

**ANALYSIS OF MELAMINE AND CYANURIC ACID BY LIQUID
CHROMATOGRAPHY WITH DIODE ARRAY DETECTION AND TANDEM
MASS SPECTROMETRY**

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Thesis Advisors: Dr. Rodney J. Bushway and Dr. L. Brian Perkins

An Abstract of the Thesis Presented
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Melamine and cyanuric acid are the compounds that caused the global incidences of kidney related disease to pets and infants in North America and China during the last two years. After a concerted research effort by U.S. laboratories, it was discovered that they were intentionally added in raw ingredients to pet foods or milk to increase nitrogen levels without providing protein. Melamine and cyanuric acid can easily combine by hydrogen bonding and produce a melamine-cyanurate complex that directly caused renal failure to pets and infants who consumed the tainted pet food or milk. Many analytical methods for the determination of melamine and cyanuric acid have been developed. In terms of sensitivity and validity, not all of the methods are useful. In the research described in this thesis, various analytical methods and techniques were explored to find better and easier methods for melamine and cyanuric acid analysis. In the first chapter, methods of enzyme immunoassay (EIA), high-performance liquid chromatography with diode array detection (HPLC-DAD), and ultra-performance liquid chromatography with

tandem mass spectrometry (UPLC-MS-MS) were studied for melamine analysis. The limits of detection (LOD) for EIA and HPLC-DAD were 0.02 and 0.1 $\mu\text{g/mL}$, respectively. The r^2 values between the EIA and HPLC-DAD methods for melamine analysis of the fortified and originally contaminated samples were 0.997 and 0.989. The r^2 values for UPLC-MS-MS with HPLC-DAD and with EIA were 0.957 and 0.949, respectively. The commercial melamine EIA kit used in this study proved to be a rapid and inexpensive alternative to the HPLC-DAD method to quantify melamine in pet foods. In the second chapter, pressurized liquid extraction (PLE) was evaluated and compared with typical extraction methods such as polytron and sonication. Recoveries obtained by the PLE method were significantly higher ($P \leq 0.05$) than those of sonication and polytron methods for dry pet food samples. For the analysis of adulterated pet foods, PLE resulted in the highest melamine content followed by sonication and polytron. PLE provided the best extraction efficiency compared to sonication and polytron. In the third chapter, liquid chromatography with tandem mass spectrometry (LC-MS-MS) was used for determination of melamine and cyanuric acid using an alkaline pH aqueous extraction solvent (0.1 N ammonium hydroxide). When melamine cyanurate complex was fortified, alkaline extraction solvent yielded better recoveries than the acetonitrile/water (50/50) mixture, which is the most commonly used solvent for extracting melamine and cyanuric acid from various sample matrices. Since similar results were obtained from the adulterated pet food samples, it is assumed that the adulterated samples were contaminated with melamine cyanurate complex rather than free melamine and cyanuric acid. Therefore, the method developed in this study is effective for the accurate determination of melamine and cyanuric acid.

DEDICATION

I dedicate this thesis to my parents who let me do whatever I wanted to do and just watched me. Without their love, support, and prayer I could not be the person where I stand now.

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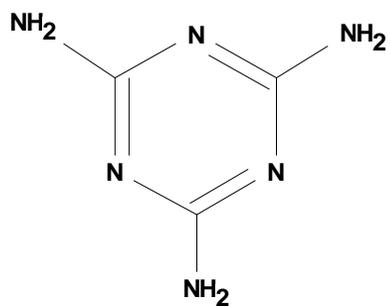
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CHAPTER 1

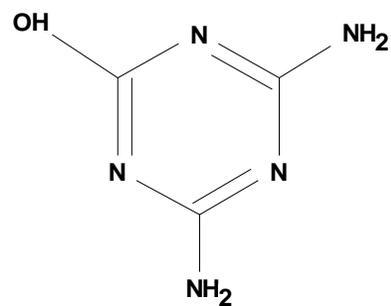
INTRODUCTION

Melamine and analogues

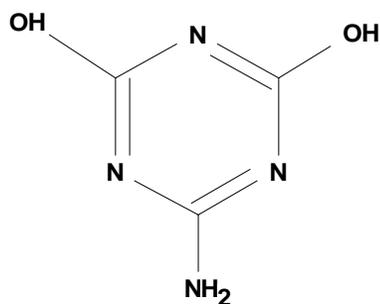
Melamine (1,3,5-triazine-2,4,6-triamine; $C_3H_6N_6$; mw = 126.13) consists of three reactive amine groups ($-NH_2$) and an aromatic s-triazine ring that provides materials derived from it with excellent thermal stability. Melamine has several synonyms such as melamine crystal; cyanurotriamide; 2,4,6-triamino-s-triazine; cyanuramide and cyanuric acid amide. Melamine can be metabolized by microorganisms to form such analogues as ammeline, ammelide, or cyanuric acid, by replacing amino groups with hydroxyl groups at each position (Wackett et al., 2002) (Figure 1). These analogues can also be produced by acidic or alkaline hydrolysis from melamine using high temperature (Bann and Miller 1958). Cyanuric acid may also be found as an impurity of melamine. Cyanuric acid is a widely used stabilizer in outdoor swimming pool water to minimize the decomposition of chlorine (sanitizer) by light (Downes et al. 1984). Melamine and cyanuric acid can bind to each other by hydrogen bonds in an aqueous environment (Seto and Whitesides 1993; Liu and Wang 2006; Ranganathan et al. 1999) (Figure 2). Melamine and cyanuric acid can create large molecules by cross linking hydrogen bonds. Using this self-assembly of melamine and cyanuric acid, melamine could be added into a swimming pool to measure the amount of cyanuric acid by measuring the turbidity caused by melamine cyanuric acid precipitation (Fernando 2002). Melamine cyanurate is almost water insoluble (2 mg/L) (Dobson et al. 2008) and used as a fire retardant because it absorbs heat energy when decomposed into melamine and cyanuric acid by fire (EFRA 2007).



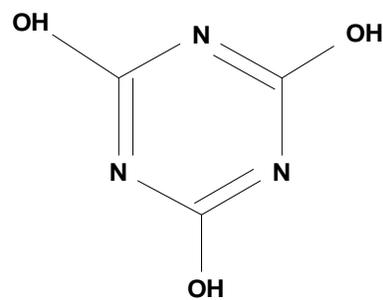
Melamine



Ammeline



Ammelide



Cyanuric acid

Figure 1. Structures of melamine, ammeline, ammelide, and cyanuric acid.

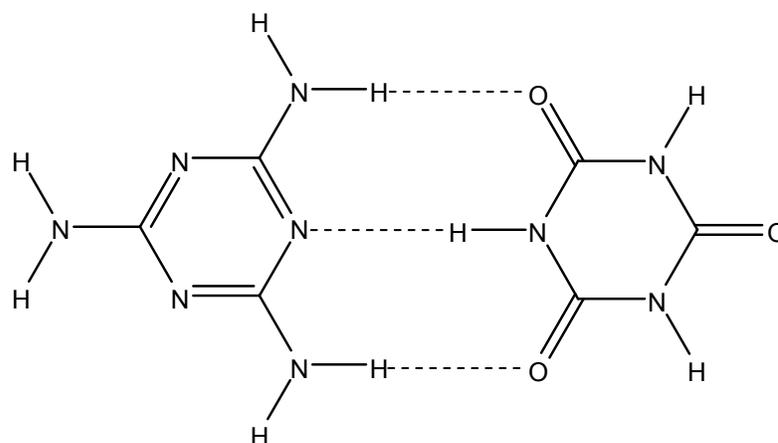


Figure 2. The complex of melamine and cyanuric acid bound by hydrogen bonds.

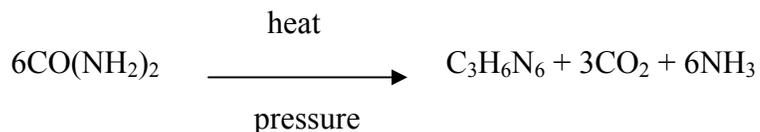
Melamine and its analogues are thermally stable and are insoluble in many solvents. Physical properties of melamine and its analogues are shown in Table 1. Melamine is slightly soluble in water, in alcohol, glycol, pyridine, and glycerol. Cyanuric acid is more soluble than melamine in water and is also soluble in hot alcohols, pyridine, concentrated HCl and H₂SO₄. Some alkali solutions such as NaOH and KOH also can dissolve cyanuric acid. Ammeline is very slightly soluble in aqueous alkalies and mineral acids. Ammelide is insoluble in water but, can be easily dissolved in alkalies, ammonia and concentrated mineral acids. (Merk 1989; Sigma-Aldrich 2008; Acros organics 2008; Bann and Miller 1958; Dobson et al. 2008).

Table 1. Physical properties of melamine, ammeline, ammelide and cyanuric acid

	Melamine	Ammeline	Ammelide	Cyanuric acid
Physical state	Powder	Powder	Powder	Powder
Color	White	White	White	White
Melting point (°C)	> 300	> 310	NA*	> 360
Solubility in water (mg/L)	3240	75	77	2000

* Not available

Melamine can be produced by processing urea, dicyandiamide or hydrogen cyanide under high temperature and pressure. Melamine was originally produced by the dicyandiamide process from the 1930's. However, current production of melamine is based on the urea process (Shawqui et al. 1989). The net reaction for melamine production from urea is shown below.



Similar to melamine, most cyanuric acid can be produced from urea by pyrolysis. However, it must be purified to be used further. Higher purity cyanuric acid can be achieved by hydrolysis of melamine, ammeline or ammelide with sulfuric acid at high temperatures (≥ 175 °C) (Bann and Miller 1958; Wegleitner et al. 1981).

Melamine is widely used in a form of melamine-formaldehyde (MF) resin for surface coatings, laminates, paper treating, molding compounds, textile treatments and concrete additives (Maxwell 2004). Since kitchen utensils made from MF resin are almost unbreakable and inexpensive, they can be found in every home. This popular use of kitchen articles made from melamine has attracted researchers, and led them to investigate migration of melamine and formaldehyde from kitchen articles such as plates and cups into food stuffs (Bradley et al. 2005; Lund and Petersen 2006). Melamine also has been used as a fertilizer because of its high nitrogen content even though its effectiveness as a fertilizer depends on the presence of microorganisms in soil such as *Klebsiella terrigena* that can metabolize it to readily available nitrogen compounds such as urea (Shelton et al. 1997).

Melamine Incidences

In March 2007, the illness and deaths of cats and dogs were reported to the United States FDA (Food and Drug Administration). The FDA's investigation revealed that they had consumed melamine-contaminated pet food (US FDA 2008a). This incidence caused a dozen deaths and hundreds of cases of kidney-related illness to cats and dogs, and led to the largest pet food recall in FDA history. Further investigation found that the melamine-tainted pet foods were also contaminated with structurally similar compounds such as cyanuric acid, ammeline and ammelide. It was revealed that some ingredients used to make pet food, such as wheat gluten and rice protein, had been intentionally adulterated with melamine and related compounds. It is assumed that these nitrogen-rich chemicals were added to make the final product appear to be protein rich, resulting in higher commercial value (WHO 2008). Interestingly, there were several outbreaks of renal failure linked to ingestion of commercial dog foods in South Korea and Taiwan in 2004. The 2004 outbreaks had identical clinical, histological, and toxicological findings with the outbreak that occurred in 2007. However, the sources of the contaminants in the 2004 outbreaks remains undetermined (Brown et al. 2007). Another case of renal failure in dogs associated with melamine tainted pet food occurred in Italy in 2008. High levels of melamine (766.8 ppm) and cyanuric acid (158.5 ppm) were found in the pet food products that were consumed by dogs. Their findings agree with the previous reports of outbreaks from Asia in 2004 and the USA in 2007 (Cocchi et al. 2009). In September 2008, an increased frequency of kidney stones and renal failure in babies was reported in China. Since then, more than 50,000 infants have been hospitalized and resulted in 6 deaths after drinking baby formula tainted with melamine.

The Chinese government announced that 52,857 babies had been treated for renal complications by September 22, 2008 (Kuehn 2009; Chan et al 2008). At the same time, 15,017 children were admitted to hospitals in Hong Kong. Five of them had kidney stones, and four of these five children had lived in China previously (Griffiths and Fok 2008). Since large quantities of milk-based products had been exported to other countries, the United Nation (UN) alerted the countries that had imported from China. The European Union (EU) banned the import of baby food from China (Parry 2008). The EU members have been asked by the European commission to conduct checks on all products from China containing more than 15% milk. The United Kingdom (UK) currently requires products from China containing more than 15% milk be subjected to documentary, identity and physical checks, including laboratory analysis. Foodstuffs with melamine levels exceeding 2.5 ppm (mg/kg) are to be destroyed (Food Standards Agency 2008). The US FDA has stricter regulations. Based on their findings, the US FDA has stated that melamine and its analogues below 2.5 ppm in regular foods and 1 ppm in infant formula do not raise public health concerns (US FDA 2008b).

Toxicity

Melamine and cyanuric acid have a low acute toxicity, and do not produce acute renal toxicity (Dobson et al. 2008; Canelli 1974). Melamine toxicity in mice and rats is both subchronic and chronic (US NTP 1983). The oral LD₅₀ in rats for melamine and cyanuric acid are 3161 and >3400 mg/kg body weight, respectively (OECD 2002; US FDA 2008c). One of the main toxic effects of melamine is calculi formation (OECD 2002; Melnick 1984). A high incidence of bladder calculi was induced in weanling rats

by Heck and Tyl 1985, but the incidence was much lower in adult rats. Dietary exposure to melamine or cyanuric acid alone did not cause renal failure in cats. However, cats dosed with a combination of melamine and cyanuric acid were euthanized after 48 hours of dosing due to acute renal failure (Puschner et al. 2007). Dobson et al. (2008) found that a mixture of melamine and cyanuric acid fed to rats caused renal damage and crystals in the nephrons which were identified as a melamine cyanurate complex. There are no melamine studies with human subjects, but, there is a study (with limited data) on oral exposure to cyanuric acid, which showed that more than 98% of the orally administered cyanuric acid is excreted, unchanged, in urine within 24 hours (Allen et al 1982). Lam et al. (2009) found that urinary melamine levels in patients who consumed melamine-tainted milk strongly correlated with the size of the renal stones. However, urinary cyanuric acid levels did not correlate with the stone size. Therefore, they hypothesized that unlike in animal cases, cyanuric acid may not be important in formation of melamine-associated renal stone disease in humans.

Analytical methods

Liquid Chromatography

Liquid chromatography is the most commonly used method for melamine and cyanuric acid analysis. Beilstein et al. (1981) used reversed-phase (RP) high performance liquid chromatography (HPLC) with UV (ultraviolet) detection for analysis of s-triazine herbicides, melamine and melamine analogues. They conducted the analysis at low temperature (2 °C) to achieve complete separation of cyanuric acid, ammelide and ammeline. Lowering the temperature provided parameters such as increased viscosity and

pressure, which were necessary to separate the compounds. Ishiwata et al. (1987) have determined the melamine content in beverages by ion-pair liquid chromatography (LC). Limit of quantitation (LOQ) achieved by their method was 50 ppb ($\mu\text{g/L}$). HPLC with electrochemical detection was employed by Pukkila et al. (1987) for melamine analysis. However, no better sensitivity was obtained than with UV detection. Melamine and its three hydrolytic products ammeline, ammelide and cyanuric acid leached from melamine resin tableware were analyzed by HPLC-UV (Sugita et al. 1990). Levels ranging from 3.0 to 9.7 ppm ($\mu\text{g/mL}$) of melamine were detected in the leachate solution and the three hydrolytic compounds were not detected at a limit of 0.02 ppm ($\mu\text{g/mL}$). Using HPLC-UV detection, analysis of cyromazine (an insecticide) and melamine as a metabolite of cyromazine, in different matrices such as poultry meats, eggs, and soil has been reported (Cabras et al. 1990; Yokley et al. 2000; Chou et al. 2003). Simultaneous detection of melamine and its analogues, ammeline, ammelide, and cyanuric acid in cereal flour was studied using HPLC-UV detection (Ehling et al. 2007). To prevent the formation of the melamine and cyanuric acid precipitate, alkaline conditions (pH 11-12) were maintained using a sodium hydroxide (0.05 M for stock standard solution, and 0.01 M for extraction solvent). Although the method developed was relatively simple and convenient, the sensitivity was not sufficient to obtain the current legal limit (2.5 $\mu\text{g/g}$). The limit of detection (LOD; three times the standard deviations of the intercepts of the calibration curves divided by the slopes of the calibration curves) was 5 ppm ($\mu\text{g/g}$) for melamine, ammeline and ammelide and 90 ppm ($\mu\text{g/g}$) for cyanuric acid. Kim et al. (2008) developed an HPLC-DAD (diode array detection) method for melamine analysis of pet food with LOD (signal to noise ratio = 3:1) of 0.1 $\mu\text{g/mL}$. Muniz-Valencia et al. (2008)

used 100% aqueous mobile phase (5 mM sodium phosphate; pH 5.0) for analysis of melamine and its analogues from rice protein and animal feed, but their method did not consider the possible formation of precipitate from the binding of melamine and cyanuric acid. Briggles et al. (1981) first used a RP-HPLC for quantitative determination of cyanuric acid in urine and swimming pool water. Modified and improved methods for cyanuric acid analysis of pool water were later developed by Cantu et al. (2000; 2001).

Quadrupole tandem mass spectrometry (MS/MS) has been successfully employed for melamine and cyanuric acid analysis, in conjunction with liquid chromatography. Due to better selectivity, sensitivity and confirmation of the target analyte, LC-MS/MS has been more widely used than HPLC-UV. The fragmentation of the melamine molecule by electron impact ionization (EI), laser desorption ionization (LDI) and collision-induced dissociation (CID) was studied by Ju et al. (1999). Fragment ions such as m/z (mass-to-charge ratio) = 43 (CN_2H_3^+), 53 ($\text{C}_2\text{N}_2\text{H}^+$), 56 (CN_3H_2^+), 68 ($\text{C}_2\text{N}_3\text{H}_2^+$), 83 ($\text{C}_2\text{N}_4\text{H}_3^+$), 85 ($\text{C}_2\text{N}_4\text{H}_5^+$), 99 ($\text{C}_2\text{N}_5\text{H}_5^+$), 110 ($\text{C}_3\text{N}_5\text{H}_4^+$) were generated from the melamine molecular ion ($m/z = 127 [\text{M} + \text{H}]^+$) by EI. Ju et al. (1999) found that $m/z = 43$ (CN_2H_3^+) and 85 ($\text{C}_2\text{N}_4\text{H}_5^+$) were the major product ions in the EI experiment. However, $m/z = 68$ ($\text{C}_2\text{N}_3\text{H}_2^+$) and 85 ($\text{C}_2\text{N}_4\text{H}_5^+$) product ions were chosen for melamine analysis by other researchers for ion monitoring by tandem mass spectrometry (Andersen et al. 2008; Filigenzi et al. 2008; Heller and Nochetto 2008; Smoker and Krynitsky 2008). Currently, two ion transitions (m/z 127 \rightarrow 85 and m/z 127 \rightarrow 68) with positive ionization are commonly monitored for quantitation and qualification of melamine. Using negative ionization, two ion transitions (m/z 128 \rightarrow 85 and m/z 128 \rightarrow 42) were monitored for cyanuric acid analysis by tandem mass spectrometry with electrospray ionization (ESI)

mode (Karbiwnyk et al. 2009; Andersen et al. 2008; Filigenzi et al. 2008; Heller and Nochetto 2008; Turnipseed et al. 2008; Smoker and Krynitsky 2008).

Matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry was studied for the analysis of melamine, ammeline, ammelide, and cyanuric acid (Campbell et al. 2007). They observed $[M + H]^+$ ions for ammelide and ammeline under positive ion conditions with sinapinic acid (SA) as the matrix. When alpha-cyano-4-hydroxy-cinnamic acid (ACHC) was used as a matrix, a matrix-melamine complex (m/z 316) was observed, indicating a complex of protonated melamine ion (m/z 127.4) plus the matrix, ACHC ($mw = 189$). However, no complex was observed when SA was used as the matrix. The major ion formed is $[M + H]^+$ at m/z 127.3 which could be the protonated melamine ion. Cyanuric acid was analyzed using SA as the matrix in the negative mode and $[M - H]^-$ was observed at m/z 128.

Gas Chromatography

Since melamine and cyanuric acid are nonvolatile and soluble in aqueous solvents (acidic or basic), liquid chromatography is often a better choice for analysis. However, there have been some studies focusing on analyzing melamine and cyanuric acid analysis by gas chromatography (GC). Melamine analysis by GC was conducted by Bardalaye et al. (1987) testing cyromazine and its product, melamine in Chinese cabbage samples. They extended their research for mass spectral properties of cyromazine and melamine using GC-MS (Toth and Bardalaye 1987) and found that the most abundant fragment ions of melamine by electron impact (EI) ionization were achieved at m/z 68 and m/z 85, which, are also the ions used for the current LC-MS-MS work. Yokely et al.

(2000) monitored the same fragment ions for melamine determination in soil. Unfortunately, cyanuric acid analysis by gas chromatography required derivatization. Fiamegos et al. (2003) have developed a GC method for trace cyanuric acid analysis using flame thermoionic detection (FTD) and mass-selective detection (MSD). LOQs (the lowest concentration calculated by a signal-to-noise ratio = 10) were found to be less than 1 and 90 $\mu\text{g/L}$ for MS and FTD, respectively. Most recently, Litzau et al. (2008) established a GC-MS method for screening melamine and its analogues from various food matrices. They derivatized melamine and its analogues by inducing trimethylsilyl derivatives, which, dramatically enhanced their chromatographic properties and provided specific mass spectra. This method can be used as a screening method with a minimum reporting level (MRL) of 10 $\mu\text{g/g}$ and above. A semi-quantitative estimation can be achieved only when a sample is positive at the MRL or above. Additional guidance is available to extend the MRL to 2.5 $\mu\text{g/g}$, the current legal limit set by the US FDA.

Other methods

Bioanalytical methods such as enzyme –linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) have been developed as a simple, sensitive and convenient screening tools for melamine. The first commercial EIA kit for melamine analysis was developed by Beacon Analytical Inc. (Portland, Maine). Kim et al. (2008) evaluated the EIA kit with other analytical methods such as HPLC-DAD and UPLC-MS-MS methods, and found that the EIA kit is a rapid and inexpensive alternative to quantify melamine in pet food samples. Garber (2008) has examined two commercial ELISA kits for melamine analysis from dog foods. A melamine plate kit from Abraxis (Varminster, Pennsylvania)

had a LOD (concentration of melamine necessary to generate a response greater than four times the standard deviation from background) of 1 $\mu\text{g/mL}$ from dog food samples.

A Surface Enhanced Raman Spectroscopy (SERS) method for melamine analysis was developed by He et al. (2008) and Lin et al. (2008). In their proposed strategy, the SERS method developed could be used first as a screening method for the presence of melamine, and the HPLC-DAD method could follow for quantification and confirmation of SERS-positive samples. Yan et al. (2009) have developed a new approach to melamine analysis using capillary zone electrophoresis with diode array detection. They successfully separated melamine from other interferences in samples by optimizing a pH (3.2) buffer solution. The achieved LOD (ratio of signal to noise = 3:1) and LOQ (the lowest levels at which melamine can be reliably detected in the extract of sample matrices) were 0.01 and 0.05 $\mu\text{g/mL}$, respectively.

Objectives

The overall goal of this research was to explore the analytical methods for the detection of melamine and cyanuric acid using various instruments and techniques.

The specific objectives of the studies were to:

1. Compare EIA, HPLC-DAD, UPLC-MS-MS methods to analyze melamine in pet food samples.
2. Study the applicability of a pressurized liquid extraction (PLE) system for melamine analysis and compare it with other typical extraction methods such as polytron and sonication.
3. Develop a LC-MS-MS method for the determination of melamine and cyanuric acid, using alkaline pH aqueous extraction for the complete dissociation of melamine-cyanurate complex in samples.

CHAPTER 2
MELAMINE ANALYSIS OF PET FOOD BY EIA, HPLC-DAD AND
UPLC-MS-MS

Chapter Abstract

Melamine in pet food (fortified or originally contaminated) was determined by enzyme immunoassay (EIA), high-performance liquid chromatography with diode array detection (HPLC-DAD), and ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS-MS). The limits of detection (LOD) for EIA and HPLC-DAD were 0.02 and 0.1 $\mu\text{g/mL}$, respectively. The linear ranges of the calibration curves for EIA and HPLC-DAD were 0.02–0.5 and 0.1–500 $\mu\text{g/mL}$, respectively. The coefficient of determinations (r^2) of the standard curves for EIA and HPLC were 0.9991 and 0.9999, respectively. Coefficient of variations (CV) from both inter-and intra-assay were <9.31%, and recovery range for all concentrations was between 71 and 105%. The r^2 values between the EIA and HPLC-DAD methods for melamine analysis of the fortified and originally contaminated samples were 0.9973 and 0.9885. The r^2 values for UPLC-MS-MS with HPLC-DAD and with EIA were 0.9566 and 0.9489, respectively.

Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is a nitrogen-based industrial chemical. It has been used in the manufacture of durable plastics such as tableware and cups. This use has led to a concern that melamine could migrate into food coming in contact with these plastics (Bradley et al. 2005; Kawai et al. 1989; Lund and Petersen 2006; Martin et

al. 1992). Melamine can also be formed from the degradation of cyromazine, a larvacide used to prevent flies from hatching (Cabras et al. 1990). High and continuous dietary exposure to melamine causes formation of bladder stones and increases incidence of urinary bladder tumors in male rats (OECD 2002; WHO 1999). To date, no studies using human subjects have been reported.

On March 15, 2007, a pet food manufacturer (Menu Foods, Ontario, Canada) alerted the United States Food and Drug Administration (FDA) to 14 animal deaths in the United States that appeared to be linked to pet food. In the following months, consumers and veterinarians reported more illnesses and deaths potentially associated with pet food. The FDA found melamine and melamine-related compounds such as cyanuric acid, ammeline, and ammelide in the pet food samples. Further investigation showed that wheat gluten and rice protein imported from China as pet food ingredients were contaminated with melamine. Melamine and melamine-related compounds are not approved to use as an ingredient in pet food (US FDA 2007a).

Melamine analysis in matrices such as Chinese cabbage (Bardalaye et al 1987), soil (Yokley et al. 2000), poultry meat (Chou et al. 2003), beverage (Ishiwata et al. 1987), and catfish (Andersen et al. 2007) has been performed by gas chromatography (GC), GC/mass spectrometry (GC/MS), high-performance liquid chromatography (HPLC) with UV detection, and LC tandem mass spectrometry (LC/MS/MS). Determination of melamine in pet food samples has been conducted by the FDA using an HPLC-UV procedure for quantitation and GC/MS for confirmation (US FDA 2007b; US FDA 2007c). However, currently there is no official method for melamine determination in pet food.

This paper describes a new enzyme immunoassay (EIA) procedure and an HPLC-diode array detection (DAD) method that can be used to determine melamine in pet food. EIA is a good screening procedure because of its simplicity and low cost per analysis, and HPLC-DAD can be used for confirmation and quantification. Ultra-performance LC coupled with MS/MS (UPLC-MS/MS) was also tested for correlation with EIA and HPLC-DAD for melamine analysis of pet food.

Materials and Methods

Apparatus

EIA analysis was conducted using Beacon melamine plate kit (Beacon Analytical Systems, Portland, ME) and a plate reader (ELx 808, BioTek Instruments, Inc., Winooski, VT). Hewlett Packard model 1050 liquid chromatograph equipped with a diode array detector and a Luna C8(2) column (150 × 4.6 mm, 3 μm, Phenomenex, Torrance, CA) were used for HPLC-DAD analysis for melamine. ACQUITY UPLC liquid chromatography coupled with a Quattro Premier XE tandem quadrupole mass spectrometer equipped with an electrospray ionization source (ESI) and a BEH C18 column (100 × 1.0 mm, 1.7 μm, Waters Corp., Milford, MA) were used and compared with the other two methods.

Materials

Blank samples (wet and dry) for controls and fortification were purchased from a local grocery store (Portland, ME). Approximate percentages of protein, fat, and carbohydrate for dry pet food (28 %, 17 %, and 48 %) and for wet pet food (42 %, 32 %, and 20 %)

and 20 %) were calculated by weight on dry matter. Originally contaminated sample products (recalled) were provided by the New York State Department of Agriculture and Markets Food Laboratory (Albany, NY). HPLC grade acetonitrile, tetrahydrofuran and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Melamine standard ($\geq 99\%$) was purchased from Fisher Scientific (Pittsburgh, PA). Sodium 1-octanesulfonate monohydrate ($\geq 99\%$), potassium chloride ($\geq 99\%$), hydrochloric acid (37 %), acetic acid ($\geq 99\%$) and ammonium acetate ($\geq 99\%$) were purchased from Sigma-Aldrich (St.Louis, MO).

Sample Fortification

To produce three concentrations of melamine-fortified samples (0.1, 1, and 3 mg/g), 14 g of each pet food were weighed into a centrifuge tube (50 mL), and solid melamine (14 and 42 mg) was added directly to the samples to make 1 mg/g and 3 mg/g, respectively. For 0.1 mg/g, a melamine solution (4 mg/mL) was prepared in a mixture of acetonitrile/water (50:50) and then added (350 μ L) to the sample (14 g). The fortified samples were homogenized by inserting a spatula into the sample tube and were stirred while vortexing for 10 min.

Melamine Analysis by EIA

An aliquot of the homogenized pet food was weighed (2 g for wet and 1 g for dry) into a 50 ml polypropylene centrifuge tube and 10 ml of methanol/water (60:40) was added. The mixture was sonicated for 3 min in an ultrasonic cleaning bath and allowed to stand for 5 min before filtering through a glass fiber filter (Grade 6; 11.0 cm; Fisher

Scientific, Pittsburgh, PA). EIA analysis of melamine in pet food was performed using a commercial kit provided by Beacon Analytical Systems. The procedure was included in the kit. In short, 150 μL of calibrating reagents (for standard curve) or sample extracts were added to the test wells followed by 50 μL of melamine HRP conjugate. The sample extracts were diluted as directed in the procedure. The plate was gently shaken for 60 sec and incubated for 30 min. The content of the wells was decanted, and the wells were washed 3 times with tap water. Once washed, the water in the wells was removed by tapping the plate onto absorbent paper. Then, 100 μL of substrate was added to each well and the plate was incubated for 30 min followed by the addition of 100 μL stop solution to each well. The absorbance was read at 450 nm using a plate reader. The absorbance (B) at 450 nm was determined within 15 min after the reaction was stopped, and compared to a zero concentration standard (B_o) to give the ratio B/B_o (mean absorbance value for the standard divided by the mean absorbance value for the zero standard).

Melamine Analysis by HPLC-DAD

Sample preparation followed the procedure for EIA extraction except that acetonitrile/water (50:50) was used as the extraction solvent. The tube was vortexed for 1 min, sonicated for 30 min, and vortexed again for 1 min. The homogenate was centrifuged at 10,000 \times g for 15 min and the supernatant was filtered through a 0.45 μm syringe filter (PTFE; 13 mm; 0.45 μm pore size; Fisher Scientific, Pittsburgh, PA). Ten microliters of this filtrate was injected into the HPLC system. Separation was achieved with a Luna C8(2) column. Mobile phase was buffer/tetrahydrofuran/0.1M ammonium acetate (70:5:25). The flow rate was 0.5 mL/min and the wavelength was monitored at

236 nm. The buffer composition was 10 mM citric acid and 10 mM sodium octanesulfonate adjusted to pH 3.0 with citric acid. From a stock solution of 1 mg/mL, ten different concentrations of melamine standard (0, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500 $\mu\text{g/mL}$) were prepared by diluting appropriate volumes in a solution of acetonitrile/water (50:50). The stock solution was sonicated for 10 min to assure the melamine was completely dissolved. The working standards were analyzed in triplicate by HPLC-DAD.

A solution of acetonitrile/water (50:50) and acidic buffer (pH 2.0) consisting of 0.2 M potassium chloride (KCl) and 0.2 M hydrochloric acid (HCl) was used to compare recovery efficiency of melamine from both dry and wet pet food. Higher relative centrifugal force (RCF; 20,000 \times g) was used for acidic buffer extraction. Melamine determination was conducted by HPLC-DAD.

Melamine Analysis by UPLC-MS-MS

Determination of melamine in pet food was conducted by ultra-performance liquid chromatography coupled with a tandem quadrupole mass spectrometer equipped with an electrospray ionization source (ESI). Initial mobile phase composition was 95:5 (0.15 % acetic acid in water/0.15 % acetic acid in methanol) with a flow rate of 70 $\mu\text{L/min}$. The mobile phase program was 0 – 1 min 95:5, 1 – 4.41 min ramp gradient to 5:95, 4.41 – 6.50 min hold 5:95, 6.50 – 8.00 min ramp gradient back to 95:5, 8.00 – 10.00 min hold 95:5. The capillary voltage was maintained at 3.35 kV. The cone voltage was 22 V. The multiplier voltage was 650 V. The flow of the desolvation gas and cone gas were set to 900 Lh^{-1} and 300 Lh^{-1} , respectively. Source temperature and desolvation gas temperature were held at 100 and 250 $^{\circ}\text{C}$, respectively. The mass spectrometer was

operated in multiple reaction monitoring mode (MRM), and the ion spray source was operated in positive ion mode. The collision energy was varied for each monitored transition. The transitions monitored for melamine were m/z 126 \rightarrow 42.8 at 25 V and m/z 126 \rightarrow 84.7 at 17 V. The ratio of transition m/z 126 \rightarrow 42.8 to m/z 126 \rightarrow 84.7 was 20 %. The dwell time for each monitored transition was 0.1 s. Sample preparation was same as for the HPLC-DAD method.

Inter- and Intra-assay Variation for HPLC-DAD Analysis

Inter-assay variation was computed from the analysis of the three concentrations of melamine-fortified samples, carried out on six different days. Intra-assay variation was calculated by analysis of six replicates of each concentration on a single day. Sample recoveries were calculated from the standard curve.

Correlation among HPLC-DAD, EIA, and UPLC-MS-MS Analysis

Two sets of pet food samples were prepared for comparison between HPLC-DAD, EIA and UPLC-MS-MS techniques to determine melamine content in the samples. The first set consisted of known concentrations of melamine-fortified samples (18 samples), and the second set consisted of commercial products which were recalled for melamine contamination (6 samples). The results of the analyses of the first set of fortified samples by HPLC-DAD and EIA was tested for correlation. After fortifying and homogenizing, each sample was divided into two tubes for HPLC-DAD and EIA analysis (36 tubes), and tested separately. The results of the analyses of the second sample set (6 commercial pet food products) were compared between all three techniques, HPLC-DAD,

EIA and UPLC-MS-MS. Each pet food product was homogenized and divided into three tubes for HPLC-DAD, EIA and UPLC-MS-MS analysis (18 tubes), and tested separately.

Results and Discussion

The limit of detection (LOD) for EIA was $0.02 \mu\text{g/mL}$, defined as the lowest melamine concentration to produce a B/B_o value of 10 standard deviation units greater than B_o . The range of linearity was from 0.02 to $0.5 \mu\text{g/mL}$, with an r^2 of 0.9991.

Melamine in the pet food was extracted with acetonitrile/water (50:50) and analyzed by HPLC-DAD. The mechanism of separation described in this paper was ion-pairing with sodium octanesulfonate (10 mM, pH 3.0) (David et al. 2005). Figure 3 shows the chromatogram of a fortified pet food sample (1 mg/g) analyzed by HPLC-DAD. It illustrates a fully resolved melamine peak at 15 min, with other observed peaks eluting earlier. Adding THF and ammonium acetate to the mobile phase solved the problems of interference and late eluting compounds. The LOD was $0.1 \mu\text{g/mL}$. Signal response was linear from 0.1 to $500 \mu\text{g/mL}$ with an r^2 of 0.9999.

In an effort to reduce the use of hazardous solvents, an acidic buffer was compared to the acetonitrile:water extractant. Results of this comparison (Table 2) indicate no significant difference between the extractants ($n = 5$, $P > 0.05$, Tukey's Studentized Range Test; SAS Institute Inc., Cary, NC) for both wet and dry pet food samples. However, the aqueous extraction solution caused a high level of wheat gluten gelatinization and resulted in high sample viscosity, making filtration difficult even using high RCF ($20,000\times g$). Therefore, acetonitrile and water mixture were chosen for the HPLC-DAD method.

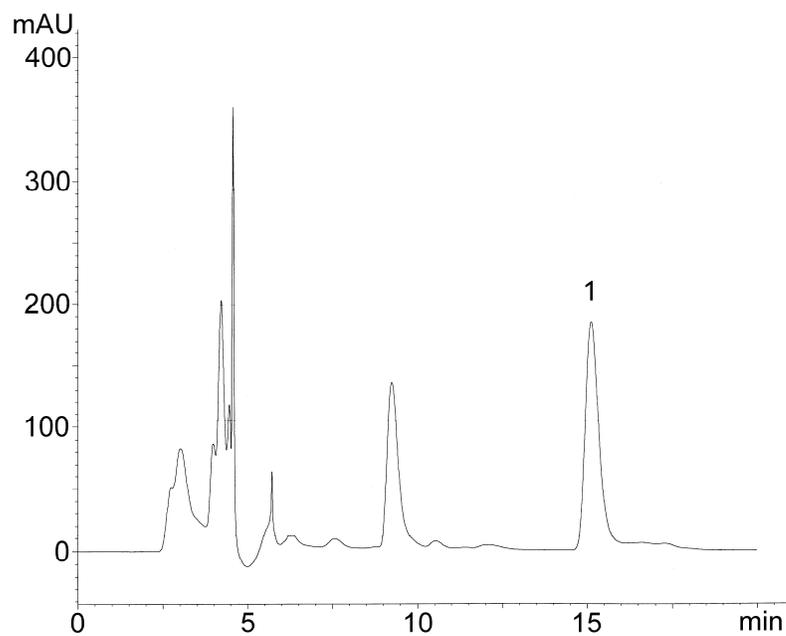


Figure 3. Chromatogram of melamine-fortified pet food sample analyzed by HPLC-DAD (1=melamine peak).

Table 2. Comparison of Two Extract Solvents for Recovery of Melamine from Dry and Wet Pet Foods.

pet food	extract solvent	measured (mg/g)	<i>n</i>	recovery (%)
Dry (0.1 %)	Acetonitrile: H ₂ O (50:50)	0.882 ± 0.004	5	88.3
	Acidic buffer	0.886 ± 0.026	5	88.6
Wet (0.1 %)	Acetonitrile: H ₂ O (50:50)	0.864 ± 0.028	5	86.4
	Acidic buffer	0.899 ± 0.029	5	90.0

Contaminated pet food was analyzed for melamine by UPLC-MS-MS. Figure. 4 shows a chromatogram generated from both melamine transitions (m/z 126 \rightarrow 42.8 and m/z 126 \rightarrow 84.7) eluting at 1.39 min. The ion ratio of m/z 42.8 to m/z 84.7 was 22% which is well within the generally accepted 10% window.

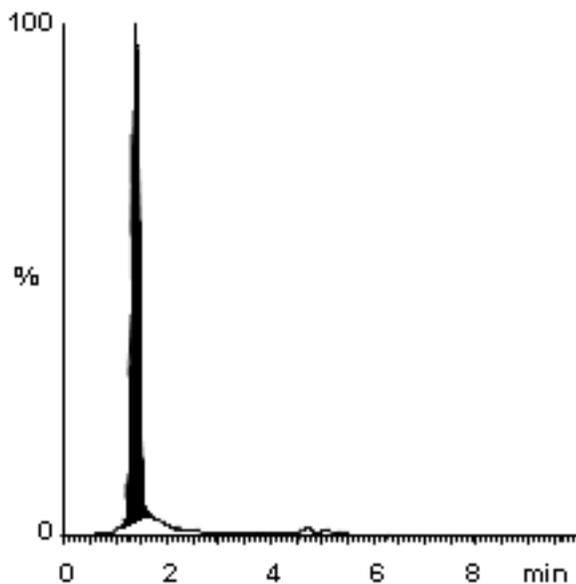


Figure 4. Chromatogram of melamine-contaminated pet food sample analyzed by UPLC-MS/MS.

Six pet food samples (A through F) fortified with three different concentrations of melamine for each sample were tested for inter- and intra-assay variation using HPLC-DAD (Table 3). The highest variations for both inter- and intra-assay (9.31 and 7.90 %, respectively) were observed at the lowest concentration (0.1 mg/g) of melamine added. Overall variability is below 10 %, indicating that the HPLC-DAD method is reliable and reproducible. The recovery range for all concentrations is between 71 and 105 %.

Table 3. Inter-assay and Intra-assay Variation of Six Pet Foods Fortified with Melamine.

Pet food	mg/g	Inter-assay ^a			Intra-assay ^b				
		<i>n</i>	Measured (mg/g)	Recovery (%)	CV (%)	<i>n</i>	Measured (mg/g)	Recovery (%)	CV (%)
A	0.1	6	0.080±0.007	80.1	9.31	6	0.085±0.003	85.5	3.65
	1	6	0.810±0.054	81.0	6.70	6	0.795±0.027	79.5	3.45
	3	6	2.323±0.048	77.4	2.05	6	2.376±0.025	79.2	1.06
B	0.1	6	0.077±0.005	76.5	6.98	6	0.081±0.005	80.7	6.15
	1	6	0.747±0.049	74.7	6.55	6	0.800±0.017	80.0	2.16
	3	6	2.256±0.074	75.2	3.26	6	2.170±0.055	72.3	2.53
C	0.1	6	0.071±0.005	70.8	7.67	6	0.091±0.007	91.3	7.90
	1	6	0.745±0.058	74.5	7.73	6	0.816±0.022	81.6	2.67
	3	6	2.347±0.063	78.2	2.69	6	2.293±0.065	76.4	2.82
D	0.1	6	0.078±0.003	77.6	4.08	6	0.075±0.001	75.1	1.71
	1	6	0.901±0.021	90.1	2.36	6	0.831±0.022	83.1	2.70
	3	6	2.514±0.047	83.8	1.87	6	2.302±0.042	76.7	1.84
E	0.1	6	0.090±0.006	89.6	6.29	6	0.085±0.006	84.5	7.45
	1	6	1.056±0.017	105	1.57	6	0.813±0.029	81.3	3.55
	3	6	2.293±0.059	76.5	2.58	6	2.280±0.023	76.0	1.02
F	0.1	6	0.079±0.005	79.2	5.89	6	0.080±0.004	80.2	5.24
	1	6	0.873±0.009	87.3	0.98	6	0.788±0.033	78.8	4.19
	3	6	2.364±0.028	78.8	1.18	6	2.324±0.045	77.5	1.94

^aInter-assay variation was determined by single test on six different days.

^bIntra-assay variation was determined by six replicates on a single day.

The EIA, HPLC-DAD and UPLC-MS-MS methods were compared using 1) artificially-fortified (18 samples; Figure 5. A) and 2) originally contaminated (6 samples; Figure 5. B, C, D) products. The coefficient of determination between EIA and HPLC-DAD (Figure 4. A) was 0.9885, signifying good agreement between methods. The slope of the correlation graph was 0.7839, indicating a slightly higher bias for the EIA method. Figure 5. B depicts the comparison of HPLC-DAD and EIA for melamine analysis from recalled pet food products (6 samples). A good correlation ($r^2 = 0.9973$) was obtained with a slope of 0.7943, again indicating a slightly higher bias for the EIA method. An evaluation of UPLC-MS-MS with HPLC-DAD (Figure 5. C) and with EIA (Figure 5. D) for melamine content of recalled pet food products resulted in good agreement between the three methods. The coefficients of determination between UPLC-MS-MS and HPLC-DAD, and UPLC-MS-MS and EIA analysis were 0.9566 and 0.9489, respectively. The same sample outlier for each method comparison skewed the coefficients.

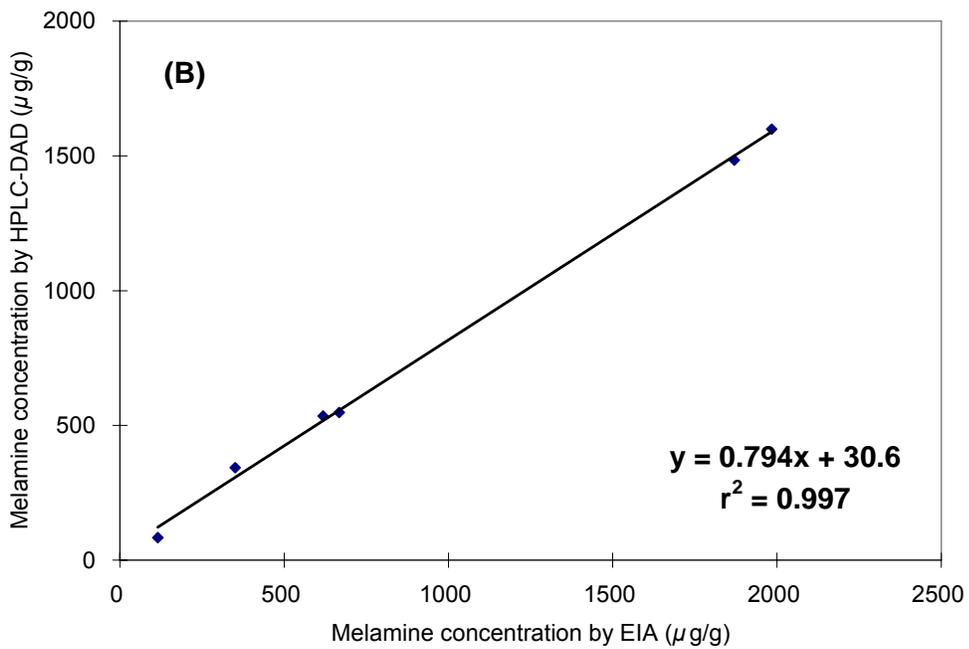
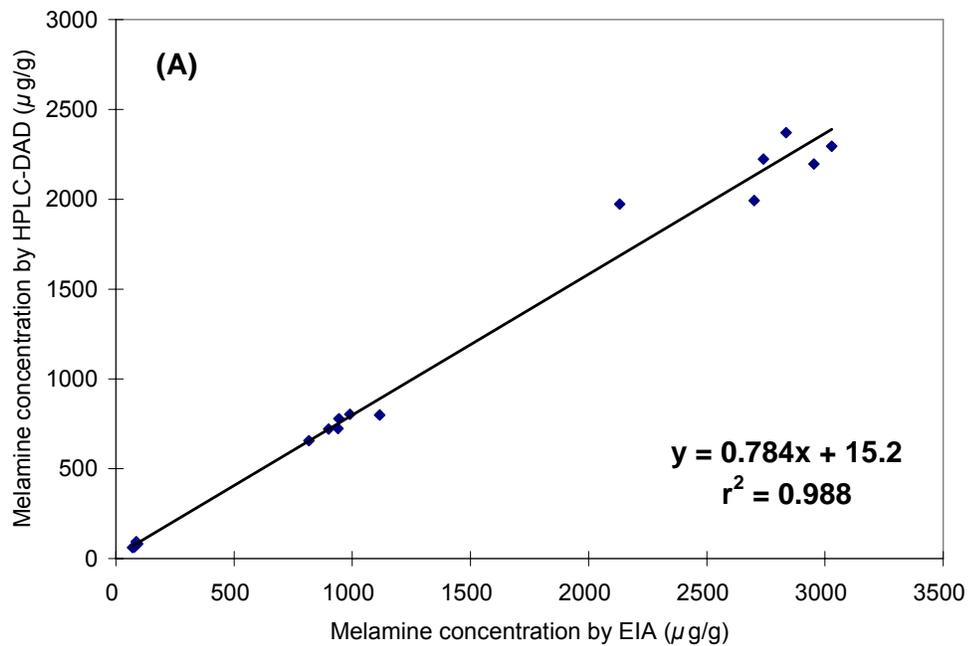


Figure 5. Correlation between HPLC-DAD and EIA determinations of melamine in (A) fortified and (B) originally contaminated pet food.

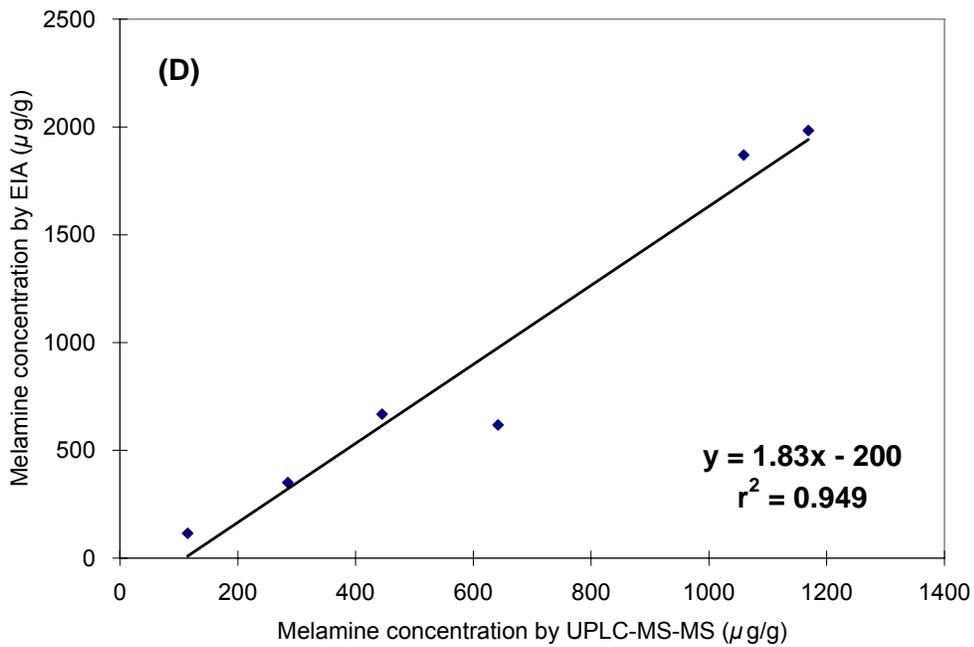
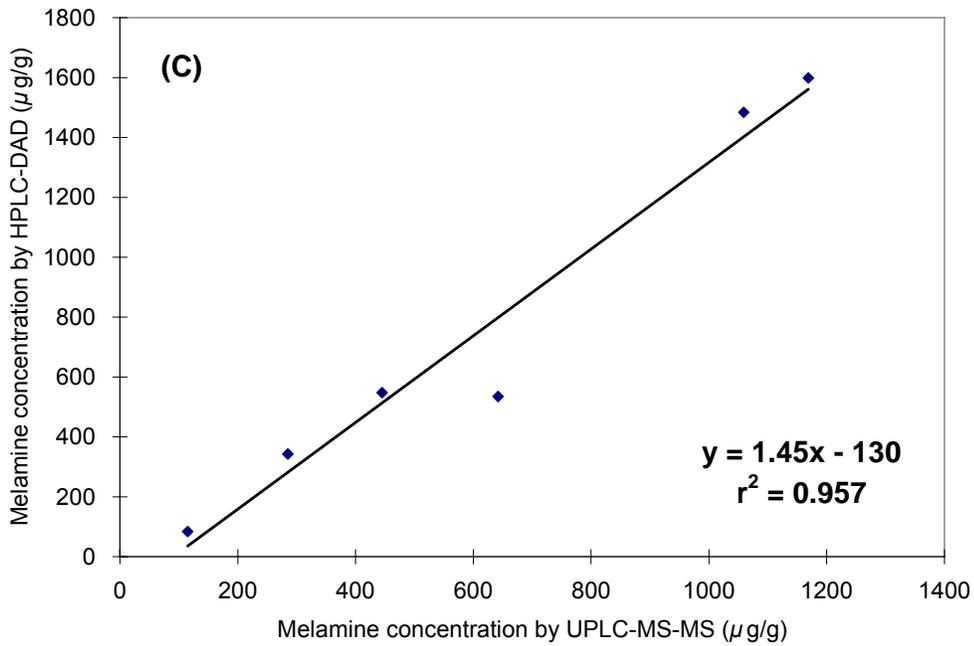


Figure 5. Correlation between (C) HPLC-DAD and UPLC-MS/MS, and (D) EIA and UPLC-MS/MS for melamine analysis from originally contaminated pet food. Each data point represents the mean of 3 determinations.

Conclusions

EIA is often considered a qualitative or semi-quantitative technique. However, the commercial melamine EIA kit used in this study proved to be a rapid and inexpensive alternative to HPLC instrumentation to quantify melamine in a variety of pet foods. The HPLC-DAD method for analyzing melamine in pet food was proven to be linear, reliable and repeatable over the intended dynamic range. Sample preparation for all three procedures was simple and required minimal clean-up procedures. Good agreement between the three methods for melamine analysis of fortified and recalled pet food samples indicates that the technologies developed in this study are all suitable for melamine analysis in pet food samples. The methodology we used in this study is adequate for analysis of free melamine. The possibility of melamine-cyanuric acid complex which is possibly formed in pet food was not considered.

CHAPTER 3

APPLICABILITY OF PRESSURIZED LIQUID EXTRACTION FOR MELAMINE ANALYSIS IN PET FOODS WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY DETECTION

Chapter Abstract

A pressurized liquid extraction (PLE) method was developed for melamine analysis in pet foods. The PLE method which utilized an accelerated solvent extraction (ASE[®]) system, was also compared with sonication and polytron extraction methods. The parameters for the optimized PLE method were temperature (75 °C for wet pet food, 125 °C for dry pet food), pressure (1500 psi), static time (10 min), flush volume (40%), purge time (1 min), and number of cycles (1). Recoveries obtained by the PLE method were significantly higher ($P \leq 0.05$) than those of sonication and polytron methods for dry pet food samples. For the analysis of adulterated pet foods, PLE resulted in the highest melamine content followed by sonication and polytron. Using PLE, samples fortified with melamine at 2.5 and 100 mg/kg resulted in recoveries ranging from 54.6 to 90.2% for wet samples and from 89.9 to 116% for dry samples. Low recovery rate from wet samples at low spike level (2.5 mg/kg) may have been caused by co-aggregation of polysaccharide with melamine due to low pH during solid-phase extraction (SPE) clean-up. Limit of detection (LOD) and limit of quantification (LOQ) values were 0.5 (mg/kg) and 1.0 (mg/kg) for dry samples. Overall, PLE had the best extraction efficiency compared to sonication and polytron, proving PLE to be a useful tool for melamine analysis of pet foods.

Introduction

In 2007, adulterated pet foods resulted in major recalls throughout the United States. It was found that imported ingredients from China such as wheat gluten and rice protein used to make pet foods were adulterated with melamine (US FDA 2008a). In 2008, baby formula contaminated by melamine caused more than 50,000 babies in China to have kidney problems, along with a few deaths related to this condition (US FDA 2008c). It is thought that the nitrogen-rich compound melamine (1,3,5-triazine-2,4,6-triamine) was intentionally added to the pet food ingredients or baby formula to increase nitrogen levels, so that the final products would appear to have a sufficient protein content (World Health Organization 2008). Melamine and cyanuric acid can combine in water to form a melamine-cyanurate complex which is considered the causative agent for the kidney disease (He et al. 2008; Cianciolo et al. 2008; Reimschuessel et al. 2008).

Recently, various analytical methods have been used to determine melamine concentrations in different matrices such as cereal flour (Ehling et al. 2007), kidney tissue (Filigenzi et al. 2008), fish tissue (Andersen et al. 2008), porcine muscle (Filigenzi 2007), and infant formula (Turnipseed 2008) by liquid chromatography with ultraviolet (UV) detection or a tandem mass spectrometry. Pet food analysis by high-performance liquid chromatography (HPLC) with diode array detection (DAD) was conducted by Kim et al. (2008). To date, sonication and/or mechanical shaking have been the main procedures used to extract melamine from sample matrices (Ehling et al. 2007; Filigenzi et al. 2008; Andersen et al. 2008; Filigenzi 2007; Turnipseed 2008; Kim et al. 2008; US FDA 2007c; Smoker and Krynitsky 2008). However, since melamine is only slightly soluble in water at room temperature or very slightly soluble in hot alcohol (Merck 1989), extraction

methods that use mild temperature such as sonication and polytron may yield poor recovery from pet foods, especially from dry samples. Pet food is a very complicated matrix comprised of many different food ingredients, so it can be difficult to extract melamine from pet food. Pressurized liquid extraction (PLE) is a relatively new technique that uses less solvent than conventional extraction methods. PLE can be conducted at high temperatures (50–200 °C) and pressures (500–3000 psi). High temperature and pressure increases the solubility of the target analytes and decrease the viscosity of solvent, allowing better penetration into the sample matrix (Mendiola et al. 2007). Also, high temperature increases diffusion rates of the solvent, and weakening and disrupting strong interactions between analytes and matrix components. High pressure is required to keep the solvent in a liquid state at elevated temperatures, far above the boiling point (Giergielewicz-Mozajska et al. 2001). Therefore, PLE may yield better recovery of melamine from pet foods. Since it is fast and automatic, preparation time can be dramatically reduced, a valuable asset when large numbers of samples need to be analyzed, such as the testing of recalled samples. To our knowledge, there is no literature describing melamine extraction by PLE from pet food.

This study evaluates PLE as a potential alternative to conventional methods such as sonication or polytron for melamine extraction from pet foods. Quantitative analysis of melamine was conducted by HPLC with a diode array detector.

Materials and Methods

Chemicals and Materials

Sodium 1-octanesulfonate monohydrate ($\geq 99\%$) and ammonium acetate ($\geq 99\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Melamine ($\geq 99\%$), acetonitrile (HPLC grade), water (HPLC grade), tetrahydrofuran (HPLC grade), diethylamine ($\geq 99\%$) and diatomaceous earth (catalog #. S75114) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dry and wet pet foods were purchased from local markets (Bangor, ME, USA). Adulterated pet foods were provided by the New York State Department of Agricultural and Markets Food Laboratory (Albany, NY, USA). Oasis MCX cartridges (6 mL, 150 mg) for sample clean-up were purchased from Waters Corporation (Milford, MA, USA).

Sample Preparation and Fortification

Dry and wet pet foods were homogenized for 10 min with a commercial kitchen blender (Magic Bullet, Emson, NY, USA). The homogenized wet pet food was pre-warmed by holding in warm water (60 °C) for 10 min before weighing. Since warmed wet pet food is less viscous, it is easier to homogenize with melamine. To produce melamine-fortified samples (2.5 and 100 mg/kg), 2 g of each pet food was weighed into a centrifuge tube (50 mL) or PLE cell, and the proper amount of melamine standard solutions (50 and 500 $\mu\text{g/mL}$) in acetonitrile/water (50:50) were directly added to the sample using a micropipette.

Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction was carried out using a Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA, USA). A 2 g portion of the dry pet food sample (fortified or adulterated) was placed in an 11 mL stainless steel cell followed by diatomaceous earth (0.5 g). The cell was shaken by hand for a few seconds to mix the ingredients. For wet pet food samples, 1 g of diatomaceous earth was placed in the cell, followed by 2 g of pet food and an additional 0.5 g of diatomaceous earth to cover the sample. Using a small spatula, the wet pet food was carefully stirred and mixed with diatomaceous earth and installed to the ASE system for extraction. After finishing the extraction procedure, the extract was collected in a collection vial, and transferred into a centrifuge tube (50 mL). Three mL of acetonitrile/water (50:50) was used for washing the collection vial and transferred into the centrifuge tube. The extract was centrifuged at 4,000×g for 15 min, and the supernatant was transferred to a 25 mL volumetric flask and brought to volume using acetonitrile/water (50:50). A solid-phase cartridge (Oasis MCX, 6 mL, 150 mg) was used for melamine clean-up, and the protocol used was slightly modified from the original procedure (Smoker and Krynitsky 2008). A cartridge was placed on a vacuum manifold and conditioned with 5 mL of acetonitrile followed by 5 mL of 4% formic acid in water. After closing the valve, two milliliters of 4% formic acid were added followed by addition of 4 mL of sample. The sample, mixed with 4% formic acid, was allowed to flow by gravity using no pressure or vacuum. After the sample had passed through the cartridge, it was washed with 5 mL of acetonitrile and 3 mL of 0.2% diethylamine in acetonitrile. Without drying the cartridge, 5 mL of 2% diethylamine in acetonitrile was added to elute melamine, and the cartridge was aspirated under vacuum

for 10 sec. The resulting solution was evaporated to 1 mL under a stream of nitrogen at 50 °C using a nitrogen evaporator (N-EVAP 111, Organomation Associates, Inc., Berlin, MA, USA). One milliliter of water was then added to bring the total volume to 2 mL. The sample was then filtered through a syringe filter (PTFE; 0.45 µm pore size; Fisher Scientific, Pittsburgh, PA, USA) for HPLC analysis.

The extraction cycle on the ASE 200 consists of the following steps: (1) initial filling of the cell with solvent; (2) application of pressure (500–3000 psi, 1 psi = 7 kPa); (3) cell heating (50–200 °C); (4) static time (1–99 min); (5) solvent flush step; (6) a nitrogen purge to displace residual solvent (20 – 300 s). PLE parameters such as temperature, solvent composition, pressure and extraction time were optimized and are described in Table 4. Fortified wet and dry pet food samples (100 mg/kg) were used for the optimization test.

Sonication

A portion of the pet food sample was weighed (2 g) into a 50 mL polypropylene centrifuge tube and 25 mL of acetonitrile/water mixture (50:50) was added. The tube was vortexed for 1 min, sonicated for 30 min, and vortexed again for 1 min. Sonication was performed in an ultrasonic cleaning bath (Branson 2200, Branson Ultrasonic Corp., Danbury, CT, USA). The samples were then centrifuged at 4,000×g for 15 min, and the supernatant was cleaned-up with a solid-phase cartridge, using same protocol as PLE. The solution was filtered through the syringe filter and collected in a vial (1.5 mL) for HPLC analysis.

Polytron

Pet food sample (2 g) and acetonitrile/water mixture (50:50, 25 mL) were added into a 50 mL polypropylene centrifuge tube and homogenized for 5 min with a polytron tissue homogenizer (PT 10-35, Kinematica Inc., Newark, NJ, USA). After centrifugation, the supernatant was cleaned up and filtered as described in the PLE and sonication sections for HPLC analysis.

Instrumental Analysis

Ten microliters of the extract was injected into the HPLC system consisting of a Hewlett Packard model 1050 liquid chromatograph equipped with a Luna C8(2) column (150 × 4.6 mm, 3 μm) (Phenomenex, Torrance, CA, USA) and a diode array detector (Agilent Technologies, Palo Alto, CA, USA). A mobile phase of buffer/tetrahydrofuran/0.1M ammonium acetate (70:5:25) was used. The flow rate was set at 0.7 mL min⁻¹ and the wavelength for melamine detection was set at 236 nm. The buffer composition was 10 mM citric acid and 10 mM sodium octanesulfonate adjusted to pH 3.0. From the melamine stock solution (500 μg/mL) dissolved in acetonitrile/water (50:50), five different concentrations of the melamine standard (0.1, 0.5, 1.0, 5.0, 10.0 μg/mL) were prepared by diluting appropriate volumes in the solution of acetonitrile/water (50:50). The stock solution was sonicated for 10 min to assure the melamine was completely dissolved. The working standards were analyzed in triplicate by HPLC-DAD. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by diluting the extract of melamine spiked pet food sample with blank sample extract until signal-to-noise (S/N) reached 3 and 10, respectively.

Results and Discussion

PLE Optimization

Since melamine is thermally stable and slightly soluble in water, it can be extracted by the PLE technique using solvents such as water and acetonitrile. The extraction solvent was investigated for melamine extraction (Figure 6). Due to the poor solubility of melamine in organic solvent, melamine recoveries were lower when the extraction solvents contained more than 50% acetonitrile (Merck 1989). However, more than 50% of water in the extraction solvent also resulted in lower recoveries. Due to increased viscosity by water, the extraction solvent was not completely removed from the extraction cell by the flushing and purging steps. This remaining solvent could contain melamine, which probably led to lower recoveries. In our preliminary studies, other organic solvents such as methanol and ethanol showed no difference compared to acetonitrile for melamine extraction (data not shown) when the same percentage mixtures with water were used. Since melamine is a relatively polar compound, keeping a constant percentage of water may be more important than changing organic solvents.

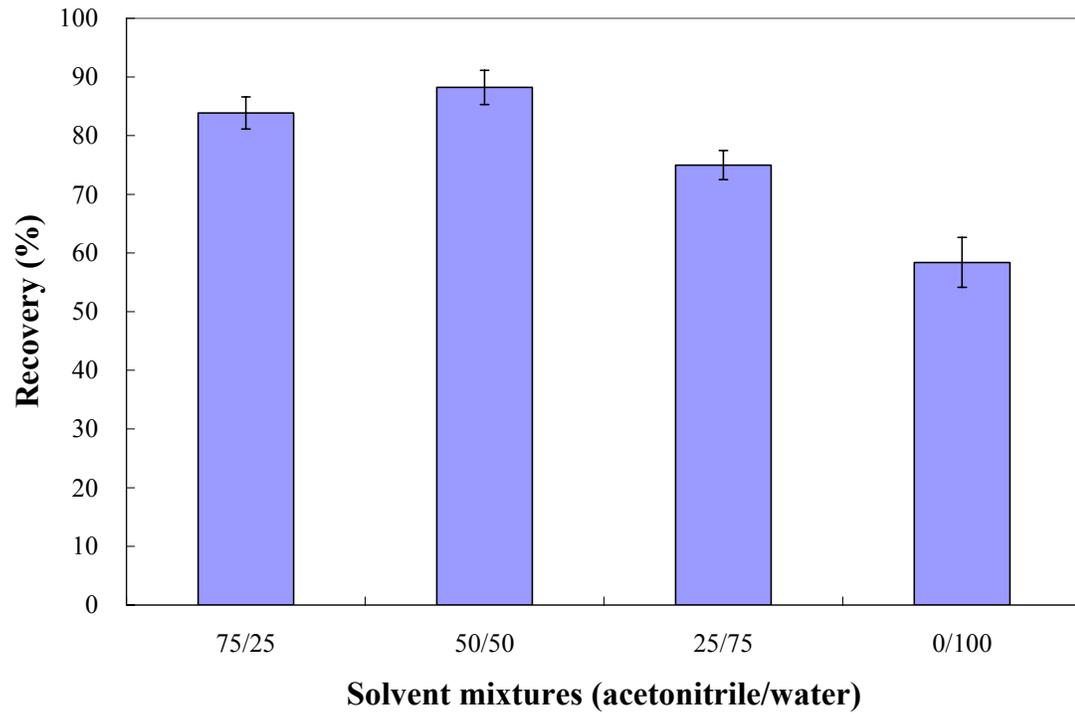


Figure 6. Recoveries of melamine by PLE extraction using different ratios of acetonitrile and water. Each bar presents the mean value (n = 3) and error bars indicate the standard deviation (+/-).

Diatomaceous earth was always used as a dispersing agent and mixed with pet food samples for melamine extraction in order to prevent clogging in the extraction cell, which is caused by gelatinization of polysaccharide (starch) or wheat gluten in the pet food. Also, the dispersion of diatomaceous earth aids in extraction efficiency because it increases the surface area contact with the extraction solvent. Pure wheat gluten was also tested for PLE applicability and was found to form a gel inside the cell, causing clogging. It is likely that the high temperature denatured and gelatinized the wheat gluten. Since wet pet food is sticky and moist, mixing well with diatomaceous earth is important to prevent clogging. Addition of diatomaceous earth first to make a bed in a cell followed by addition of wet pet food and another layer of diatomaceous earth was shown to provide good extraction and minimized clogging problems.

Operation parameters such as pressure, temperature, static time, and flush volume were also optimized for melamine extraction from spiked wet and dry samples (100 mg/kg) (Table 4). Results of varying pressure from 500 to 2000 psi in an extraction cell showed that although higher pressure appeared to give better extraction, levels higher than 1500 psi did not give better results. Higher temperature resulted in better melamine recovery. It is well known that higher temperatures decrease viscosity and surface tension of solvents so that extraction is improved by easier access of solvent to the sample matrix (Mendiola et al. 2007). However, dry pet food required higher temperature (125 °C) than wet pet food (75 °C) for the best extraction. We assume that some high molecular weight compounds such as polysaccharides and protein contained in gravy from wet pet food may interfere with melamine extraction at high temperature because of gelatinization. Several static times between 5 and 20 min were also tested to find the best time for

melamine extraction. Ten minutes of static time was chosen because melamine recovery from 10 min was nearly the same as that for longer time, indicating that 10 min is long enough for the solvent to reach and extract the melamine in the sample matrix. Flush volume after extraction was chosen as 40% of the cell volume, the lowest level resulting in melamine extraction equivalent to higher flushing volumes.

Table 4. Selected ASE 200 operating conditions for pressurized liquid extraction of melamine from dry and wet pet food samples (100 mg/kg).

Extraction solvent	ACN*/H ₂ O (50:50 v/v)
Pressure (psi)	1500
Temperature (°C)	125 (dry), 75 (wet)
Static time (min)	10
Flush volume (%)	40
Purge time (min)	1
Number of cycles	1
Cell volume (mL)	11

* Acetonitrile

Comparison of PLE, Sonication and Polytron Extraction

Extraction methods for melamine from pet foods were compared. Dry pet foods were fortified with melamine at a level of 2.5 mg/kg and extracted by PLE, sonication or polytron (Table 5). Each extraction method was tested three times ($n = 3$) and each test was run in triplicate. Overall, recoveries ranged between 82.6 and 93.3%. Recovery differences among the three methods were statistically tested by Tukey's studentized range test at a 95% confidence level. Recoveries obtained by the PLE method were significantly higher ($P \leq 0.05$) compared with sonication and polytron methods. Sonication and polytron extractions resulted in similar recovery rates. Gfrerer et al. (2004) showed an accelerated solvent extraction (same as pressurized liquid extraction) gave higher extraction efficiency for pesticides from animal feed than other extraction techniques due to better contact of the solvent with the matrix at high temperature and high pressure. PLE extraction had the highest relative standard deviation (RSD) followed by sonication and polytron. High temperature was used for PLE, which could denature protein in the sample, a problem mostly due to wheat gluten added to pet food to give a desirable texture. This may interfere with melamine extraction, causing increased variability in recoveries. Wet pet food extracts were aggregated when mixed with 4% formic acid for solid-phase extraction and the flow of extracts were slowed. Build-up of aggregated material was observed on the frit of the cartridge and likely attracted melamine from the extract solution during aggregation, resulting in low melamine recovery. The aggregated material could be wheat gluten or starch in the gravy from wet pet food, which is denatured and aggregated by low pH (pH 2.0) of the formic acid.

Table 5. Recovery and relative standard deviation (RSD) of melamine by PLE, sonication and polytron extraction methods in fortified dry pet food samples.

Extraction	Fortification level (mg/kg)	Recovery (%)*	RSD (%)
PLE	2.5	93.3 ^a	3.51
Sonication	2.5	82.6 ^b	2.33
Polytron	2.5	84.4 ^b	1.65

Values are calculated after three repeated experiments (n = 3).

PLE was conducted with the conditions in Table 1.

*Different lowercase letters (a and b) are significantly different.

Adulterated wet pet foods were also extracted and analyzed for melamine (Figure 7). Four different samples (1–4) were tested three times and each test was run in triplicate. All of the samples contained melamine exceeding the legal limit (2.5 mg/kg). Sample 1 contained less melamine compared to other samples. The melamine in the sample 1 extracted by PLE was significantly higher ($P \leq 0.05$) than those from sonication and polytron. There was no significant difference ($P \geq 0.05$) between sonication and polytron extractions. However, in sample 2, 3, and 4, melamine contents analyzed from all three different extractions were significantly different each other ($P \leq 0.05$). PLE extraction resulted in the highest melamine content followed by sonication and polytron extraction. The melamine content of samples 2 and 3 were similar, with a concentration range from 600 to 780 mg/kg. Sample 4 contained the highest level of melamine (greater than 1400 mg/kg). Based on these two experiments, it appears that PLE extraction gives better melamine recovery than the sonication and polytron extraction methods. For adulterated samples, sonication resulted in higher melamine extraction than the polytron. Since there

is a possibility of melamine binding to chemicals such as cyanuric acid resulting in the formation of a melamine cyanurate complex and/or nonspecific binding to the pet food matrix, physical forces such as high temperature, pressure, and ultrasonic impact may release bound melamine to the solvent. This explains why PLE and sonication extraction produced the higher levels of melamine than polytron. Melamine in the fortified pet foods is in a free (unbound) state. Therefore, melamine extraction from adulterated samples is probably more difficult than from fortified samples. This could be a reason that no differences were found between sonication and polytron extraction methods for fortified samples, but significant recovery difference ($P \leq 0.05$) between these methods were observed for adulterated samples except sample 1.

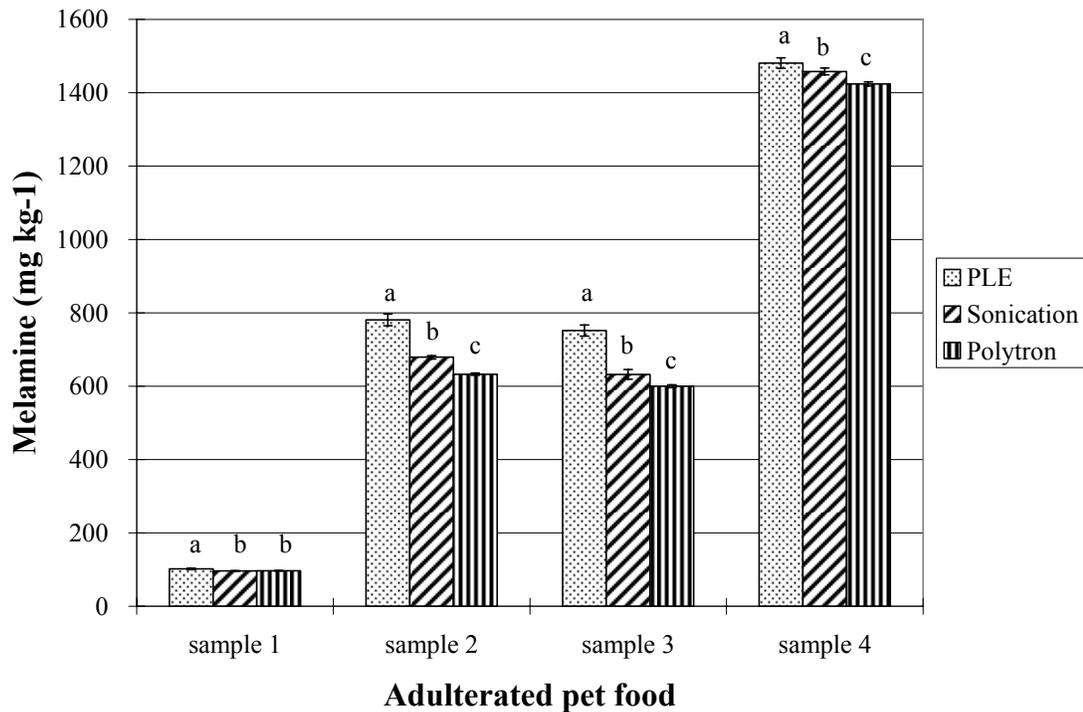


Figure 7. Determination of melamine from adulterated pet food samples (1–4) extracted by three different methods. Each bar presents the mean value ($n = 3$) and error bars indicate the standard deviation (+/-). *Different lowercase letters (a and b) are significantly different.

Recoveries, RSDs, LOD, and LOQ by PLE Extraction for Various Pet Food Samples

Three wet (A, B, and C) and dry (D, E, and F) pet food samples were spiked with two different levels of melamine (2.5 and 100 mg/kg) for each sample. Since the contamination levels of melamine in the adulterated samples were very high, ranging from 96 to 1480 mg/kg, we chose to fortify at 100 mg/kg. After extraction by PLE, all the samples were analyzed three times on different days (Table 6).

Table 6. Recovery and relative standard deviation (RSD) of melamine spiked in different pet food samples with different levels after PLE extraction.

Pet food	Fortification level (mg/kg)	Recovery (%)	RSD (%)
A (wet)	2.50	54.6	7.96
	100	86.2	1.42
B (wet)	2.50	60.4	4.88
	100	87.1	1.79
C (wet)	2.50	56.9	10.4
	100	90.2	1.34
D (dry)	2.50	89.9	3.29
	100	93.1	12.1
E (dry)	2.50	93.5	14.1
	100	116	11.4
F (dry)	2.50	91.7	10.3
	100	112	8.98

Values are calculated after repeating experiments in triplicate (n = 3).
Extraction conditions in Table 1 were used.

Overall recovery range was 54.6–90.2% for wet samples and 89.9–116% for dry samples. Low recovery rates were observed from wet pet food samples at low spike level (2.5 mg/kg). As mentioned previously, polysaccharide in gravy from wet pet food might interfere with melamine recovery co-aggregating with melamine and removing it from the extract solution. Overall RSD range was 1.34–10.4% for wet samples and 3.29–14.1% for dry samples. High temperature used for dry pet food extraction could denature protein, which may interfere with the extraction procedure, resulting in greater variation. LOD and LOQ values were obtained only for dry pet food samples extracted by PLE due to low recovery problem from wet pet food samples by aggregation. LOD and LOQ values for dry samples were 0.5 (mg/kg) and 1.0 (mg/kg). Overall, PLE had the best extraction efficiency compared to sonication and polytron. It is apparent that PLE can be used for melamine analysis in pet foods.

Conclusions

The PLE method developed in this study allows for reliable analysis of melamine in pet food. Due to the poor solubility and potential binding of melamine to cyanuric acid or sample matrix, an aggressive extraction method such as PLE is a better choice for melamine analysis of pet foods. Since the PLE method is fully automated, total analysis time is shortened, which is desirable for testing in laboratories.

CHAPTER 4

**DETERMINATION OF MELAMINE AND CYANURIC ACID BY LIQUID
CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY USING
ALKALINE AQUEOUS EXTRACTION FOR DISSOCIATION OF MELAMINE-
CYANURATE COMPLEX IN PET FOOD**

Chapter Abstract

A method using a liquid chromatography with tandem mass spectrometry for melamine and cyanuric acid analysis was developed in conjunction with alkaline aqueous extraction. Alkaline pH was maintained throughout sample extraction and clean-up. Ammonium hydroxide solution (0.1 N) was successfully adopted as an alkaline extraction solvent. Recoveries range for melamine from dry and wet samples (0.1, 1, and 100 $\mu\text{g/g}$) ranged from 70.1 to 94.3% and from 90.6 to 105%, respectively. However, cyanuric acid from low levels of fortified samples (0.1 and 1 $\mu\text{g/g}$) showed poor recovery due to pyroglutamic acid interference. Cyanuric acid and pyroglutamic acid could not be chromatographically resolved, sharing the same ion transition (m/z 128 \rightarrow 85). Ammonium hydroxide extraction and acetonitrile/water mixture (50/50) extraction methods were compared using adulterated samples, as well as samples fortified with melamine, cyanuric acid and melamine cyanurate complex. It appeared that the acetonitrile/water mixture underestimated the true contamination level of melamine and cyanuric acid. Much lower levels of melamine (22 – 38.2%) and cyanuric acid (9.92 – 11.6%) were determined by acetonitrile/water (50/50) extraction from the adulterated samples. When samples were fortified with melamine cyanurate complex, the

acetonitrile/water resulted in extraction gave significantly lower recoveries for both melamine (29.5%) and cyanuric acid (13.0%). These results indicate that the adulterated samples were contaminated with a mixture of melamine, cyanuric acid and melamine cyanurate complex, rather than with individual melamine and cyanuric acid. Based on the results of this study, there is a high probability that at least some of the melamine-contaminated pet foods sold in the U.S. were adulterated with a melamine cyanurate complex, possibly in the form of a fire retardant.

Introduction

A recall of pet foods tainted with melamine in March, 2007 triggered investigation into the contamination of melamine and its analogues in various food products from many countries. In October 2008, melamine-contaminated infant formula caused kidney disease in more than 50,000 babies in China, and resulted in some deaths. Melamine was also found in many other food products containing milk-based ingredients. Melamine and cyanuric acid were found in renal tissues from the animals killed by the pet food-associated outbreak in 2007 (Brown et al 2007). It is known that melamine and cyanuric acid can combine by hydrogen bonding and form crystals in aqueous medium (Seto et al. 1993; Ranganathan et al. 1999; He et al. 2008; Reimschuessel et al. 2008). It has also been experimentally proven that animals fed both melamine and cyanuric acid together develop renal crystals (Cianciolo et al. 2008; Reimschuessel et al. 2008). Therefore, it is believed that melamine and cyanuric acid in food or feed caused the sickness and deaths. It is also suspected that melamine tainted food or feed was intentionally produced for the purpose of boosting nitrogen levels, resulting in false

protein content. The nitrogen content by mass of melamine and cyanuric acid is 66.64% and 32.56%, respectively (Merk 1989). Therefore, for the purpose of boosting nitrogen levels, adding melamine is more effective than adding cyanuric acid, leading to the question: Why does tainted food or feed often contain significant levels of both melamine and cyanuric acid instead of melamine, alone? Although there is a possibility of biodegradation of the added melamine into cyanuric acid by bacteria (Wackett et al. 2002), this is unlikely to occur because the raw ingredients such as rice protein and wheat gluten, which are the source of melamine contamination for pet food, have water activities too low to support bacterial growth. Therefore, we assume that melamine cyanurate complex could be the original contaminant for the tainted pet food. We further postulate that a melamine cyanurate-based product such as fire retardant could be the alternative source because it may be more cost effective and more accessible than pure melamine. Since melamine cyanurate complex is very insoluble in both aqueous and organic solvent at neutral pH, it is difficult to analyze. To our knowledge, there is no analytical method to measure the melamine cyanurate complex itself. Our preliminary work showed that highly alkaline solutions dissociate melamine cyanurate into melamine and cyanuric acid, and prevent formation of melamine cyanurate as well. In this study, we present the analytical method for quantification of melamine and cyanuric acid in pet food by alkaline aqueous extraction method for dissociation of melamine cyanurate complex, using liquid chromatography with tandem mass spectrometry.

Materials and Methods

Materials

Melamine (99+%), cyanuric acid (98%) and DL-pyrroglutamic acid (DL-2-pyrrolidone-5-carboxylic acid; 98%) were purchased from Fisher Scientific (Pittsburgh, PA). Melamine cyanurate, DL-glutamic acid and sodium phosphate monobasic (reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile was used (Fisher Scientific, Pittsburgh, PA). Concentrated ammonium hydroxide and hydrochloric acid were ACS grade. Water was deionized and purified to 18.2 M Ω cm (Millipore, Bedford, MA). Supelclean ENVI-Carb cartridges (6 mL, 500 mg) for solid-phase extraction were purchased from Supelco (Bellefonte, PA, USA) and Oasis MCX cartridges (6 mL, 150 mg) were obtained from Waters corporation (Milford, MA).

Standard Solutions

A stock solution of melamine or cyanuric acid was prepared by dissolving 100 mg in 100 mL of acetonitrile/water (50/50). The stock solutions were prepared fresh, monthly. Intermediate solutions of 1 μ g/mL melamine and 10 μ g/mL cyanuric acid were prepared by diluting appropriate aliquots of the stock solutions with the acetonitrile/water solution in volumetric flasks. The intermediate solutions were prepared weekly from the stock solutions. Five-point standard curves of melamine and cyanuric acid were prepared from the intermediate solutions, daily. Appropriate aliquots of the intermediate solutions of 1 μ g/mL melamine and 10 μ g/mL cyanuric acid were diluted with acetonitrile/water solution to make the concentrations of 1, 5, 10, 50, and 100 ng/mL for melamine and 50, 100, 500, 1000, and 2500 ng/mL for cyanuric acid.

Sample Preparation

Adulterated pet foods were provided by the New York State Department of Agriculture and Markets Food Laboratory (Albany, NY). Blank samples (dry and wet) for controls and fortification were purchased from a local grocery store (Bangor, ME). Dry and wet (including gravy) pet foods were blended with a kitchen chopper. Two grams of sample was weighed into a 50 mL centrifuge tube. For fortified samples (0.1, 1 and 100 $\mu\text{g/g}$), the proper volume of melamine or cyanuric acid solutions (10, 100, and 1,000 $\mu\text{g/mL}$) in acetonitrile/water (50:50) were directly added to the sample using a micropipette. Twenty mL of 0.1 N ammonium hydroxide in water was added into the tube. The tube was vortexed for 2 min, sonicated for 30 min, and vortexed again for 2 min. The homogenate was centrifuged at 4,000 x g for 15 min at room temperature. The supernatant (10 mL) was removed to a new 50 mL centrifuge tube, and mixed with 5 mL of hexane by vortexing for 2 min. After standing for 10 min, the tube containing the supernatant and hexane was centrifuged at 4,000 x g for 20 min at room temperature. Using a Pasteur pipet, a portion of the aqueous lower phase was carefully removed by and used for solid-phase extraction.

Solid-phase cartridge (Supelclean ENVI-Carb, 6 mL, 500 mg) was used for both melamine and cyanuric acid clean-up. For melamine, the cartridge was conditioned with 5 ml of methanol followed by 5 ml of 0.1 N ammonium hydroxide in water using gravity. Five milliliters of the aqueous phase extract was added and allowed to flow no faster than 0.5 mL/min. After the extract passed through the cartridge, it was washed with 5 ml of methanol by gravity. Without drying the cartridge, 10 mL of 0.1 N ammonium hydroxide in methanol/water (50/50, v/v) was added with a gravity drip to elute melamine, and the

cartridge was aspirated under vacuum for 10 sec. The collected eluate was evaporated to 5 mL under a stream of nitrogen at 60 °C using a nitrogen evaporator (N-EVAP 111, Organomation Associates, Inc., Berlin, MA, USA), and five milliliters of acetonitrile was added to bring the total volume to 10 mL. The final solution was then sonicated for 5 min and filtered through a syringe filter (PTFE; 0.45 μ m pore size; Fisher Scientific, Pittsburgh, PA, USA) for analysis. For cyanuric acid, the cartridge was conditioned in the same way as melamine. Sample loading (5 mL) was conducted as melamine with slower than 0.5 mL/min flow rate, and the cartridge was aspirated under vacuum for 10 sec. The cyanuric acid was eluted with 15 mL of 0.1 N ammonium hydroxide in methanol with a gravity drip. The solution was evaporated to 0.5 mL, under a stream of nitrogen, at 50 °C, using the nitrogen evaporator. Acetonitrile/water (50/50) was added to a final volume of 2.5 mL. The final solution was sonicated for 5 min and filtered through a syringe filter (PTFE; 0.45 μ m pore size; Fisher Scientific, Pittsburgh, PA, USA) for analysis.

An extraction solvent of acetonitrile/water (50/50) was used for comparison with the aqueous alkaline solution described above. Twenty mL of the solvent (50/50) was added to the tube containing 2 g sample. The tube was vortexed for 2 min, sonicated for 30 min, and vortexed again for 2 min. The homogenate was centrifuged at 4,000 X g for 15 min at room temperature. The supernatant was used for solid-phase extraction. An Oasis MCX cartridge (6 mL, 150 mg) was used for melamine clean-up, and the protocol used was slightly modified from the original procedure (Smoker and Krynitsky 2008). The cartridge was conditioned with 5 mL of acetonitrile followed by 5 mL of 4% formic acid in water using gravity. After closing the valve, 2 mL of 4% formic acid followed by 4 mL of extract was added. The valve was opened to allow flow by gravity through the

column. The column was washed with 5 mL of acetonitrile and 3 mL of 0.2% diethylamine in acetonitrile by gravity. Without drying the cartridge, 5 mL of 2% diethylamine in acetonitrile was added to elute melamine, and the cartridge was aspirated under vacuum for 10 sec. The eluate was evaporated to 1 mL under a stream of nitrogen at 50 °C using the nitrogen evaporator and water was added to bring the final volume to 2 mL. The solution was sonicated for 5 min and filtered through the syringe filter for analysis.

LC-MS/MS Analysis

The analysis was performed with an Alliance 2695 Separation Module (Waters, Milford, MA) coupled to a Micromass Quattro *micro* API triple-quadrupole mass spectrometer (Waters, Milford, MA) with MassLynx software (Version 4.0.0). The analytical column was a Luna 3u Phenyl-Hexyl (150 mm × 2.0 mm, 3 μm, Phenomenex, Torrance, CA). The mobile phase consisted of 95 % water and 5% acetonitrile with the pH adjusted to 10.0 with concentrated ammonium hydroxide, and eluted isocratically with a flow rate of 0.12 mL/min. The electrospray ionization (ESI) source was operated in both positive (melamine) and negative (cyanuric acid) modes. Data acquisition was performed in the multiple reaction monitoring (MRM) mode. Tuning of the mass spectrometers were conducted by direct infusion of a 5 μg/mL melamine or cyanuric acid in 0.1 N NH₄OH. The parameters were selected as follows: capillary voltage, 2.5 kV; cone voltage, 35 V (melamine) and 20 V (cyanuric acid); extractor, 1 V; RF lens, 0.5 V; source temperature, 120 °C; desolvation temperature, 300 °C; desolvation gas, 600 L/h; cone gas, 50 L/h; LM resolution 1, 11 (melamine) and 14 (cyanuric acid); HM resolution

1, 11 (melamine) and 14 (cyanuric acid); ion energy 1, 0.2 (melamine) and 0.1 (cyanuric acid); entrance, 0; exit, 5; LM resolution 2, 14; HM resolution 2, 14; ion energy 2, 1 (melamine) and 0.5 (cyanuric acid); multiplier, 650 V. Using the CID (collision induced decomposition) valve, flow of collision gas (argon) was adjusted to keep a pressure of 2.99×10^{-3} mbar in the collision cell. Two ion transitions for each compound were monitored (m/z 127 \rightarrow 85; collision energy = 17 eV and 127 \rightarrow 68; collision energy = 25 eV for melamine, and m/z 128 \rightarrow 85; collision energy = 10 eV and m/z 128 \rightarrow 42; collision energy = 10 eV for cyanuric acid).

Quantification of melamine and cyanuric acid from adulterated samples was performed using the external standard method. A five-point standard curve was made every day using 1, 5, 10, 50, and 100 ng/mL of melamine and 50, 100, 500, 1000, and 2500 ng/mL of cyanuric acid standard solutions. Measurement for each concentration was conducted in triplicate. Ion transitions of m/z 127 \rightarrow 85 and m/z 128 \rightarrow 42 were used for quantification of melamine and cyanuric acid, respectively. For fortified samples, standard solutions for quantification were made from a blank extract solution after SPE clean-up and used to calculate the recovery rate.

Results and Discussion

Considering the potential existence of melamine cyanurate complex and/or possible formation from melamine and cyanuric acid, an alkaline solution was used for the extraction of melamine and cyanuric acid from pet foods. Ammonium hydroxide (0.1 N, pH 11.0) was successfully adopted as an alkaline extraction solvent that can be used with a tandem mass spectrometer. Another strong alkaline reagent, diethylamine (DEA)

which also dissociates melamine cyanurate into individual melamine and cyanuric acid, was not compatible with the analytical column (Luna 3u Phenyl-Hexyl) because the amine group from DEA solution interferes the column's binding mechanism for melamine and cyanuric acid. Furthermore, diethylamine is a relatively unstable reagent, and its health hazard rating requires careful handling. Since pH changes during the clean-up procedure of ion exchange solid-phase extraction cartridge may cause melamine cyanurate complex formation (if a sample contains both melamine and cyanuric acid), ion exchange SPE cartridge use (either cation or anion) was not considered for melamine and cyanuric acid clean-up. The polar character of melamine and cyanuric acid causes insufficient retention on reversed-phase columns (Ehling et al. 2007). Karbiwnyk et al. (2009) showed that a non-porous graphitized carbon black column gives improved retention time for cyanuric acid in aqueous solvent compared to C₁₈-cation exchange mixed mode SPE cartridges. In our preliminary study, the graphitized carbon black column (SupelcleanTM ENVI-CarbTM) gave good retention of melamine and cyanuric acid in alkaline aqueous solvent. We also found that cyanuric acid eluted before melamine through the graphitized carbon black column which makes it possible to use one SPE cartridge for both melamine and cyanuric acid clean-up.

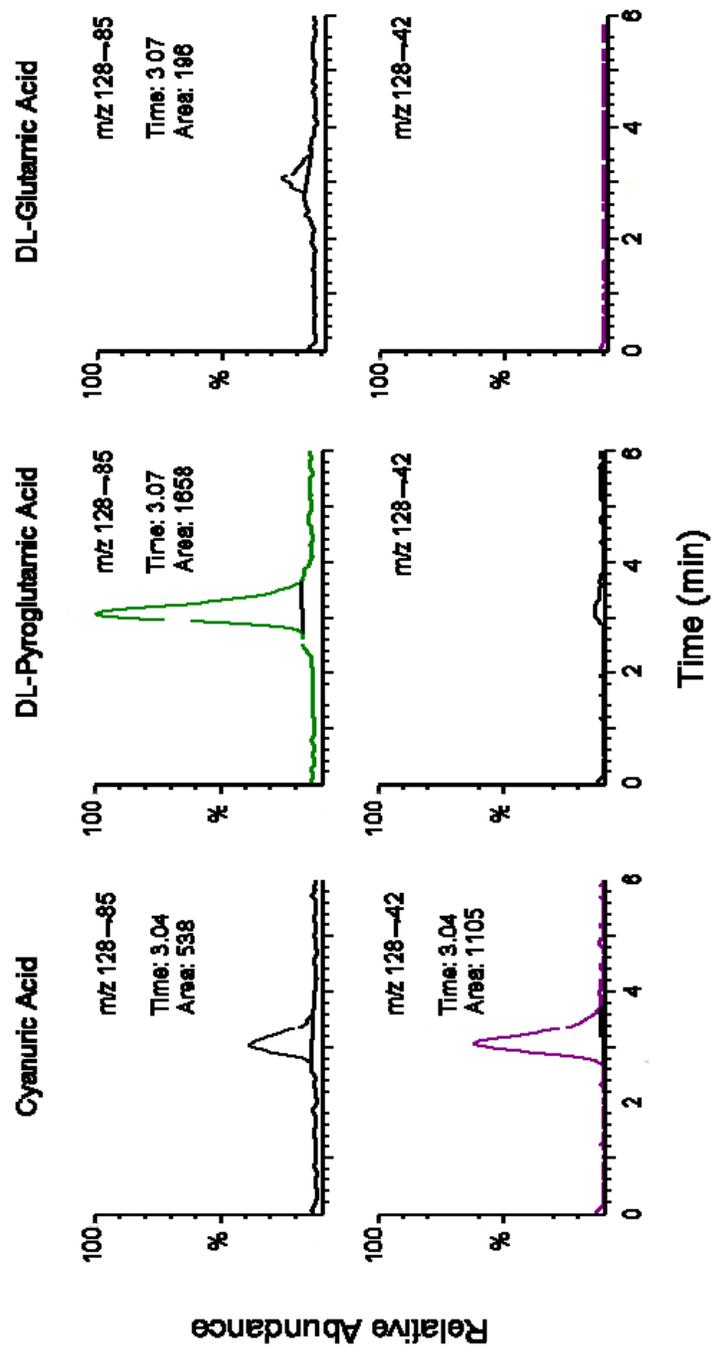


Figure 8. Ion chromatograms of cyanuric acid (1 µg/mL), pyroglutamic acid (50 µg/mL) and glutamic acid (50 µg/mL).

Pet food is a difficult matrix to clean up due to the complexity of ingredients. An unknown peak from blank pet food samples was found to have the same retention time as cyanuric acid and shared one of the two transitions (m/z 128 \rightarrow 85). Initially identified as the cyanuric acid peak, the other ion transition (m/z 128 \rightarrow 42) was suppressed by matrix effects. We confirmed that the unknown peak as pyroglutamic acid and/or glutamic acid, which has the ion transition (m/z 128 \rightarrow 85) when the ionization parameters for cyanuric acid is used. Figure 8 shows selected ion chromatograms for cyanuric acid (1 $\mu\text{g/mL}$), pyroglutamic acid (50 $\mu\text{g/mL}$) and glutamic acid (50 $\mu\text{g/mL}$). Since pyroglutamic acid has almost same molecular weight (129.11) as cyanuric acid (129.07) and is a highly polar due to the presence of carboxyl group (Figure 9), it is not surprising that pyroglutamic acid has same retention time and shares one of the two ion transitions with cyanuric acid.

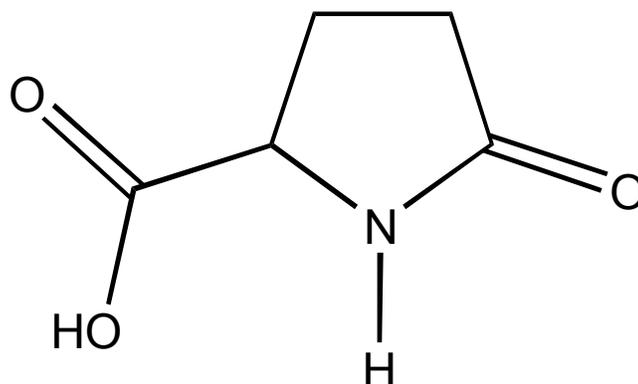


Figure 9. Structure of DL-pyroglutamic acid.

It is not completely understood why glutamic acid ($m_w = 147.13$) provides the transition ($m/z\ 128 \rightarrow 85$), but one theory is that a product ion at $m/z\ 128$, possibly due to the loss of H_2O from the precursor ion at $m/z\ 146$, corresponding to the $[M - H]^-$ for glutamic acid, might be further fragmented into smaller ion $m/z\ 85$. Since pyroglutamic acid can be produced by heat processing of fruits or vegetables (Haard 1985; Schneider et al. 2003), it is very possible that pyroglutamic acid was formed from glutamic acid and glutamine during pet food thermal processing i.e.; extrusion. The unresolved pyroglutamic acid and cyanuric acid peaks make it difficult to analyze low levels of cyanuric acid in the samples. Additional experiment is being undertaken to solve the problem.

Table 7 shows recovery of melamine and cyanuric acid fortified in dry and wet pet food samples with different fortification levels (0.1, 1, and 100 $\mu g/g$). Recoveries for melamine from dry and wet samples ranged from 70.1 to 94.3% and from 90.6 to 105%, respectively. The wet samples tend to have higher recovery than dry samples. Low levels of cyanuric acid (0.1 and 1 $\mu g/g$) could not be calculated from wet or dry samples because of the pyroglutamic acid problem mentioned previously. However, once the proper separation conditions are found, low cyanuric acid levels will be determined with a good recovery.

Table 7. Recovery of melamine and cyanuric acid from fortified pet food samples.

Sample type	Fortification ($\mu\text{g/g}$)	Melamine		Cyanuric acid	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Dry	0.1	70.1	3.70	NA	NA
	1	82.1	11.2	NA	NA
	100	94.3	3.79	105	3.52
Wet	0.1	90.6	7.22	NA	NA
	1	83.0	4.73	NA	NA
	100	105	1.70	99.9	7.81

Table 8. Contents of melamine and cyanuric acid in adulterated pet foods tested using two different extraction solvents.

Sample	Melamine				Cyanuric acid			
	0.1N NH ₄ OH		ACN/H ₂ O (50/50)		0.1N NH ₄ OH		ACN/H ₂ O (50/50)	
	Content (μg/g)	RSD (%)	Content (μg/g)	RSD (%)	Content (μg/g)	RSD (%)	Content (μg/g)	RSD (%)
A	1583	6.95	384	6.65	599	14.3	59.4	16.2
B	1594	10.8	441	11.9	561	3.39	56.0	12.6
C	1484	5.75	497	4.76	680	13.3	60.7	19.9
D	1533	4.38	446	11.84	546	9.57	63.1	17.8
E	1449	8.88	553	11.7	561	13.6	61.8	19.2
F	1423	4.42	1171	2.78	99.7	12.7	63.2	5.07
G	1077	3.59	715	7.24	186	2.42	47.8	4.37
H	1158	2.61	731	4.57	182	9.79	57.7	9.34
I	0.395	3.13	0.090	15.9	ND ^a	-	ND	-
J	0.500	4.15	0.110	13.6	ND	-	ND	-
K	0.081	9.85	0.028	8.59	ND	-	ND	-
L	0.464	14.7	0.177	7.75	ND	-	ND	-
M	0.656	3.12	0.165	10.3	ND	-	ND	-
N	0.409	11.5	0.142	10.0	ND	-	ND	-
O	1.22	10.1	0.407	10.2	ND	-	ND	-

Fifteen adulterated pet food samples were analyzed for melamine and cyanuric acid content using two different extraction methods (Table 8). Melamine contamination levels tested by the ammonium hydroxide extraction method ranged from 0.081 to 1594 ($\mu\text{g/g}$). Conversely, the levels tested by acetonitrile/water (50/50) extraction method ranged from 0.058 to 1171 ($\mu\text{g/g}$). The acetonitrile/water (50/50) extraction method resulted in 22.0 – 82.3% of melamine as compared to ammonium hydroxide extraction. The cyanuric acid determination followed a similar pattern. Contamination levels of cyanuric acid tested by ammonium hydroxide extraction method ranged from 99.7 to 680 ($\mu\text{g/g}$). The same samples tested by the acetonitrile/water (50/50) extraction method ranged from 47.8 to 63.2 ($\mu\text{g/g}$). The acetonitrile/water (50/50) extraction method resulted in 9.92 – 63.4% of cyanuric acid levels determined by ammonium hydroxide extraction. If samples F – H are not considered, those ranges would be narrowed to 22.0 – 38.2% for melamine and 9.92 – 11.6% for cyanuric acid. These values indicate that the extraction method with acetonitrile/water (50/50) tends to underestimate the contamination level of melamine and cyanuric acid in pet foods. There are two possible explanations for this phenomenon. The neutral pH of acetonitrile mixture with water (50/50) allowed melamine and cyanuric acid in the pet foods to bind with each other, and/or failed to dissociate the existing melamine cyanurate complex into melamine and cyanuric acid. Melamine and cyanuric acid bind to each other, via hydrogen bonding forming a melamine cyanurate crystal at neutral pH of water or aqueous solution (He et al. 2008; Reimschuessel et al. 2008). However, once melamine and cyanuric acid are dissociated, they do not recombine to form melamine cyanurate complex in acetonitrile/water mixture (Filigenzi et al. 2008). This is one of the reasons that final

solvent before injecting into the LC system for analysis is always an acetonitrile/water mixture. Percent RSDs between the two extraction methods (ammonium hydroxide and acetonitrile mixture) are similar and range from 2.61 – 14.7% and 2.78 – 15.9% for melamine, and 2.42 – 14.3% and 4.37 – 19.9% for cyanuric acid. For samples A through H, with high contamination levels of melamine and cyanuric acid, the SPE clean-up procedure was deemed unnecessary, and the sample extracts were simply diluted to 10 – 1000 times with acetonitrile/water (50/50) before LC analysis.

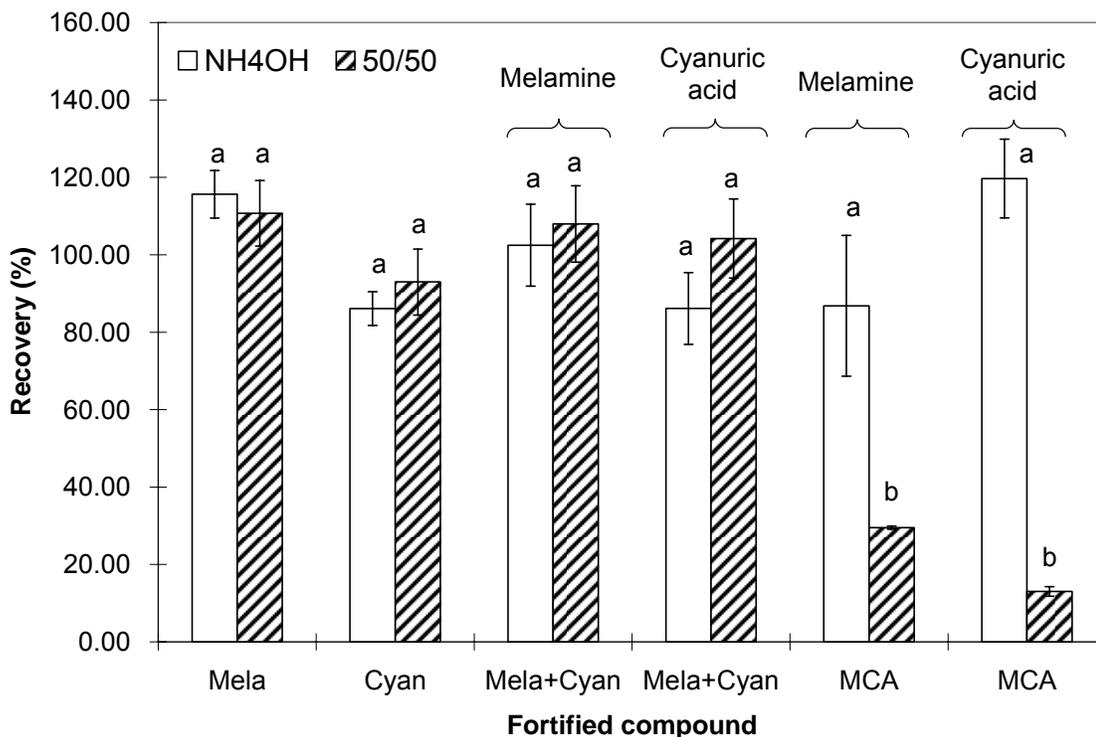


Figure 10. Recovery of melamine and cyanuric acid by ammonium hydroxide (0.1 N) and acetonitrile/water (50/50) extractions. Fortification level of melamine and cyanuric acid was 1000 $\mu\text{g/g}$, individually. Fortification level of melamine cyanurate complex was 2000 $\mu\text{g/g}$. Within each fortification, different letters indicate significant differences.

Two different extraction solvents (0.1 N ammonium hydroxide and acetonitrile/water mixture) were compared for recoveries of melamine and cyanuric acid when fortified individually or together (1000 $\mu\text{g/g}$ for each compound). Melamine cyanurate complex was also fortified for the comparison test (2000 $\mu\text{g/g}$). Proper amounts of each compound were directly added to 20 g of blank wet pet food sample, and mixed well using a spatula. For the combined melamine cyanuric acid test (Mela + Cyan), 1 g of each melamine or cyanuric acid fortified sample was mixed together for the extraction. Figure 10 shows the melamine and cyanuric acid recoveries from each fortification such as melamine only fortification (Mela), cyanuric acid only fortification (Cyan), melamine + cyanuric acid fortification (Mela + Cyan), and melamine cyanurate complex fortification (MCA) pet food samples. When only either melamine or cyanuric acid was fortified, the recoveries of melamine by ammonium hydroxide and acetonitrile/water extraction were 116% and 111%, respectively. The recoveries of cyanuric acid by ammonium hydroxide and acetonitrile/water extraction were 86.1 and 92.9%, respectively. There was no significant difference ($P \geq 0.05$) for both melamine and cyanuric acid recovery between the two extraction methods as calculated by the paired t-test. Similarly, when both melamine and cyanuric acid were fortified together, no significant difference ($P \geq 0.05$) in recovery was found between the methods. It appears that acetonitrile/water mixture gave better recovery for both melamine and cyanuric acid. This is quite reasonable because acetonitrile/water (50/50) does not promote melamine and cyanuric acid binding, and may have better penetration into sample matrix because of organic portion (acetonitrile) in the solvent. However, when the samples were fortified with melamine cyanurate complex, the acetonitrile/water mixture gave significantly

lower ($P \geq 0.05$) recoveries for both melamine (29.5%) and cyanuric acid (13.0%) as expected. This result indicates that acetonitrile/water mixture neither promotes melamine and cyanuric acid binding or completely dissociates the melamine cyanurate complex into individual melamine and cyanuric acid. Interestingly, melamine and cyanuric acid levels recovered by acetonitrile/water mixture are 33.9% and 10.9% of those levels recovered by ammonium hydroxide method. These results are very similar to the previous adulterated sample test in which the acetonitrile/water mixture resulted in 22.0 – 38.2% of melamine, and 9.92 – 11.6% of cyanuric acid levels as compared to ammonium hydroxide. These results imply that the adulterated samples, with the exception of sample F through H, could be contaminated with melamine cyanurate complex. A preliminary study showed that melamine cyanurate complex could be completely dissolved in 0.1 M hydrochloric acid (major component of gastric acid in the stomach) with 1 hr of sonication. The solution consisted of approximately 1:1 ratio of melamine and cyanuric acid which means that 0.1 M hydrochloric acid can dissolve the melamine cyanurate and also dissociates it into melamine and cyanuric acid. Therefore, it is possible that melamine cyanurate complex in adulterated sample could be dissolved and dissociated in the stomach after consumption, and recombined somewhere in the body which has enough water and appropriate pH for formation of melamine cyanurate crystals such as the bladder or kidney. This is a possible scenario for the mass death of cats and dogs in 2007.

We theorize that there could be vectors for contamination. First, melamine and impurities such as cyanuric acid, ammeline and ammelide might be added, and melamine cyanurate complex formed during pet food processing. However, the amount of cyanuric

acid found in some adulterated samples (A – E) is too high to support the cyanuric acid impurity theory. Second, melamine cyanurate complex might have been added to the sample (raw ingredients). However, cyanuric acid is not detected in some samples (I – O), and has relatively lower content than melamine which should approximately be a ratio of 1:1 if pure melamine cyanurate complex was added. It is quite possible that multiple sources of melamine were used to adulterate pet foods, including semi-pure melamine and a melamine cyanurate complex, such as fire retardant.

In summary, a method using an alkaline aqueous solution was developed for melamine and cyanuric acid analysis. During the entire procedure, alkaline pH was maintained until injection. The sample preparation method described in this paper gave better recoveries than the acetonitrile/water mixture method for both melamine and cyanuric acid. Also, experimental results achieved from this study strongly imply that melamine and cyanuric acid could exist in the form of melamine cyanurate complex that can not be completely dissociated and analyzed by the acetonitrile/water mixture method. Melamine cyanurate complex is commonly used for fire retardant. It could be easily added to increase nitrogen levels because it is inexpensive and is easily acquired. Such a fire retardant would give a false negative result of melamine and cyanuric acid analysis due to incomplete dissociation. Therefore, the method developed in this study will promote more accurate analysis of melamine and cyanuric acid.

CONCLUSIONS AND FUTURE DIRECTIONS

Various analytical methods and techniques were studied for melamine and cyanuric acid analysis. The study of each chapter was successfully conducted, and provided very useful information. EIA, HPLC-DAD, and UPLC-MS methods were developed and evaluated in comparison to each other. Excellent agreement among the three methods was found. The EIA method was proven to be a rapid and inexpensive screening method for melamine analysis. The pressurized liquid extraction (PLE) system is a relatively new technique and provides many advantages over traditional methods. As expected, the better recoveries from both adulterated and fortified pet food samples were obtained by PLE. In the last chapter of this research, melamine and cyanuric acid in the adulterated samples was found to exist in the form of melamine cyanurate complex which can not be completely dissociated and analyzed by the acetonitrile/water mixture method that is most widely used now. This indicates that an alkaline environment similar to the one developed in this study is necessary to dissociate melamine cyanurate complex into individual melamine and cyanuric acid so that more accurate analysis can be conducted. The methods developed in this study can provide basic information about melamine and cyanuric acid analysis, and will contribute to the current literature, allowing more accurate determination of melamine and cyanuric acid.

Development of chemical analogs as an internal standard for the analysis of melamine and cyanuric acid by LC-MS-MS may be helpful. Currently, isotopically labeled analogs such as $^{15}\text{N}_3$ -labeled melamine and $^{13}\text{C}_3$ -labeled cyanuric acid are sometimes used for melamine and cyanuric acid analysis. However, the isotopically labeled analog is usually expensive. Since it is unstable radioactive compound, it requires a special permit and skill

to use it. Indeed, there is a chance to form melamine cyanurate complex if $^{15}\text{N}_3$ -labeled melamine and $^{13}\text{C}_3$ -labeled cyanuric acid were added in the sample combining with existing melamine and cyanuric acid. Therefore, finding a chemical analog which has desirable characteristics such as a similar retention time to the analytes, good stability, good chromatographic resolution and none of the sample components would provide researchers with easier and more reliable methods for melamine and cyanuric acid analysis.

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APPENDICES

APPENDIX A

**COMPARISON OF THE EFFICACY OF SODIUM ACID SULFATE AND CITRIC
ACID TREATMENTS IN REDUCING ACRYLAMIDE FORMATION IN
FRENCH FRIES**

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Abstract

Two acidulant food additives, sodium acid sulfate (SAS) and citric acid were investigated for their effectiveness in reducing acrylamide formation in French fries. Acrylamide concentration was determined by gas chromatography-mass spectrometry (GC-MS) after cleanup of French fry extracts by passage through a C-18 column and derivatization by bromination. At a frying temperature of 180 °C, both acidulants appeared ineffective, possibly due to the rapid rate of acrylamide formation which surpassed the capacity of the acidulants to protonate acrylamide intermediates. At the lowest frying temperature tested (160 °C), 3% SAS and 3% citric acid significantly inhibited acrylamide formation as compared to the control. However, 3% SAS appeared to inhibit acrylamide formation more effectively than citric acid at 160 °C, as well as at frying temperatures of 170 and 180 °C. Our results indicate that acrylamide formation

during frying can be reduced by treatment of potatoes with 3% SAS or citric acid. but SAS, a stronger acid with a lower pK_a , is the more effective acidulant.

Introduction

Acrylamide is a neurotoxic chemical compound, and a probable human carcinogen (US EPA 1990; Friedman 2003). In April 2002, Swedish researchers found high levels of acrylamide in various fried and oven baked foods (Tareke et al. 2002). Currently, there are several ways to reduce acrylamide formation in foods including removing asparagine and reducing sugars (which are precursors for acrylamide formation), adding free amino acids (other than asparagine), or decreasing pH. When the pH is lowered, the Schiff base, a crucial intermediate required for acrylamide formation, will attract protons from the acidic environment. This protonated Schiff base will regenerate asparagine and reducing sugars (Yaylayan et al. 2005), rather than being converted to acrylamide. The inhibitory effect of pH on acrylamide formation has previously been demonstrated in a model system using citric acid (Vleeschouwer et al. 2006; Jung et al. 2003).

Citric acid has long been used as an acidulant in food processing, and is known as an effective treatment for inhibiting acrylamide formation. However, citric acid imparts a sour taste and tougher texture on dip treated French fries when applied in concentrations greater than or equal to 2 %. Sodium acid sulfate (SAS) is a relatively new acidulant approved by the FDA in 1998 for use in the food industry. With a lower pK_a than citric acid (1.99 vs 3.14 respectively), SAS dip treatments have a lower pH and may be used to reduce production of acrylamide in fried potatoes. According to a flavor

profile prepared by Sensory Spectrum, Inc. (Chatham, NJ), SAS may reduce the sour taste produced by low concentration citric acid treatments. The inhibitory effects of SAS on acrylamide formation in carbohydrate-rich foods have not been investigated to date. A comparison of the effects of SAS and current citric acid treatments could facilitate development of a novel food safety product and improved potato treatment and processing protocols. The objective of this research is to measure the comparative reduction of acrylamide formation in potatoes treated with SAS and citric acid and subsequently cooked at various frying temperatures. Acidulant concentrations will also be investigated to determine optimum treatment conditions. Acrylamide will be quantified using a GC-MS analytical method developed by Tareke et al. (2002), and modified in our laboratory.

Materials and Methods

All the chemicals used in this experiment were purchased from Fisher Scientific (Pittsburgh, PA). Potato (Russet Norkotah) was peeled and cut into French fry strips using a French fry cutter (3/8 inch, Vollrath Co., Sheboygan, WI). The fries were treated with distilled water, citric acid (1 and 3 %; w/v), or sodium acid sulfate (1 and 3%; w/v) solutions by dipping for 5 min. After freezing the potato strips for 1 hr at -20 °C, they were fried in a fryer (Hotpoint Co., Peterborough, UK) using vegetable oil obtained from a local grocery store for 5 min at a temperature of 160, 170 or 180 °C. After cooling for 10 min, French fries were frozen at -20 °C and blended using a kitchen blender (Handy Chopper Plus, Black & Decker, Towson, MD).

The analytical method used to quantify acrylamide by GC-MS was as described by Tareke et al. (2002), with slight modifications. The homogenized sample (5 g) was diluted into 35 mL of water, and mixed for 2 min using a polytron (CH-6010, Kinematica Inc., Bohemia, NY). After centrifugation for 20 min at 4,000 X g, the supernatant was filtered using a Whatman #50 filter with suction. For final purification, filtered extract (15 mL) was loaded on a graphitized carbon black column (1,000 mg, 15 mL, Alltech, Deerfield, IL), or a C18-U or C18-E column (500 mg, 6 mL, Strata, Phenomenex, Torrance, CA). C18-U and C18-E columns were conditioned with 5 mL of methanol followed by 5 mL of water before loading the extract. The graphitized carbon black column did not require pre-conditioning.

The acrylamide in the purified extract (25 mL) was derivatized through bromination by adding potassium bromide (3.75 g), hydrobromic acid (acidification to pH 1 – 3), and saturated bromine water (1.25 mL). The sample was kept at 4 °C for 24 hours. Excess bromine was decomposed by adding sodium thiosulfate (1 M), in single drops until the yellow color disappeared. Sodium sulfate (7.5 g) was added and the solution stirred vigorously for 10 min, transferred to a separatory funnel and extracted by shaking with 5 mL of ethyl acetate for 2 min. The ethyl acetate extraction was repeated and the two organic fractions combined in a 25 mL volumetric flask.

The acrylamide in French fry samples was quantified using an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA). The GC column was a DB-5 fused silica capillary column (30 m X 0.25 mm i.d., 0.25 µm film thickness; Varian Inc., Walnut Creek, CA). Two microliters of sample were injected with a splitless mode, facilitating injection of the

entire sample volume (2 μL) for analysis. The temperature program was as follows: isothermal for 1 min at 65 $^{\circ}\text{C}$, increased to 250 $^{\circ}\text{C}$ at a rate of 15 $^{\circ}\text{C}/\text{min}$, and isothermal at 250 $^{\circ}\text{C}$ for 10 min. The analysis was performed using electron ionization (70 eV) and selected ion monitoring. The ions used to facilitate identification of the target analyte (2,3-dibromopropionamide) were $[\text{C}_3\text{H}_5^{81}\text{BrNO}]^+ = 152$ (100 %), $[\text{C}_3\text{H}_5^{79}\text{BrNO}]^+ = 150$ (100 %), and $[\text{C}_2\text{H}_3^{79}\text{BrNO}]^+ = 106$ (65 to 70 %) using 150 (m/z; mass-to-charge ratio) for quantification. Quantification was performed by comparison of peak areas on a chromatogram with a five point standard curve prepared with acrylamide solutions in water at concentrations of 0, 10, 50, 100, and 1000 $\mu\text{g}/\text{L}$.

Statistical Analysis

Significant differences ($P \leq 0.05$) between the mean value of each treatment at the same frying temperature and mean value of each cartridge column were calculated using Duncan's multiple range tests with SAS software 9.1 (SAS Institute Inc., Cary, NC).

Results and Discussion

Three different columns (graphitized carbon, C18-U, and C18-E) were compared for acrylamide purification efficiency (data not shown). The graphitized carbon column efficiently removed all interfering co-extractants and resulted in the highest acrylamide recovery. The lowest acrylamide content was measured when no clean-up column was used. These results indicate that compounds in the French fry extract interfere with acrylamide derivatization and can affect acrylamide detection by GC-MS. There were no significant differences in acrylamide content quantified by the graphitized carbon and

C18-U columns ($P \geq 0.05$). C18-U would be the most accurate column for acrylamide clean-up based on standard deviations calculated for each column. The inhibitory effects of SAS and citric acid on acrylamide formation are shown in Figure A1. GC-MS analysis detected the greatest acrylamide content in the control samples (A) and dramatically reduced acrylamide peaks with the citric acid and SAS treatments (Figure A1, B and C).

Figure A2 shows the effect of acidulants on acrylamide formation at various frying temperatures (160, 170 and 180 °C) and at different concentrations. Results show acrylamide formation increased with increasing frying temperatures. No significant inhibitory effect ($P \geq 0.05$) from treatment with acidulants was noted at the two highest frying temperatures (170 and 180°C), although both decreased acrylamide formation at 170 °C, as compared to the control. Acrylamide levels were similar for all treatments at 180 °C, suggesting that there are no inhibitory effects at higher temperatures. The rate of acrylamide formation may have accelerated beyond the acidulants' capacity to effectively protonate the intermediate Schiff base at the higher frying temperature rendering the acidulants less effective in inhibiting acrylamide formation. When potatoes were fried at 160 °C, 3% citric acid and 3% SAS significantly decreased acrylamide formation ($P \leq 0.05$) as compared to the acrylamide levels measured in the extract of potatoes fried at the higher temperatures. Three percent SAS had a greater inhibitory effect on acrylamide formation than citric acid and control samples at frying temperature of 160 and 170 °C.

As expected, the SAS solutions had a lower pH than citric acid solutions of the same concentration due to lower pK_a values. One percent solutions of SAS and citric acid had pH values of 1.61 and 2.21, respectively. The pH of 3% SAS was 1.33, and the pH of 3% citric acid was 1.98. Based on the lower pH and confirmed by GC-MS analysis, a

SAS dip treatment is more effective for inhibiting acrylamide formation in French fries than citric acid.

Summary

The inhibitory effect of SAS and citric acid on acrylamide formation in French fries was investigated in this work. Initial comparison of three cleanup columns indicated a C18-U column was the most effective for separating acrylamide based on analytical results, least variance and the lower cost for this column. At the highest frying temperature (180 °C), the tested acidulants were less effective, possibly due to the rapid rate of acrylamide formation which surpassed the acidulants' capacity to protonate acrylamide intermediates. However, at the lowest frying temperature (160 °C), 3% SAS and 3% citric acid significantly ($P \leq 0.05$) inhibited acrylamide formation. Frying temperature strongly influenced the effectiveness of these acidulants. Overall, SAS was more effective than citric acid in reducing acrylamide formation in French fries. However, further study is needed to confirm these results and sensory testing is required to determine flavor acceptability of SAS treated potatoes.

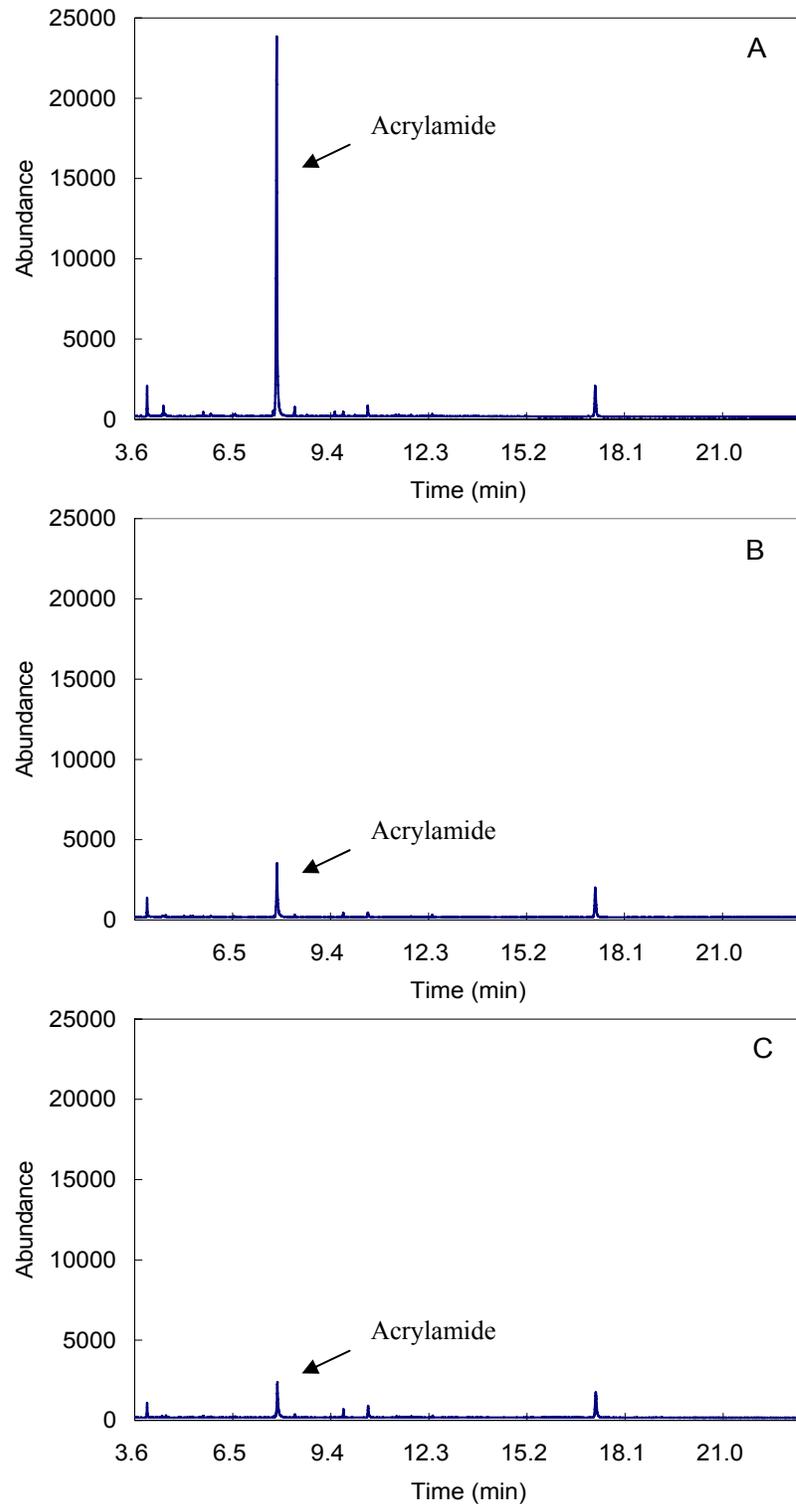


Figure A1. GC-MS chromatogram of acrylamide in French fries dip-treated with distilled water or acidulants before frying. (A) Distilled water. (B) 3% citric acid. (C) 3% SAS treatment. Potatoes were dipped for 5 min., then fried at 160 °C for 5 min.

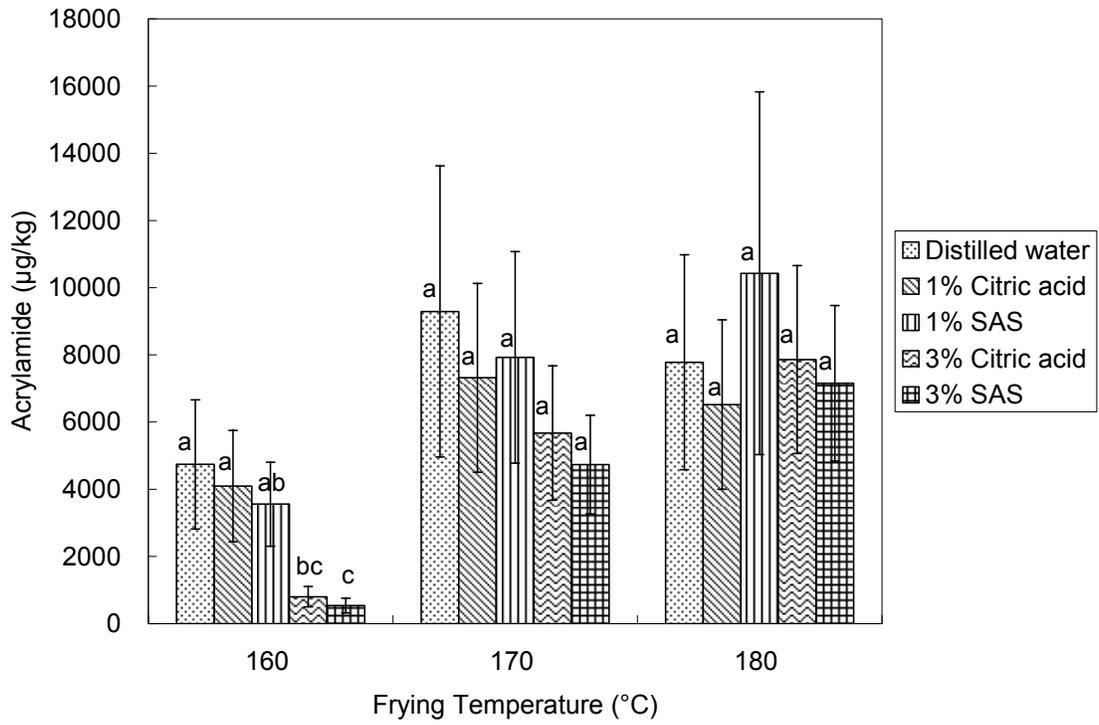


Figure A2. Effect of acidulants on acrylamide formation in French fries at various frying temperatures and treatment concentrations. Within each frying temperature, different letters indicate significant differences. Means were calculated from triplicate analysis for each column.

APPENDIX B
DETERMINATION OF BISPHENOL A IN BABY BOTTLES BY EIA AND
HPLC/FLD

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Abstract

Baby bottles are often comprised of polycarbonate (PC) which is produced from the polymerization of 2,2-bis(4-hydroxyphenyl)propane (bisphenol A). Since bisphenol A (BPA) is considered a potential endocrine disrupter and can migrate from the bottle to baby formula, using a baby bottle made of polycarbonate is a potential health risk to babies. High-performance liquid chromatography (HPLC) has been widely used to detect bisphenol A in various sample matrices using tandem mass spectrometry (MS/MS) or fluorescent (FLD) detection. An enzyme immunoassay (EIA) method was recently developed for the quantitation of bisphenol A in baby bottles. Baby bottles were cut into small pieces and ground with a grinding mill to produce fine powder for the extraction of bisphenol A. Ten baby bottles were extracted with methanol and assayed by EIA and HPLC/FLD. The concentrations of bisphenol A found in the baby bottles ranged from 5.2

to 1,060 ng/g. Recoveries for bisphenol A-fortified baby bottles ranged from 94.2 to 100 % for the four different spiking levels. A good correlation ($r^2 = 0.9795$) between the EIA and HPLC/FLD methods was obtained. The EIA kit used in this study proved to be an inexpensive alternative to HPLC instrumentation to quantify bisphenol A in baby bottles.

Introduction

Due to desirable properties such as light weight, optical clearness and heat resistance, polycarbonate is widely used for making baby bottles. Although polycarbonate is stable at high temperatures, hydrolysis of polycarbonate can occur on the surface of a bottle, and release free BPA into baby formula. The harmful effects of BPA on human health is still argued. BPA can influence mammary gland development (Markey et al. 2001) and cause urethral malformations (Timms et al. 2005). Another study indicates that there is no detectable effects in toxicological tests (Ashby 2003). However, the potential risk from BPA in baby bottles should be avoided. Indeed, the potential health risk of BPA to babies could be greater than adults. Many studies on the migration of BPA from PC baby bottles have been reported. Migration of BPA from cut portions of PC baby bottles into food simulants has been determined by HPLC/FLD and confirmed by GC/MS (Biles et al. 1997). Another study was also conducted by HPLC/FLD and GC/MS for determination of residual BPA from PC baby bottles (Wong et al. 2005). An enzyme immunoassay (EIA) method was recently developed for the quantitation of BPA in baby bottles. This EIA method is simple and easy for the quantitation of BPA. In this study, we compared the EIA method to HPLC/FLD for BPA analysis from baby bottles.

Materials and Methods

Ten baby bottles were purchased from a local grocery store. They were cut into small pieces and ground by a Wiley mill. Samples of the ground baby bottles were weighed (0.1 g) and put into a glass vial. One milliliter of methanol was added followed by vortexing and sitting for 30 min. It was filtrated with 0.45 μm pore size PTFE syringe filter. Another 1 ml of methanol was added and extracted again. After filtration, those solutions were combined and analyzed with EIA and HPLC/FLD. Analysis by EIA was conducted by the manufacturer's manual (Beacon Analytical Systems Inc., Portland, ME). Hewlett Packard model 1100 liquid chromatograph equipped with a fluorescence detector and a Gemini C6-Phenyl column (250 \times 4.6 mm, 5 μm , Phenomenex, Torrance, CA) were used for HPLC/FLD analysis. Mobile phase used was acetonitrile/water (50:50). The flow rate was 1 mL/min, and the wavelengths were monitored at 235 nm (excitation) and 317 nm (emission).

Results and Discussion

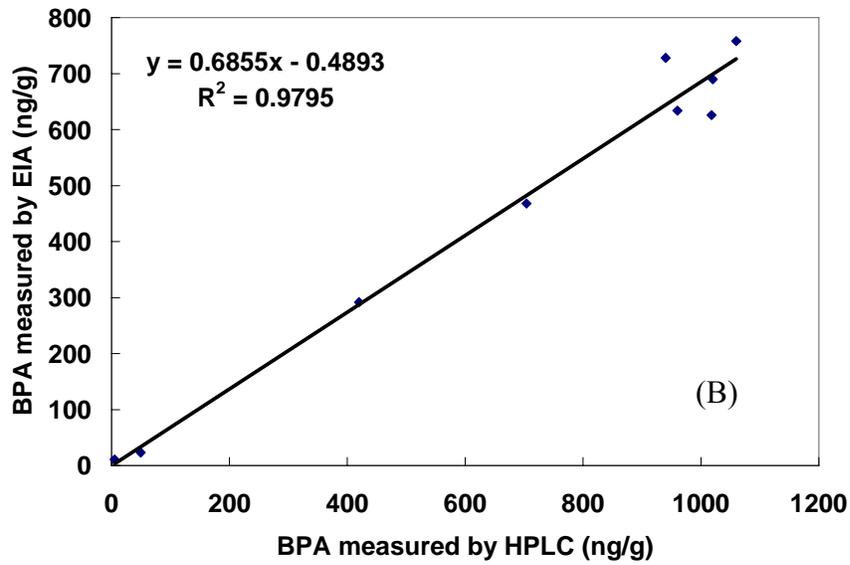
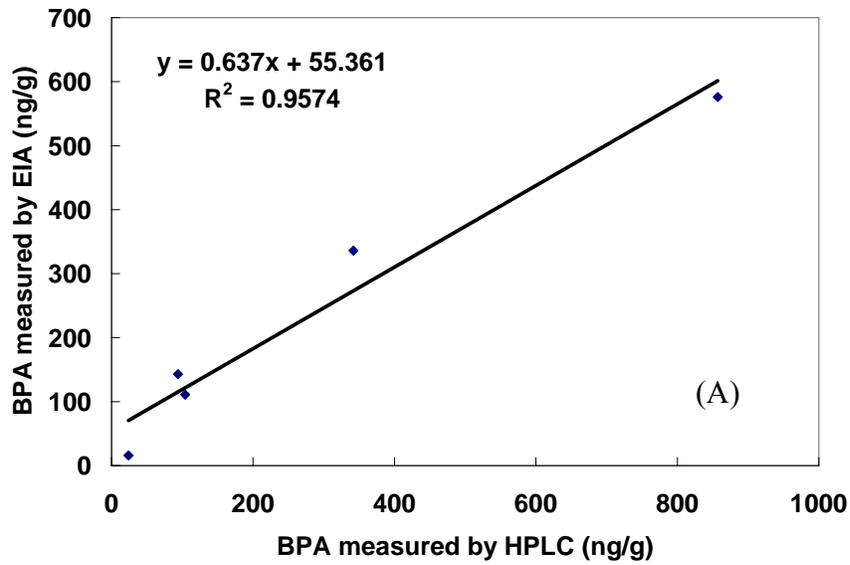


Figure B1. Comparison of EIA and HPLC/FLD methods for BPA analysis from baby bottles. (A) Same samples but extracted separately. (B) Same samples and same extracts tested.

The concentrations of bisphenol A found in the baby bottles ranged from 5.2 to 1,060 ng/g. Good correlations between the EIA and HPLC/FLD methods were obtained ($r^2 = 0.9574$ and 0.9795) (Figure B1). The EIA method developed is very specific towards BPA with 100% reactivity. Cross reactivity to other BPA-related substances are very low <10%.

APPENDIX C

AQUEOUS CHLORINE DIOXIDE AS A SANITIZER FOR CONTROLLING FIVE FOODBORNE PATHOGENS, YEASTS AND MOLDS ON BLUEBERRIES

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Abstract

Aqueous chlorine dioxide (ClO₂) was studied for its effectiveness in controlling foodborne pathogens such as *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Yersinia enterocolitica* as well as natural flora on blueberries such as yeasts and molds. All five pathogens were spot-inoculated on the skin surface of blueberries. A sachet (2g size) containing all necessary chemicals for generation of ClO₂ was used to provide approximately 320ppm of ClO₂ in 7.6 liter of distilled water. The efficacy of different concentrations (1, 3, 5, 10, and 15ppm) of ClO₂ and various contact times (10 sec, 1, 5, 10, 20, 30 min, 1 h, and 2 h) were studied. Reductions of all pathogens inoculated on blueberries were achieved by the application of ClO₂ treatments. Aqueous ClO₂ was most effective in reducing *L. monocytogenes* (4.88 log CFU/g) as compared to the other pathogens. *P. aeruginosa* was reduced by 2.16 log CFU/g ($P < 0.05$) after 5min when treated with 15ppm of ClO₂.

Relatively short treatment time (20 or 30 min) was more effective in reducing *S. Typhimurium* than longer treatment time (1 or 2 h) for most concentrations. The highest reduction (4.67 log CFU/g) in the population of *S. aureus* was achieved with a 15ppm treatment of ClO₂ for 30 min. When treated for 2 h with 5ppm ClO₂, the reduction of *Y. enterocolitica* (3.49 log CFU/g) was not significantly different than the reductions at 10 and 15ppm (3.70 and 3.54 log CFU/g, respectively). In general, longer treatment times did not significantly reduce pathogen counts ($P > 0.05$) as compared to shorter treatments. Fifteen ppm of ClO₂ reduced natural yeasts and molds by 2.82 log CFU/g after 1 h. Results indicate that aqueous ClO₂ shows promise as a sanitizer for reducing foodborne pathogens as well as yeasts and molds. In addition, concentrations of ClO₂ were shown to decrease over time when stored at room temperature. When exposed to blueberries, ClO₂ concentrations were further reduced, showing significant degradation ($P < 0.05$) and suggesting a need for further study of the effect of organic materials on aqueous ClO₂ residues after treatments.

Keywords: Aqueous chlorine dioxide; Foodborne pathogens; Blueberries

Introduction

Bacterial foodborne outbreaks have been associated with raw fruits and vegetable products (Burnett and Beuchat 2001). Although bacterial foodborne illnesses have not been linked to consumption of fresh or raw blueberries to date, blueberries are vulnerable to bacterial contamination in the field due to hand harvesting, field packing, and manure fertilization. In industries, many produces including blueberries are washed or sprayed with chlorinated water containing 50 to 200ppm of active chlorine to reduce microorganisms (Brackett 1992; Cherry 1999). However, chlorine may not be effective in reducing microorganisms on fruits and vegetables at high concentrations (Brackett 1992; Zhang and Farber 1996; Taormina and Beuchat 1999). Indeed, some chemical by-products formed when chlorine is used for reducing microorganisms in food processing are considered as mutagenic or carcinogenic (Richardson et al. 1998).

Researchers have focused on chlorine dioxide (ClO_2) as an alternative sanitizer since it has 2.5 times the oxidation capacity of chlorine and is less reactive to organic compounds (Benarde et al. 1967; Richardson et al. 1998; Beuchat et al. 2004; Han et al. 2004; Lee et al. 2004; Kaye et al. 2005). Most of the investigators working on sanitization use gaseous ClO_2 because it offers greater penetration than aqueous ClO_2 and is therefore considered more effective in reducing microorganisms on fruits and vegetables (Han et al. 2001; Lee et al. 2004). For applications in the food industry, however, use of gaseous ClO_2 may be limited because gaseous ClO_2 treatment must be conducted in a firmly and safely sealed chamber (Lee et al. 2004), high concentrations of the gas are potentially explosive (US EPA 1999), and numerous mechanical devices or steps are necessary to handle ClO_2 gas as well as to provide precise concentrations for

sanitization (Han et al. 2004; Lee et al. 2006). Aqueous ClO₂ offers several advantages for food sanitization, especially for processing of vegetables and fruits, such as blueberries. A special chamber is not required for the sanitizing process, handling is easier than with gaseous ClO₂, and the liquid may be easily applied to the existing process during washing without modifying subsequent steps. In addition, using an aqueous solution may provide advantages to producers who wish to preserve the appearance of their sanitized produce. Generating chlorine dioxide traditionally requires either reaction with acid or on-site instrumentation such as an applicator or generator to generate and apply the liquid. Therefore, it is very inconvenient, relatively expensive, and requires technical expertise. For application of ClO₂ in the food industry, simple and inexpensive sanitizing procedures are necessary. Recently, a small and dry chemical pouch method was developed for generating ClO₂ (Intellectual Capital Associates (ICA) TriNova, LLC, Forest Park, GA). This method is easy to use when compared with machinery systems. Applying this new pouch method as an aqueous sanitizer to reduce microorganisms on produce has not been investigated.

This study was conducted to evaluate the bactericidal effectiveness of aqueous ClO₂ generated by a chemical pouch on five foodborne pathogens, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Yersinia enterocolitica* inoculated onto the surface of blueberries. Molds and yeasts that naturally occur on blueberries were also investigated.

Materials and Methods

Bacterial Strains

A cocktail mixture of two strains for each pathogen was utilized in this study. Each species was studied individually. Two strains of *Listeria monocytogenes* (ATCC 7644 and ATCC 19115), *Pseudomonas aeruginosa* (ATCC 9027 and ATCC 10145), *Salmonella* Typhimurium (ATCC 14028 and ATCC 13311), *Staphylococcus aureus* (ATCC 25923 and ATCC 12600), and *Yersinia enterocolitica* (ATCC 27729 and ATCC 9610) were obtained from the Food Microbiology Laboratory in the Department of Food Science and Human Nutrition at University of Maine (Orono, ME), and used to inoculate the surface of blueberries. Each strain of *L. monocytogenes*, *P. aeruginosa*, *S. Typhimurium*, *S. aureus*, or *Y. enterocolitica* was cultured in brain heart infusion (BHI; Difco, Becton Dickinson, Sparks, MD) broth at 37 °C for 24 h. Cultures were kept under refrigeration (4 °C) as stock cultures and transferred weekly to maintain viability.

Preparation of Inoculum

One loop of each strain culture was taken from the stock culture in refrigeration, transferred in 5 ml of BHI broth, and incubated at 37 °C for 24 h. After 24 h, 0.1 ml of the culture was inoculated into 100 ml of BHI in a 250 ml centrifuge bottle, followed by incubation at 37 °C for 24 h. Cells of the strains cultured in each centrifuge bottle were harvested by centrifugation (15,300 × g, 15 min, 4 °C), washed two times with 100 ml of sterile buffered peptone water (BPW; Difco), and resuspended in 10 ml of BPW. Two suspensions of each pathogen were combined to give 30 ml of a cocktail mixture for each pathogen. Each cocktail mixture contained approximately 10⁸ to 10⁹ CFU/ml of cells.

Before inoculation on the blueberries, populations of each mixture were determined by serially diluting suspensions in sterile 0.1% peptone water (Difco) and spread plating 0.1 ml on the appropriate selective agar.

Inoculation of Blueberries

Blueberries were purchased at a local grocery store (Bangor, ME), and stored at 4 °C for a maximum of 2 days before use. The berries were placed in single layers on plastic trays. Each layer of blueberries was supported by two glass rods that held both ends of each blueberry so that the middle skin surfaces were not in contact with the plastic trays. A 150 µl of cell suspension for each pathogen was spot-inoculated on the skin surface of each blueberry using a micropipette. Half (75 µl) of the cell suspension was deposited onto blueberry skin in 12 locations and dried for 2 h in a laminar flow hood. The remaining 75 µl of cell suspension was deposited onto the uninoculated side of the blueberry, and dried for 2 h.

Preparation of Aqueous Chlorine Dioxide

A chlorine dioxide solution sachet (ICA TriNova, LLC) consisting of two components was shaken to mix the chemicals, and the mixed sachet was placed in 7.5 L of distilled water in a container. The container was stored in a dark location at room temperature for 3 days to allow the release of ClO₂ into the water. After 3 days, the container was refrigerated at 4 °C and stored as a stable ClO₂ stock solution. The concentration of ClO₂ was determined by a DPD method (*N,N*-diethyl-*p*-phenylenediamine) using a Hach DR/820 Colorimeter (Hach, Loveland, CO). The

concentration of ClO₂ in the stock solution was measured and diluted to precise concentrations prior to every test.

Treatment and Measurement of Chlorine Dioxide

Five blueberries (10 g ± 0.2) inoculated with each pathogen were aseptically placed in sterile French square bottles, treated with 200 ml of sterile distilled water (control) or 1, 3, 5, 10, or 15ppm of ClO₂ solutions (pH 4.2 for all concentrations) for 10 sec, 1, 5, 10, 20, 30 min, 1, and 2 h. The French square bottles were stored at room temperature in the dark during treatment. After each treatment time, the treatment solutions were decanted, and 40 ml of Dey-Engley neutralizing broth (D/E neutralizing broth, Difco, Sparks, MD) was immediately added to stop the action of the treatment solutions.

Eight uninoculated blueberries (10 g ± 0.2) were placed in sterile French square bottles for yeasts and molds experiments using the same concentrations of ClO₂ and the same treatment intervals. To measure the stability of ClO₂ over time (10 sec, 30 min, 2, 6, 12, and 24 h), ten French square bottles were wrapped with aluminum foil to prevent light exposure. Equal amounts of blueberries (10 g ± 0.2) were added to five of the wrapped bottles. The other 5 bottles had no berries added. Two hundred ml of ClO₂ (1, 3, 5, 10 or 15ppm) was added to each of the 10 bottles prepared. The bottles were stored at room temperature for treatment with the ClO₂ sampled and measured in triplicate for each concentration at each interval.

Microbial Enumeration

Blueberries and D/E neutralizing broth in the French square bottle were drained and placed in a stomacher bag (Seward Medical, London, England), and homogenized for 2 min using a stomacher (Tekmar Company, Cincinnati, OH). Homogenates were serially diluted in sterile 0.1% peptone water, and spread-plated in duplicate on modified Oxford agar (Neogen, Lansing, MI), Pseudomonas isolation agar (Neogen, Lansing, MI), Xylose Lysine Desoxycholate (Neogen, Lansing, MI), Baird Parker agar (Neogen, Lansing, MI), Yersinia selective agar (Neogen, Lansing, MI), and Dichloran Rose Bengal Chloramphenicol agar (Neogen, Lansing, MI) to enumerate *L. monocytogenes*, *P. aeruginosa*, *Salmonella* Typhimurium, *S. aureus*, *Y. enterocolitica*, and yeasts and molds, respectively. Bacteria were grown at 37 °C for 24 h, and yeasts and molds were grown at room temperature for 5 days.

Statistical Analysis

All experiments were replicated three times. Mean values were analyzed to determine significant differences in populations of pathogens or yeasts and molds on blueberries subjected to ClO₂ treatment at varying time intervals and concentrations. Data were subjected to the Statistical Analysis System (SAS Institute Inc., Cary, NC) for analysis of variance and Duncan's multiple range tests to determine significant differences ($P < 0.05$).

Results and Discussion

Stability of Chlorine Dioxide during Treatment

Aqueous chlorine dioxide solutions containing blueberries or not were monitored for a 24 h period. Over time all concentrations of ClO₂ tested (1, 3, 5, 10, and 15ppm) decreased regardless of whether the solution contained blueberries because aqueous ClO₂ is volatile, and releases gaseous ClO₂ into the atmosphere at room temperature (boiling point: 11 °C). However, the concentration of the aqueous ClO₂ where blueberries were added decreased significantly more ($P < 0.05$) than the concentration of the aqueous ClO₂ where no blueberries were added (Figure C1) with the exception of the 10 second (1, 3, and 15ppm) and 30 min (15ppm) treatment times. Low concentrations of aqueous ClO₂ (1, 3, and 5ppm) were completely degraded within 24 h when exposed to blueberry samples. After 24 h exposure to the blueberries, 15ppm of ClO₂ was degraded by approximately half (7.55ppm). Since the oxidizing power of aqueous ClO₂ is diminished by contact with organic matter (Lindsay et al. 2002; Reina et al. 1995), the blueberries used in this study may have affected the concentration of aqueous ClO₂. Further research is necessary to accurately describe the relationship between organic matter and aqueous ClO₂ concentration or residues.

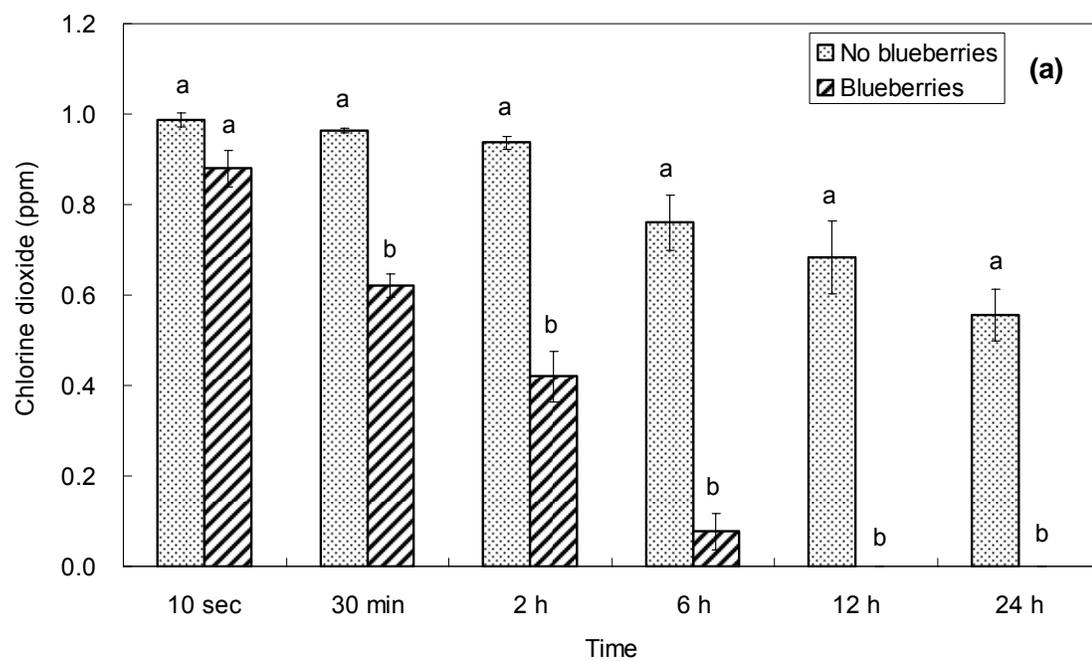


Figure C1. Decrease of chlorine dioxide (ClO₂) concentrations over time with blueberries or without. (a) 1ppm

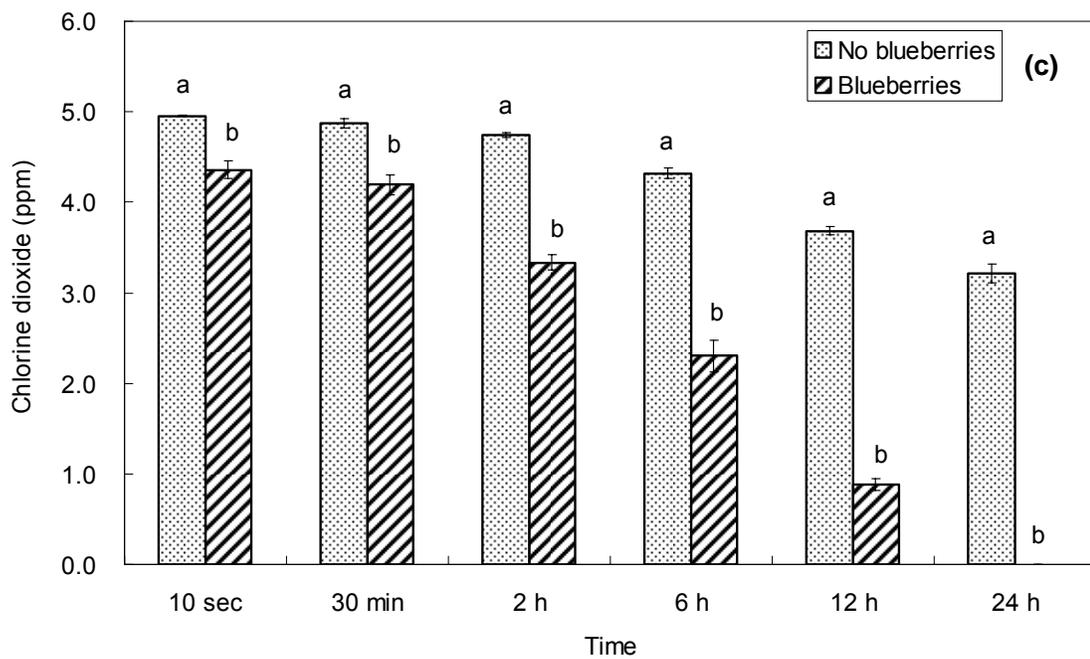
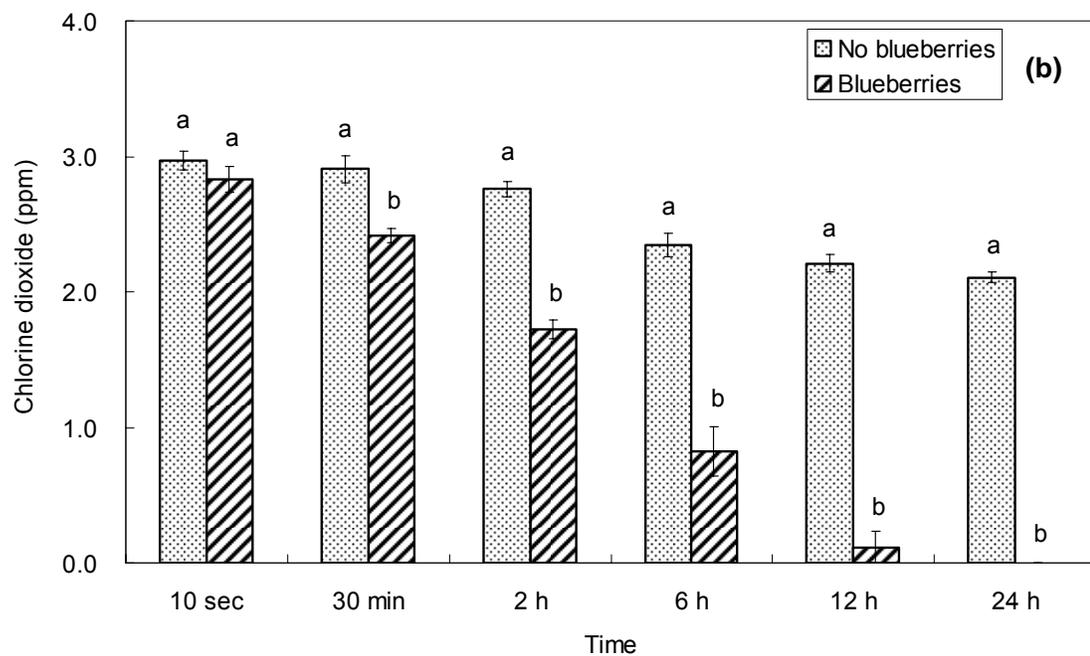


Figure C1. Continued. (b) 3ppm, (c) 5ppm.

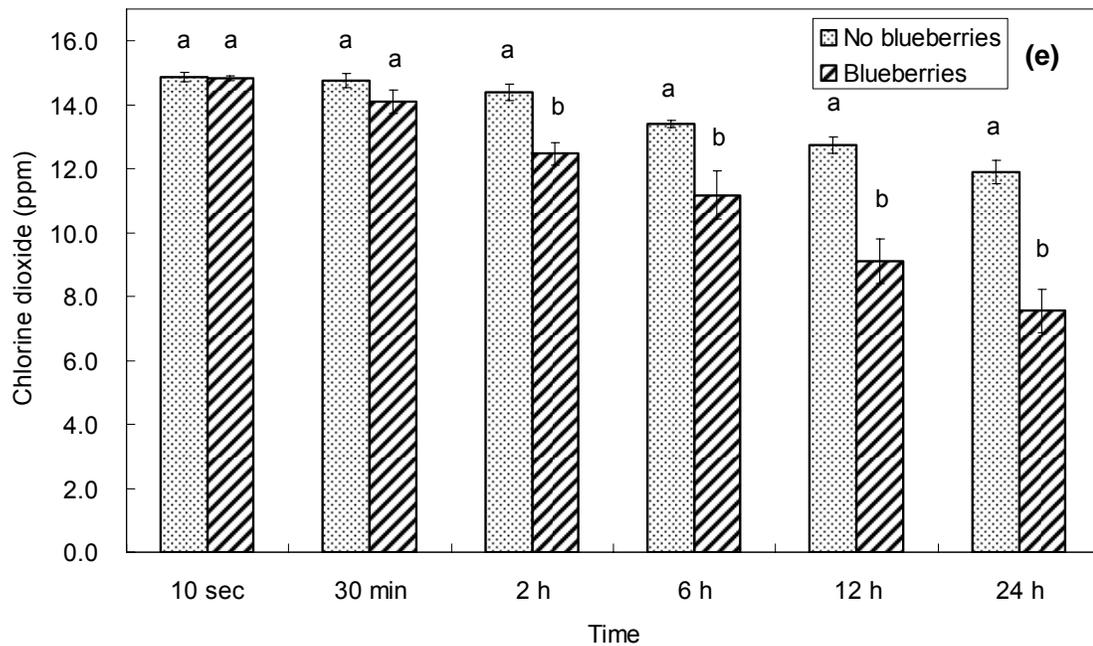
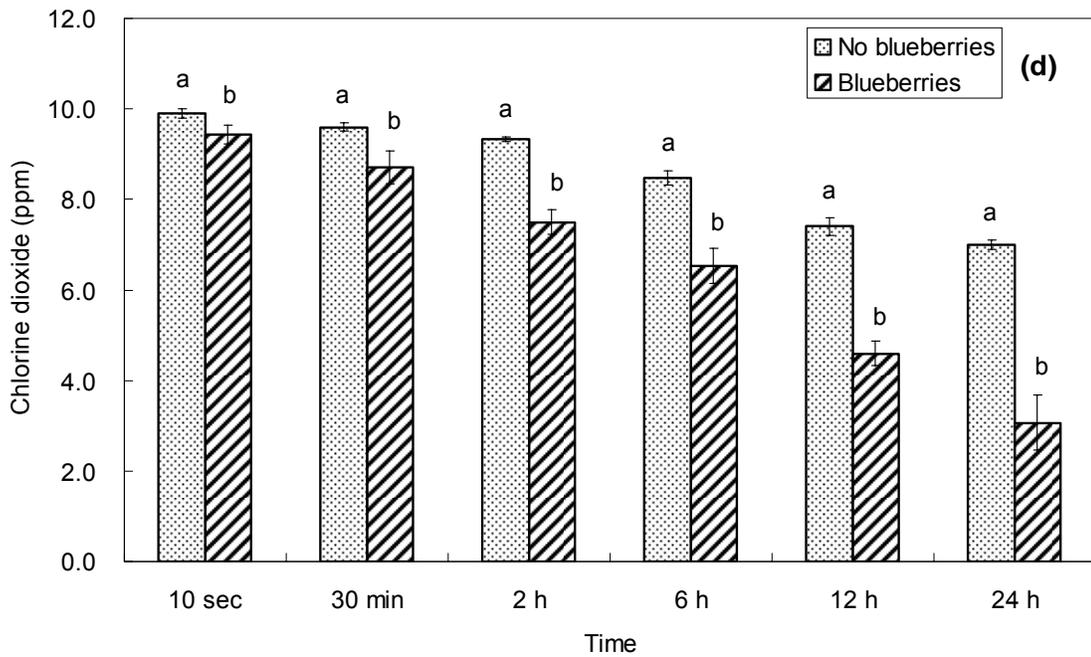


Figure C1. Continued. (d) 10ppm, and (e) 15ppm of ClO₂. Concentrations of ClO₂ at each measurement time labeled with different letters are significantly different ($P < 0.05$).

Effect of Chlorine Dioxide Treatment for Pathogens, and Yeasts and Molds on Blueberries

Five pathogens, and yeasts and molds were treated with ClO₂ to evaluate reductions in their number by aqueous ClO₂ treatment (Table C1). The effect of ClO₂ concentration and treatment time in reducing *L. monocytogenes* on blueberries was investigated. Longer treatment time resulted in an increased reduction of the populations of *L. monocytogenes*. The highest ClO₂ concentration (15ppm) and longest treatment time (2 h) caused a 4.88 log CFU/g reduction of *L. monocytogenes*. The 15ppm ClO₂ treatment reduced the population of *L. monocytogenes* more than 10ppm when treated for 30 min or longer. However, the reductions by 5 or 10ppm ClO₂ treatments after 30 min, 1 h, and 2 h were not significantly different ($P > 0.05$). At the treatment interval of 30 min, 1 h, or 2 h, all concentrations of ClO₂ (1, 3, 5, 10 and 15ppm) resulted in significant reductions ($P < 0.05$) of the population of *L. monocytogenes*. Aqueous ClO₂ was more effective in reducing *L. monocytogenes* than in reducing numbers of the other pathogens.

With *P. aeruginosa*, the greatest reduction (4.48 log CFU/g) was achieved in samples treated for 2 h with 15ppm ClO₂. However, this result was not significantly different ($P > 0.05$) from the reductions at 30 min and 1 h. These non-significant differences during varying treatment times at the same concentration of ClO₂ were also found at all other concentrations of ClO₂ (1 through 10ppm). The reductions resulting from 20 minute or longer treatment times with 3 or 10ppm ClO₂ were not significantly different from each other. In addition, 5ppm did not reduce the population of *P. aeruginosa* further ($P > 0.05$) with a treatment time longer than 10 min. Therefore, ClO₂ treatments exceeding 20 min provide no further reduction of *P. aeruginosa* populations at all ClO₂ concentrations except 15ppm.

Reduction of *S. Typhimurium* was also investigated. The most effective concentration and treatment time for *S. Typhimurium* reduction were 15ppm and 20 min, reducing *S. Typhimurium* by 3.32 log CFU/g. Longer treatment times up to 2 h did not significantly reduce the *S. Typhimurium* population ($P > 0.05$). There were no significant differences in reductions between 10 and 15ppm for 10 min or longer treatment times. Since the highest reductions were achieved at 20 and 30 min for 15 and 10ppm ClO₂ concentrations, respectively, longer treatments are not necessary considering the potential for damaging the appearance of the blueberries.

The most effective treatment time for the reduction of *S. aureus* was 30 min at concentrations of 10 and 15ppm ClO₂. However, the reductions from increased treatment times such as 1 or 2 h were not significantly different ($P > 0.05$) when compared with the reductions after the 30 min treatment at the same ClO₂ concentrations. There was no significant difference between the 10 and 15ppm treatments at any treatment time even though the mean values of bacterial reductions at 15ppm were higher than at 10ppm. As with *P. aeruginosa*, every concentration of ClO₂ treatment on *S. aureus* (other than 1ppm) showed no further reduction in the population ($P > 0.05$) even with treatment times of 20 min or more.

Application of a low concentration of ClO₂ (3ppm) resulted in a 2.88 log CFU/g reduction on *Y. enterocolitica* when treated for 2 h. This was the highest reduction as compared to reductions in other pathogens such as *L. monocytogenes* (2.07), *P. aeruginosa* (1.39), *S. Typhimurium* (1.02) and *S. aureus* (2.06) when treated with the same concentration. Similar to *S. Typhimurium*, there were no significant differences in the reductions resulting from 10 and 15ppm treatments when applied for 10 min or longer.

When treated with 15ppm, at treatment times from 20 min to 2 h, *Y. enterocolitica* displayed no significant differences ($P > 0.05$) in population reductions. In addition, 10ppm treatment yielded no significant differences ($P > 0.05$) on reductions during treatment times of 30 min to 2 h.

The effect of aqueous ClO_2 on yeasts and molds naturally occurring on blueberries were also investigated. The most effective conditions for reducing yeasts and molds (2.86 log CFU/g) on blueberries was 15ppm for 2 h even though a 1 h treatment resulted in a similar reduction of yeasts and molds (2.82 log CFU/g). As with the bacteria, results indicate that longer treatment times will not yield further reductions in population. For example, a 10ppm ClO_2 treatment yielded no significant differences in reductions of yeasts and molds ($P > 0.05$) for treatment times of 20 min through 2 h. However, treatments of 1 and 2 h significantly reduced the population of yeasts and molds ($P < 0.05$) compared with shorter treatment times (10 sec through 30 min) with the highest concentration of ClO_2 (15ppm).

Aqueous chlorine dioxide was approved by the Food and Drug Administration for washing fruits and vegetables in 1998 (FDA 1998). Richardson et al. (1998) has shown that the number of by-products formed by chlorination is much higher than the number of by-products produced by treatment with chlorine dioxide. Indeed, our experimental results strongly support the effectiveness of aqueous ClO_2 in reducing the number of microorganisms on blueberries.

In conclusion, aqueous chlorine dioxide (ClO_2) can be used as a sanitizer for controlling foodborne pathogens as well as yeasts and molds on blueberries. We expect that brief treatment intervals (20 or 30 min) and degradation of ClO_2 over time and by

organic materials (blueberries) provide advantages to food processors who seek to preserve the appearance of their product and reduce ClO₂ residues while enhancing food safety with novel sanitization procedures.

Table C1. Reductions of *L. monocytogenes*, *P. aeruginosa*, *S. Typhimurium*, *S. aureus*, *Y. enterocolitica*, and yeasts and molds after treatment with aqueous chlorine dioxide (ClO₂)

Pathogen	Treatment time	Reduction (log CFU/g)				
		1ppm ^f	3ppm	5ppm	10ppm	15ppm
<i>Listeria monocytogenes</i>	10 sec	A ^g 0.03 a ^h	C 0.04 a	D 0.08 a	D 0.04 a	F 0.07 a
	1 min	A 0.00 a	C 0.00 a	D 0.00 a	D 0.12 a	F 0.17 a
	5 min	A 0.07 c	C 0.05 c	D 0.31 c	C 0.87 b	E 1.39 a
	10 min	A 0.19 d	C 0.03 d	C 1.00 c	B 2.38 b	D 3.16 a
	20 min	A 0.17 c	C 0.58 c	B 1.61 b	B 2.66a	CD 3.46 a
	30 min	A 0.02 e	B 1.30 d	A 2.24 c	A 3.46 b	BC 3.95 a
	1 h	A 0.16 e	B 1.44 d	A 2.31 c	A 3.28 b	AB 4.25 a
	2 h	A 0.19 e	A 2.07 d	A 2.57 c	A 3.57 b	A 4.88 a
	10 sec	A 0.02 a	B 0.06 a	C 0.11 a	D 0.24 a	D 0.15 a
	1 min	A 0.03 a	B 0.06 a	C 0.13 a	D 0.25 a	D 0.08 a
<i>Pseudomonas aeruginosa</i>	5 min	A 0.05 c	B 0.18 c	BC 0.31 c	C 0.99 b	C 2.16 a
	10 min	A 0.24 b	B 0.12 b	ABC 1.50 a	B 2.20 a	C 2.36 a
	20 min	A 0.06 d	A 1.34 c	ABC 1.81 bc	A 2.96 ab	B 3.54 a
	30 min	A 0.17 d	A 1.31 c	AB 2.09 bc	A 2.98 ab	AB 3.85 a
	1 h	A 0.22 c	A 1.52 b	ABC 1.80 b	A 3.03 a	AB 3.81 a
	2 h	A 0.41 d	A 1.39 cd	A 2.36 bc	A 3.01 b	A 4.48 a
	10 sec	B 0.00 a	AB 0.10 a	C 0.00 a	E 0.00 a	D 0.12 a
	1 min	B 0.00 a	B 0.00 a	C 0.00 a	E 0.00 a	D 0.23 a
	5 min	AB 0.24 b	AB 0.43 b	C 0.27 b	DE 0.60 b	CD 1.16 a
	10 min	AB 0.21 b	AB 0.66 ab	BC 0.57 ab	CD 1.55 ab	BC 2.03 a
<i>Salmonella Typhimurium</i>	20 min	B 0.09 c	A 1.57 bc	A 1.93 ab	AB 2.86 ab	A 3.32 a
	30 min	AB 0.25 c	AB 1.52 bc	A 1.80 ab	A 3.21 a	AB 3.13 a
	1 h	AB 0.29 c	AB 1.41 bc	AB 1.47 bc	A 3.11 a	AB 2.43 ab
	2 h	A 0.42 c	AB 1.02 bc	AB 1.58 ab	BC 1.86 a	ABC 2.28 a

Table C1. Continued.

Pathogen	Treatment time	Reduction (log CFU/g)				
		1ppm ^f	3ppm	5ppm	10ppm	15ppm
<i>Staphylococcus aureus</i>	10 sec	B ^g 0.03 a ^h	C 0.10 a	C 0.27 a	D 0.19 a	D 0.21 a
	1 min	AB 0.15 c	C 0.18 c	C 0.50 bc	CD 1.01 a	D 0.98 ab
	5 min	AB 0.06 c	BC 0.39 bc	BC 1.06 ab	BC 1.85 a	CD 1.47 a
	10 min	B 0.01 b	AB 1.53 ab	AB 2.55 a	B 2.20 ab	BC 2.71 a
	20 min	B 0.02 d	AB 1.66 cd	AB 2.36 bc	A 3.46 ab	AB 4.24 a
	30 min	AB 0.09 d	A 1.92 c	AB 2.70 bc	A 3.92 ab	A 4.56 a
	1 h	A 0.26 d	A 1.73 c	A 3.07 b	A 3.82 ab	AB 4.37 a
	2 h	AB 0.19 d	A 2.06 c	A 3.11 b	A 3.87 ab	AB 4.33 a
	<i>Yersinia enterocolitica</i>	10 sec	A 0.33 a	C 0.09 b	F 0.10 b	CD 0.40 a
1 min		A 0.12 a	C 0.11 a	F 0.06 a	D 0.15 a	D 0.18 a
5 min		A 0.16 b	C 0.11 b	F 0.21 b	D 0.22 b	BC 0.86 a
10 min		A 0.27 b	C 0.51 b	E 0.82 ab	C 0.86 ab	B 1.35 a
20 min		A 0.17 c	C 0.64 bc	D 1.38 b	B 2.38 a	A 3.25 a
30 min		A 0.19 c	C 0.62 c	C 1.97 b	A 3.17 a	A 3.33 a
1 h		A 0.30 d	B 1.75 c	B 2.91 b	A 3.63 a	A 3.69 a
2 h		A 0.12 c	A 2.88 b	A 3.49 ab	A 3.70 a	A 3.54 a
Yeasts and Molds		10 sec	A 0.26 a	A 0.27 a	C 0.20 a	D 0.41 a
	1 min	A 0.25 b	A 0.25 b	BC 0.67 b	D 0.54 b	C 1.82 a
	5 min	A 0.33 b	A 0.46 b	AB 0.85 b	CD 0.87 b	C 2.07 a
	10 min	A 0.26 d	A 0.52 cd	AB 0.85 bc	BCD 1.13 b	C 1.79 a
	20 min	A 0.16 c	A 0.57 c	AB 1.14 b	ABC 1.70 a	C 1.89 a
	30 min	A 0.34 b	A 0.43 b	AB 1.20 ab	ABC 1.92 a	BC 2.21 a
	1 h	A 0.40 d	A 0.52 cd	AB 1.17 c	AB 1.95 b	AB 2.82 a
	2 h	A 0.29 c	A 0.81 c	A 1.43 b	A 2.42 a	A 2.86 a

^f Concentrations of ClO₂.

^g Within the same microorganism, mean values in the same column with different capital letters (A through F) are significantly different among various treatment times for each ClO₂ concentration.

^h Within the same microorganism, mean values in the same row with different lowercase letters (a through e) are significantly different among various concentrations for each treatment time at a 95% confidence level.

APPENDIX D
REAL TIME DETECTION OF *Escherichia coli* O157:H7 DURING
ENRICHMENT BY A QUARTZ CRYSTAL MICROBALANCE
IMMUNOSENSOR

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Introduction

Escherichia coli O157:H7 is one of the most dangerous foodborne pathogens. Outbreaks of *E. coli* O157:H7 have been considered as serious social problems. Recently, two multistate outbreaks of *E. coli* infections occurred in the United States causing serious health problem such as hemolytic-uremic syndrome (CDC 2006). Therefore, rapid detection of *E. coli* O157:H7 in food is very important for public health protection preventing wide distribution of contaminated foods. Many methods have been investigated for rapid and sensitive detection of *E. coli* O157:H7.

Quartz crystal microbalance (QCM) immunosensors have been reported for rapid and sensitive detection of *E. coli* O157:H7 (Kim and Park 2003; Su and Li 2004; Spangler and Tyler 1999; Su and Li 2005). Since QCM immunosensor is directly detecting target bacteria without the need of labeled antibodies, it is much simpler and easier in structure and operation than sandwich-type immunosensors (Fung and Wong

2001; Su and Li 2005). However, the QCM immunosensor has not yet been successfully adapted for commercial use due to vulnerable antibodies which specifically react with target bacteria. In order to detect target bacteria in a sample, it does not have to contain inhibiting materials against antibodies which are immobilized on the QCM chip surface. However, there are a lot of unknown inhibiting factors in real food samples. This limits direct use of QCM immunosensor for foods. Immunomagnetic separation technique has been used to separate and concentrate *E. coli* from enrichment broth (Chapman and Ashton 2003; Varela-Hernandez et al. 2007; Carney et al. 2006). Antibodies, which are specific to *E. coli*, are coated on magnetic particles, and these coated antibodies react with *E. coli* in enrichment media. Therefore, the antibodies against *E. coli* are quite stable in enrichment media and can maintain their activities. Real time monitoring of cell growth using a mass detecting device has been investigated. Continuous monitoring of endothelial cell growth on the QCM chip surface has been investigated (Zhou et al. 2000). Mass detecting devices such as piezoelectric cantilever and micromechanical oscillator have been used to detect the growth of *E. coli* (Detzel et al. 2006; Gfeller et al. 2005). However, the technique they used is not specific to a particular pathogen. Although their suggestion is to use selective medium for the detection of target bacteria (Detzel et al. 2006), its specific detection for target bacteria would be limited because injured target cells may not grow on selective media due to limited nutrients which cause false negative. Therefore, use of antibodies can provide better specificity than selective media for detection of target bacteria. In this paper, we propose that selective detection of *E. coli* O157:H7 during enrichment in real time is possible using QCM immunosensor coupled with flow cell so that more volume of medium can be run.

Materials and Methods

Reagents and Instruments

Affinity purified antibodies to *E. coli* O157:H7 were purchased from Biodesign International (Saco, ME). Polyethyleneimine (PEI), 50% w/v in water, glutaraldehyde (GA), 25% in water and phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals used were of analytical grade or better quality. QCM200 quartz crystal microbalance system was obtained from Stanford research systems Inc. (Sunnyvale, CA). This system includes a controller, crystal oscillator electronics, crystal holder, flow cell, and quartz crystal.

Antibody Immobilization

Anti-*E. coli* O157:H7 antibodies were immobilized on the gold surface of AT-cut quartz crystals (25.4 mm diameter) which had a polished titanium/gold electrode and 5 MHz of frequency. The crystals were pretreated with Piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide) for 30 sec to obtain clean electrode surface. After treatment, the crystals were rinsed with distilled water and dried in a stream of nitrogen gas. Then, 10 μ L of a methanol solution containing 2% PEI was dispersed onto the surface of the electrode and air dried. The crystal was then immersed in a 5% GA solution for 1 h, rinsed with distilled water and air dried. Twenty μ L of anti- *E. coli* O157:H7 solution (1mg/ml) in PBS was applied and spread over the entire gold electrode. Then, the crystal was incubated at 4 °C for 24 h under buffer (PBS) humidity. After 24 h, the crystal was rinsed with PBS to remove excess antibodies, rinsed again with distilled water and stored at 4 °C for later use.

Microbial Tests

Generic *E. coli* (ATCC 25922) and *E. coli* O157:H7 (ATCC 35150) were both obtained from the Food Microbiology Laboratory in the Department of Food Science and Human Nutrition at University of Maine (Orono, ME). Generic *E. coli* and *E. coli* O157:H7 were cultured in brain heart infusion (BHI; Difco, Sparks, MD) broth at 37 °C for 20 h before use, and the number of bacteria was determined by the conventional surface plating count method. Cell solutions were serially diluted in sterile 0.1% peptone water, and spread-plated in duplicate on tryptic soy agar (TSA; Neogen, Lansing, MI). Cultures were kept under refrigeration (4 °C) as stock cultures and transferred weekly to maintain viability.

Measurements of Frequency and Resistance

The QCM immunosensor was tested in flow mode for the detection of *E. coli* O157:H7 using a flow cell. The antibody coated crystal was mounted inside a flow cell and BHI broth was pumped through with flow rate of 0.015 ml/min at room temperature. After stabilization of the signals (ca. 10 min), 100 µL of cell dilution (5 log CFU/ml) was inoculated into the BHI broth (30 ml) and pumped into the flow cell. Frequency and resistance were recorded every 10 s for 24 h incubation at room temperature.

Fluorescence Microscope Observation

After 24 h incubation, the fluorescence images of the QCM chips were taken on Olympus BX60 microscope (Olympus; Japan). Before observation using a fluorescence microscope, SYBR-18 dye was used to stain bacteria's double strand DNA for better

observation. The dye was diluted 1,000 times with distilled water before use. The diluted dye solution was dropped on the QCM chips followed by a 15 min incubation period. Then, the chips were washed with sterile distilled water and dried with air flow gently.

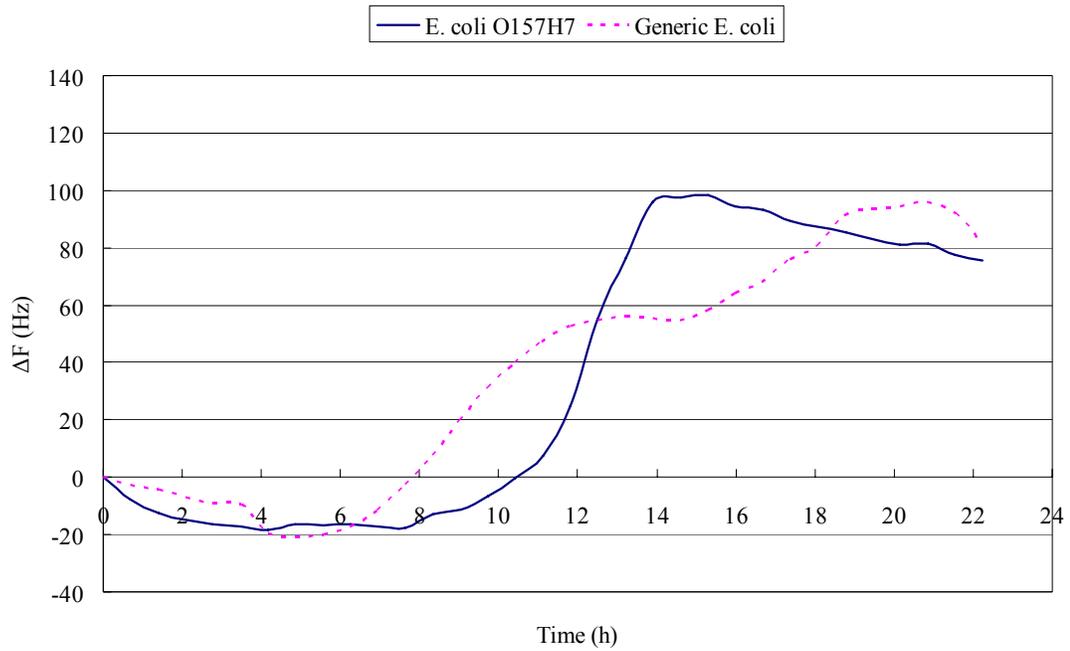
Results and Discussion

The QCM immunosensor was tested in a flow system for direct detection of *E. coli* O157:H7 in BHI media during enrichment. Responses of frequency (ΔF) and resistance ($\Delta\Omega$) to *E. coli* O157:H7 and generic *E. coli* are shown in Figure D1. Initial cell concentration for both *E. coli* O157:H7 and generic *E. coli* was 3 log CFU/ml. For frequency response, initially the frequency of both *E. coli* O157:H7 and generic *E. coli* decreased. Since BHI medium contains many proteins for growth of microorganisms, they can bind onto chip surface through glutaraldehyde which can react with any kind of proteins. Therefore, the initial frequency decrease can be attributed to nonspecific binding of proteins in BHI onto the chip surface by glutaraldehyde which was originally used for antibody immobilization. Around 10 h of circulation, frequency response of *E. coli* O157:H7 dramatically increased and then decreased slightly. However, frequency response of generic *E. coli* slightly increased after 10 h circulation. At this moment, we are not sure this moderate increase of frequency by generic *E. coli*. We will need more experiments to make sure this result. The resistance response of *E. coli* O157:H7 is very similar with frequency response of *E. coli* O157:H7. But, it is more stable compared to the frequency response in the plain period. The time to begin increase of both frequency and resistance is same. That means the dramatic increases of both frequency and resistance can indicate sudden change on QCM surface. We assume this sudden change is

associated with cell accumulation on the QCM chip surface by interaction with immobilized antibodies because generic *E. coli* did not provide this dramatic increase of signal. Generic *E. coli* does not bind with the antibodies immobilized on the chip surface (Figure D2). Fluorescence images of QCM chip surfaces were taken before and after circulation of cell suspensions for both *E. coli* O157:H7 and generic *E. coli* (Figure D2). Before circulation of cell suspension, no cells were observed on both chips. After circulation of both cell suspensions, we observed some cells on both chip surfaces. However, we found more cells of *E. coli* O157:H7 observed compared to generic *E. coli*. This means antibodies immobilized on the chip surfaces react with *E. coli* O157:H7 better than generic *E. coli*. Indeed, the binding of few generic *E. coli* cells on the chip surface is not enough to generate dramatic increase of resistance. Therefore, our QCM immunosensor system can detect *E. coli* O157:H7 in BHI medium within 14 h when initial cell concentration is 3 log CFU/ml. Theoretically, it is possible to detect 1 cell with our system because of enrichment of the cell. However, it will need more time to detect.

In this study, we found several things. First, we proved that the antibodies we used for capturing *E. coli* O157:H7 worked well in BHI medium at room temperature. Second, we can shorten entire detection time to confirm presence of target bacteria in our sample because presumptive test and enrichment step can be conducted simultaneously. Third, it is possible to apply our system for solid food sample because we can recover cells with BHI medium from the food sample by pulsifier without having sample matrices in the medium. Then, we can circulate it into the system for real time monitoring. Our next experiments will include specificity test for other pathogens such as *S. Typhimurium* and *L. monocytogenes*, real food sample test, and optimal operation test.

(A)



(B)

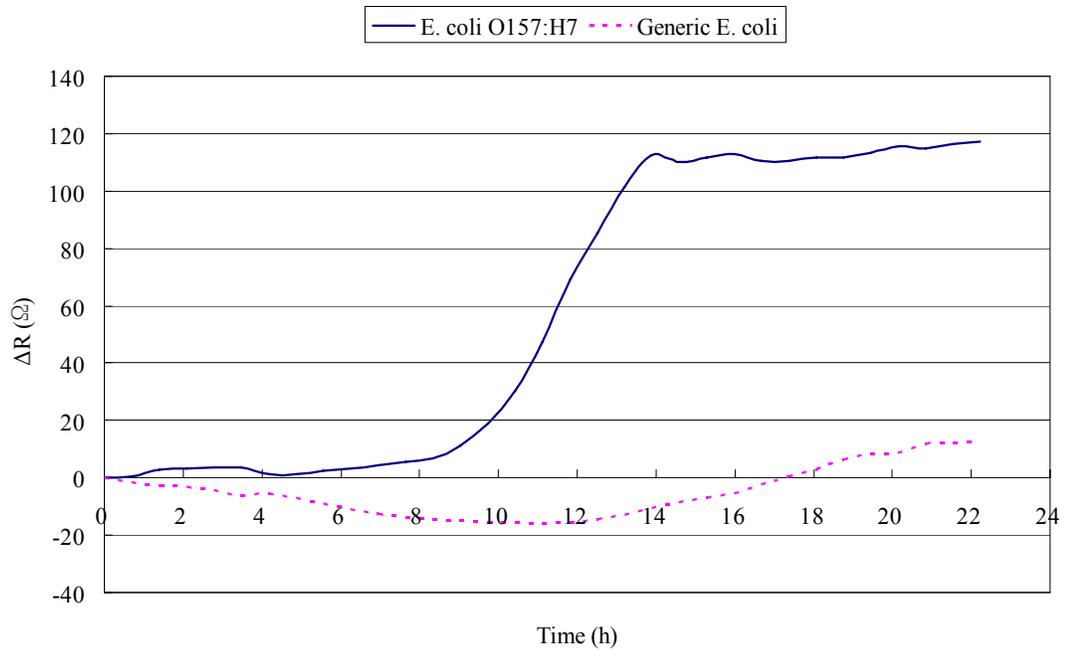
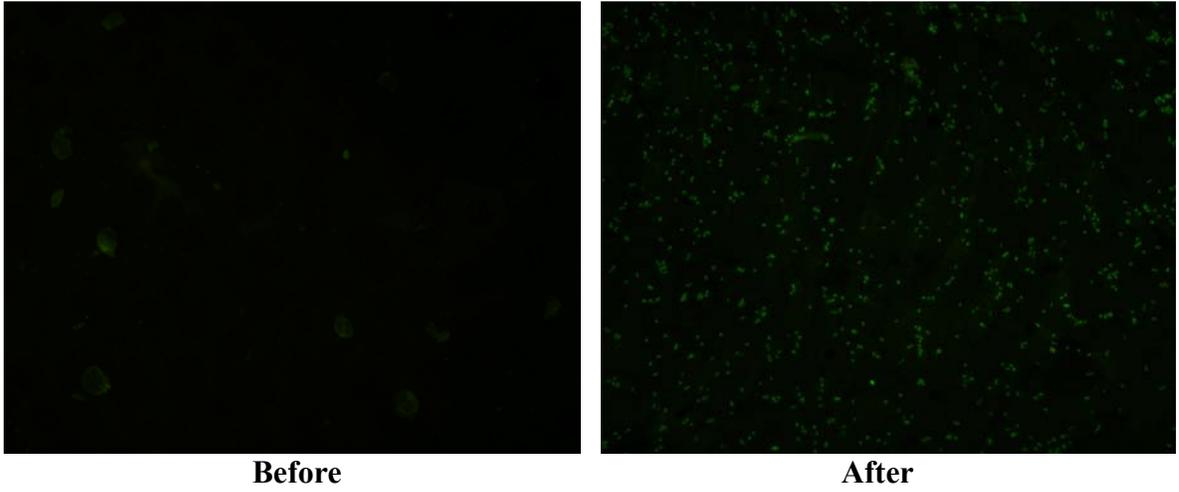


Figure D1. Responses of frequency (A) and resistance (B) to *E. coli* O157:H7 and generic *E. coli* during enrichment in BHI by circulation.

(A)



(B)

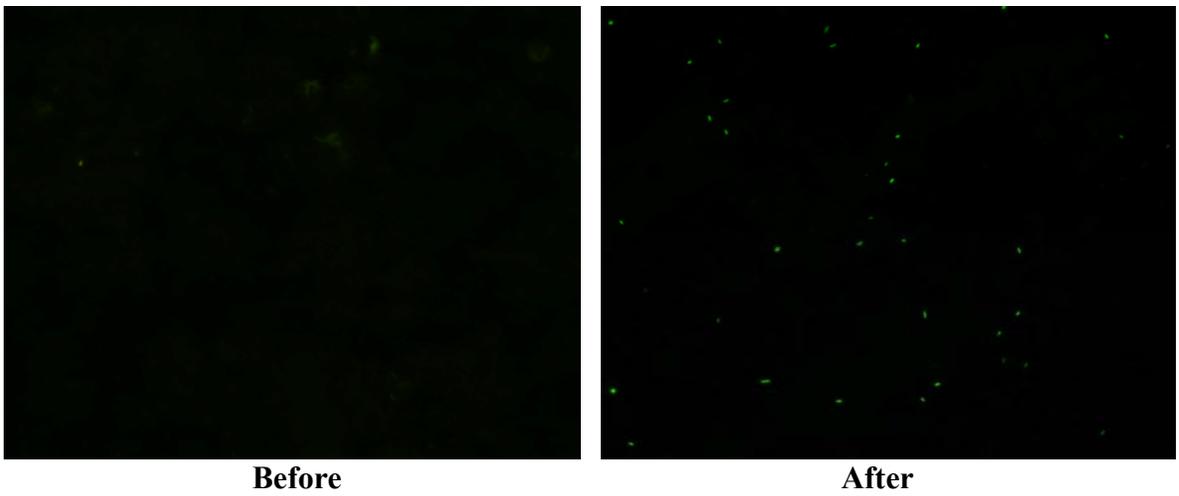


Figure D2. Fluorescence image of QCM surfaces with bound *E. coli* O157:H7 (A) and generic *E. coli* (B) before and after circulation of cell suspension.

BIOGRAPHY OF THE AUTHOR

Byungchul Kim was born in Busan, South Korea on December 3, 1973. He was raised in Busan and graduated from Pukyong National University with a Bachelor's degree in Food Engineering in 1998. He continued his education at the same university for a Master's degree in Food Science in 2000. He decided to study abroad and started at the University of Arkansas where he received a second Master's degree in Food Science in 2004. In January 2005, Byungchul began his Ph.D. study in the Department of Food Science and Human Nutrition at the University of Maine.

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