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Analytical Methods

Melamine and cyanuric acid do not interfere with Bradford and Ninhydrin assays for protein determination

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ABSTRACT

In the fall of 2007 pet food contaminated with melamine and cyanuric acid caused kidney stones in thousands of animals. In the summer of 2008, a more serious outbreak of adulterated dairy food resulted in the deaths of six infants and sickened about 290,000 children in China. In all cases, melamine was likely concentrations added to inflate the apparent protein content of the foods. To determine if we could measure protein without interference from melamine and cyanuric acid we tested these compounds in the Bradford and Ninhydrin assays, two common dye-based assays for protein, as well as by ammonia release, the most common assay used in the food industry. Neither compound was detected in the Ninhydrin and Bradford assays at concentrations of >100 μg/ml. The ammonia assay detected melamine but was inconclusive with respect to cyanuric acid. To develop an accurate test for food that would not detect either chemical as a protein, assays were run on cat food and reconstituted milk powder. The Bradford assay readily measured the protein content of each food, and importantly, the addition of melamine or cyanuric acid to reconstituted milk did not affect the readings. The protein concentrations obtained for reconstituted milk powder were as expected, but those for the cat food were 10–30-fold lower, due to its low solubility. We conclude that dye-binding assays can be employed to detect protein in food without interference from melamine and cyanuric acid, thus reducing the incentive to use them as additives.

1. Introduction

In March 2007, there was a major case of melamine poisoning caused by contaminated pet foods. Hundreds and perhaps thousands of animals, primarily cats, were sickened and killed by wheat gluten from China that was contaminated with melamine and cyanuric acid (Barboza, May 4, 2007). Despite worldwide media attention to the problem, a more serious outbreak occurred in the summer of 2008, when melamine was found in many Chinese dairy products, including infant formula and milk-chocolate. The 2008 outbreak caused the death of at least six infants and sickened about 290,000 Chinese children, about 51,000 of whom were hospitalised with kidney stones. As a result, many countries placed restrictions on Chinese dairy products and several people were convicted (Barboza, September 30, 2008; Barboza, January 23, 2009). Melamine and cyanuric acid were likely added as non-protein nitrogen additives (NPN) to the wheat gluten used in pet foods, and later to the powdered milk, in an illegal effort to increase the apparent level of protein in these products (Barboza & Barrionuevo, April 30, 2007). Both melamine and cyanuric acid are by-products of coal processing and are not approved for use as food additives for humans, although cyanuric acid is used as a NPN additive in some cattle feeds.

Until the recent outbreaks, melamine was considered harmless to humans and it is still used to make Formica counter tops in kitchens and even plastic eating utensils. Cyanuric acid is often added to swimming pools to stabilise the chlorine from UV light. Melamine rapidly forms hydrogen bonds with cyanuric acid to form melamine cyanurate, a highly ordered insoluble crystal. In fact, melamine precipitation is the basis of tests to measure the cyanuric acid content in swimming pools. When tested on cats, neither chemical is toxic individually; however, when combined they may crystallise in the kidneys to form melamine cyanurate and, thereby, cause renal failure (Puschner, Poppenga, Lowenstine, Filigenzi, & Pesavento, 2007; Xin & Stone, 2008).

The food industry uses primarily two methods to measure protein content, the Kjeldahl method and the Dumas method (Thompson, Owen, Wilkinson, Wood, & Damant, 2002). Neither of these methods measures protein directly, but instead measures the nitrogen content of samples. The first step in these procedures is to hydrolyse food, with sulphuric acid (Kjeldahl) or through combustion (Dumas), to break down proteins and release nitrogen. The second step is to measure the nitrogen released (Robyt & White, 1987; Rosenberg, 1996). Melamine and cyanuric acid are detected as proteins in these tests because of the large amounts

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of nitrogen they contain, which includes the nitrogen in the amino groups on melamine.

There are other assays for proteins based on dye-binding. The Bradford assay, developed by Marion M. Bradford in 1976, is a common protein assay widely used in molecular biology laboratories, but rarely in the food industry. It determines the amount of protein in a substance by using Coomassie Brilliant Blue Dye, which turns from red to blue when it binds to proteins. The dye is protonated by the amino groups of the basic amino acids lysine and tryptophan and then binds to hydrophobic regions in proteins and turns blue (Bradford, 1976). By measuring absorbance at 595 nm in a spectrophotometer and comparing samples to a standard protein such as bovine serum albumin, the amount of protein in a sample can be quantified. The ninhydrin assay is a common laboratory procedure used to detect amino acids. Ninhydrin binds amino groups in amino acids, and fluoresces. When heated, the bound dye turns purple. The assay is used in protein sequencers and in thin layer chromatography applications to identify amino acids (Robyt & White, 1987).

Dye-binding assays differ from the Kjeldahl method and the Dumas method in that they measure protein content directly, instead of indirectly through nitrogen content. We hypothesised that dye-binding assays could be used instead of the ammonia release assays to determine protein content without interference from melamine and cyanuric acid. Thus, we tested melamine and cyanuric acid in Bradford and ninhydrin assays, and found that neither compound was detected. When the Bradford assay was used with cat food pellets, the protein concentrations were 10–30-fold lower than reported by the manufacturer, but when the Bradford assay was used with reconstituted milk, the assay yielded concentrations within 2-fold of expected values. Thus the Bradford assay is a rapid and inexpensive substitute for nitrogen release assays. We conclude that there would be less incentive to adulterate food, especially milk, if the food industry employed dye-based assays to measure protein.

2. Materials and methods

Melamine was purchased from Fluka and cyanuric acid was purchased from Aldrich; both were prepared as 25 mM solutions, 3.15 mg/ml of melamine or 3.23 mg/ml of cyanuric acid dissolved in water. A 5% cat food solution in water was prepared by grinding and sonicating Royal Canon Urinary SO cat food pellets (kibble). Carnation milk powder was purchased from a local supermarket and suspended at a concentration of 11.5 g in 240 ml of water according to the procedure recommended for reconstitution. The sample was diluted 20-fold in water for assays.

2.1. Sample hydrolysis/digestion

Some samples were treated with acid to promote the release of nitrogen. Where indicated, melamine, cyanuric acid, and cat food were digested as follows: 100 μl of 97% H_2SO_4 or 37% HCl, as indicated, was added to 900 μl of each sample solution and then incubated at 100 °C for 1 h. To neutralise the acid, 100 μl of a 10 M solution of NaOH was added to each sample. To digest samples with proteinase, where indicated (see figures), 1 ml of the 5% cat food solution was combined with 50 μl of a 20 mg/ml proteinase K solution and then incubated at 37 °C in a water bath for 1 h.

2.2. Ninhydrin assay

To run a Ninhydrin test, 2 μl of each sample was spotted on a Flexible Thin layer Chromatography (TLC) Plate. After the samples dried, the plate was sprayed with a 0.2% ninhydrin solution prepared by dissolving 0.2 g of ninhydrin in 100 ml of methanol. The plate was air dried and then heated in an oven at 70 °C for 10 min.

2.3. Bradford protein assay

To perform a Bradford assay, test samples were added to microcentrifuge tubes and brought to a volume of 800 μl with water. Next, 200 μl of 5× Bradford reagent (Bio-Rad laboratories catalogue number 500-0006) was added to each sample to bring it to a volume of 1 ml. The samples were then analysed in a Beckman spectrophotometer to determine their absorbance at 595 nm. A standard curve was prepared with bovine serum albumin (BSA) as a control for all experiments. To do this, 0, 2, 5, 10 or 20 μl of BSA at a concentration of 1.4 mg/ml or in some assays 2.0 mg/ml, was added to 800 μl of water and then used in the assay.

2.4. Ammonia assay

Ammonia assays were performed using a kit from Sigma (catalogue number AA0100), in which ammonia reacts with α-ketoglutaric acid and NADPH in the presence of L-glutamate dehydrogenase to form L-glutamate and NAD+. The oxidation of NAD+ is read as a decrease in absorbance at 340 nm. Where indicated, the samples were treated with H_2SO_4, HCl or proteinase K. For the assay, samples were brought to 100 μl with water and then 1 ml of the ammonia assay reagent was added. The samples were incubated for 5 min at 18–35 °C and their absorbance was then measured at 340 nm. Next, 10 μl of L-glutamate Dehydrogenase solution was added to each sample and after 5 min, the absorbance was measured again at 340 nm. A standard curve was generated using an ammonia standard and 1 ml of the ammonia assay reagent. The blank was 100 μl of water and 1 ml of the reagent. The data are presented as the change in the absorbance compared to the standard curve with ammonia. Experiments in this study were repeated 2–3 times each.

3. Results

3.1. Melamine and cyanuric acid do not interfere with Bradford assays

Melamine and cyanuric acid are added to food as a means to boost the apparent protein content. To test how these compounds react in protein assays, three assays were employed: the Bradford assay, the Ninhydrin assay, and an ammonia release assay similar to the Kjeldahl method. We first tested samples of melamine and cyanuric acid in the Bradford assay. To calibrate the assay, we used bovine serum albumin (BSA) as a typical protein to create a standard curve (Fig. 1A). Fig. 1B shows the results of the Bradford assay run on samples of melamine and cyanuric acid. Unlike BSA, the Bradford assay did not detect either compound although almost 10 times as much sample was tested as BSA.

The lack of positive results from the Bradford assay of melamine and cyanuric acid prompted additional tests, which involved treatments to release nitrogen as ammonia for analysis. We treated samples with HCl and H_2S0_4, the most commonly used means of hydrolysing proteins in the food industry. Even after these treatments there was no change in the apparent protein as measured by the Bradford assay (Fig. 2A, data not shown for cyanuric acid). We conclude that neither melamine nor cyanuric acid is detected in the Bradford assay, even after hydrolysis.

3.2. A Ninhydrin test to measure protein in test substances

The second protein test we employed on melamine and cyanuric acid was a ninhydrin test. Ninhydrin reacts with amino acids,