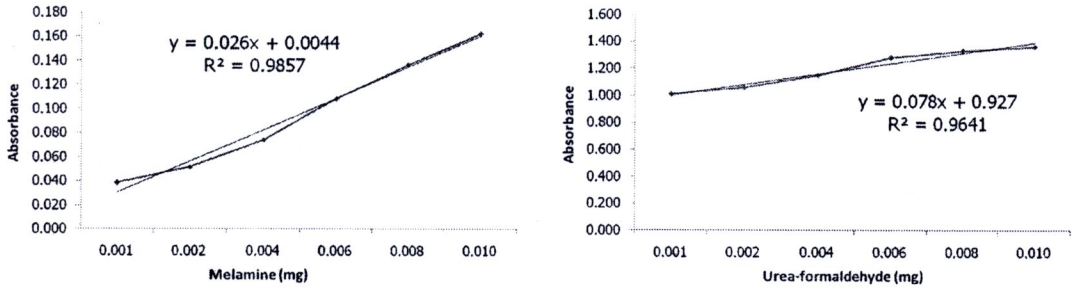


**Figure 3.1** Standardization curve of melamine and UF

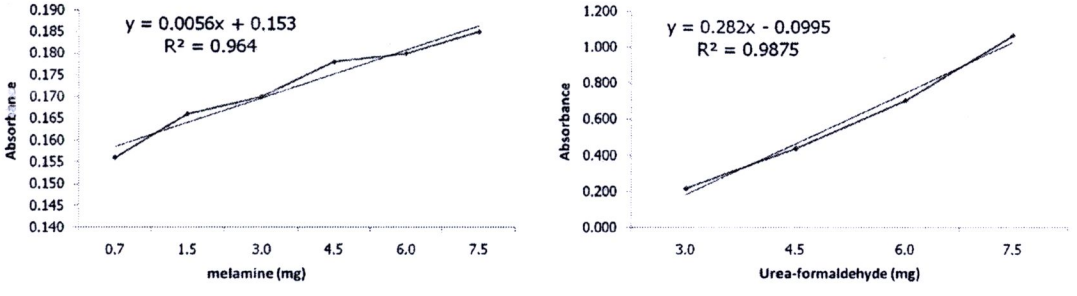


**Figure 3.2** Standard curve of melamine and UF in SBM

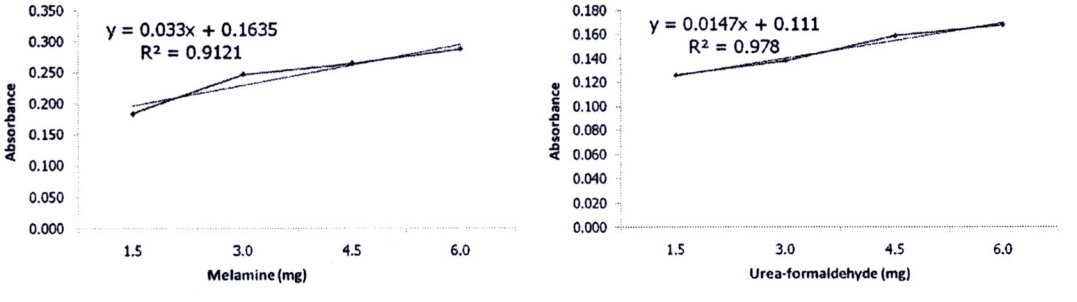


**Figure 3.3** Standard curve of melamine and UF in FFSB

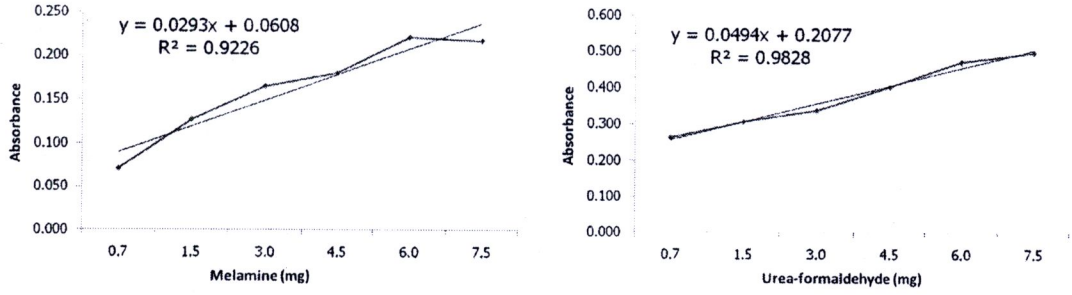


**Figure 3.4** Standard curve of melamine and UF in SBM : FFSB (1:1)

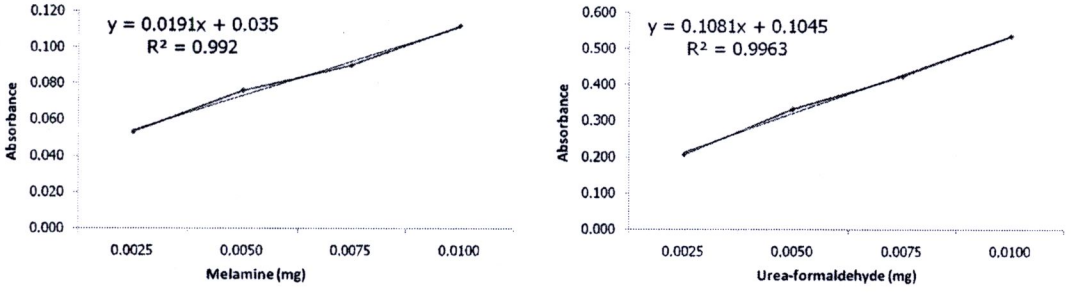


**Figure 3.5** Standard curve of melamine and UF in broiler feed

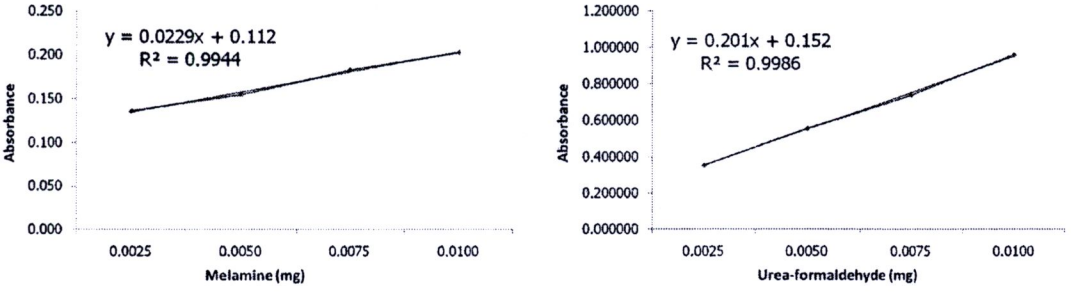


**Figure 3.6** Standard curve of melamine and UF in layer feed