LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-JRNL-408913

Quantitative Analysis of Tetramethylenedisulfotetramine ("Tetramine") Spiked into Beverages by Liquid Chromatography Tandem Mass Spectrometry with Validation by Gas Chromatography Mass Spectrometry

J. Owens, S. Hok, A. Alcaraz, C. Koester

November 20, 2008

Journal of Agricultural and Food Chemistry

Disclaimer

This document was prepared as an account of work sponsored by an agency of the United States government. Neither the United States government nor Lawrence Livermore National Security, LLC, nor any of their employees makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States government or Lawrence Livermore National Security, LLC. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States government or Lawrence Livermore National Security, LLC, and shall not be used for advertising or product endorsement purposes.

Quantitative Analysis of Tetramethylenedisulfotetramine ("Tetramine") Spiked into Beverages by Liquid Chromatography Tandem Mass Spectrometry with Validation by Gas Chromatography Mass Spectrometry

JANEL OWENS*, SAPHON HOK, ARMANDO ALCARAZ, AND CAROLYN KOESTER

Forensic Science Center, Lawrence Livermore National Laboratory, 7000 East Ave, L-091

Livermore, CA 94550

TITLE RUNNING HEAD: Tetramine by LC/MS/MS and GC/MS

*Corresponding author:

Phone: (925) 422-8914

Fax: (925) 423-9014

E-mail: owens33@llnl.gov

Disclaimer

This document was prepared as an account of work sponsored by an agency of the United States government. Neither the United States government nor Lawrence Livermore National Security, LLC, nor any of their employees makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States government or Lawrence Livermore National Security, LLC. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States government or Lawrence Livermore National Security, LLC, and shall not be used for advertising or product endorsement purposes.

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

ABSTRACT

Tetramethylenedisulfotetramine, commonly known as tetramine, is a highly neurotoxic rodenticide (human oral LD_{50} = 0.1 mg/kg) used in hundreds of deliberate food poisoning events in China. Here we describe a method for quantitation of tetramine spiked into beverages, including milk, juice, tea, cola, and water and cleaned up by C8 solid phase extraction and liquid-liquid extraction. Quantitation by high performance liquid chromatography tandem mass spectrometry (LC/MS/MS) was based upon fragmentation of *m/z* 347 to *m/z* 268. The method was validated by gas chromatography mass spectrometry (GC/MS) operated in SIM mode for ions *m/z* 212, 240, and 360. The limit of quantitation was 0.10 μg/mL by LC/MS/MS versus 0.15 μg/mL for GC/MS. Fortifications of the beverages at 2.5 μg/mL and 0.25 μg/mL were recovered ranging from 73 – 128% by liquid-liquid extraction for GC/MS analysis, 13 – 96% by SPE and 10 – 101% by liquid-liquid extraction for LC/MS/MS analysis.

Keywords: Tetramethylenedisulfotetramine, tetramine, liquid chromatography, gas chromatography, mass spectrometry

INTRODUCTION

Tetramethylenedisulfotetramine (**Figure 1A**), a heteroadamantane more commonly known as tetramine, is a lethal neurotoxic rodenticide that was once used worldwide. It is an odorless, tasteless, white, crystalline powder that dissolves easily in water (*1*). The production, use, and sale of tetramine have been banned since the 1970s, though it is still widely used and sold in China (*2*) under such names as 'Dushuqiang,' 'Meishuming,' and 'Shanbudao' (*1*). Nearly 50% of the 116 rodenticides surveyed from free markets in China contained both tetramine and fluoroacetamide [also known as 1081 (*3*)], another highly toxic rodenticide, indicating the wide availability of tetramine.

It has been estimated that there have been thousands of tetramine poisonings through food in China, resulting in hundreds of human deaths from 1977 – 2002 (*2, 4*). A 7 – 10 mg primary dose of tetramine is considered lethal for humans (*1*), though secondary and even tertiary poisonings are possible. Newspapers in China have warned against consuming suspect dog meat being sold in local markets for fear that the dogs had eaten rats poisoned by tetramine (*4*). Tetramine acts by binding non-competitively and irreversibly to the chloride channel on the γ-aminobutyric acid type A receptor complex of the neuronal cell membrane (*5*). The clinical manifestations of tetramine poisoning are dose-dependent and no known antidote exists for poisoning (*1*). Treatment is mainly supportive and may include gastric lavage and use of activated charcoal (*6*), hemoperfusion, and hemofiltration (*7*).

There has been only one reported case of tetramine poisoning within the United States. In May 2002 in New York City, a 15-month old female was exposed by accidental ingestion of tetramine that was used as an indoor rodenticide. Tetramine is not registered by the U.S.

Environmental Protection Agency for use in the U.S.; and its importation, manufacture, and use in the United States are illegal (*8*).

Given the extremely high toxicity of tetramine and the long history of deliberate food poisoning events in China with this toxic rodenticide, the development of quantitative methods for clean-up of tetramine from food matrices with instrumental analysis is of high priority. Tetramine analysis in samples of tissue, blood, urine, and rice has been previously investigated using gas chromatography coupled with various detectors (*9-13*). More recently, tetramine extraction from foods by direct immersion and headspace solid phase micro-extraction was optimized for a variety of food matrices, including juice, peanut butter, and potato chips (*14*).

To date, no methods for tetramine analysis by high performance liquid chromatography have been identified in the literature. Only a few methods of analysis by liquid chromatography-mass spectrometry (LC/MS or LC/MS/MS) of chemicals with the adamantane structure have even been reported (*15-17*). The objective of this work was to develop methods for quantitative extraction of tetramine from various beverage samples utilizing both solid-phase extraction (SPE) and liquid-liquid extraction protocols with analysis by LC/MS/MS and validation by gas chromatography (GC/MS). The target method detection limits (MDL) for these methods was 0.3 μg/mL. The MDL was calculated based upon the oral LD_{50} value of 0.1 mg/kg for humans multiplied by 70 kg, an adult weight, and normalized by both beverage portion size (236 mL) and a sensitivity factor to account for exposure by sensitive populations.

MATERIALS AND METHODS

Chemicals and Reagents

Tetramethylenedisulfotetramine was obtained from two different sources: as a gift from the U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition (CFSAN; College Park, MD, with > 98% purity and hereafter referred to as CFSAN tetramine) and synthesized at LLNL (73% purity and hereafter referred to as LLNL tetramine). Tetramine was synthesized at LLNL from 1,3,5-trioxane (Sigma-Aldrich, St. Louis, MO, ≥ 99% purity) and sulfamide (Sigma-Aldrich, St. Louis, MO, ≥ 99% purity) using the protocol described by Esser et al. (*18*). In-house synthesis of tetramine was required to obtain a standard with sufficient amount of the tetramine dimer (**Figure 1B**), the only known tetramine byproduct to form under mild conditions with acids (*19*).

Formic acid, sodium chloride, and anhydrous sodium sulfate were from Sigma-Aldrich (St. Louis, MO). HPLC-grade or GC-grade solvents, including acetone, acetonitrile, methanol, ethyl acetate, and dichloromethane, were from Fisher Scientific (Fair Lawn, NJ) as were all other chemicals unless specified otherwise.

Beverages, including whole milk, bottled iced tea, bottled water, orange juice, juice drink (containing less than 15% juice), and cola were purchased from a local store.

Preparation of Standards

Assessment of Standard Purity

The two sources of tetramine were analyzed on an Agilent Technologies (Santa Clara, CA) 6890 Gas Chromatograph coupled with 5973 Mass Selective Detector (GC/MS) controlled by ChemStation software. Stock solutions of the CFSAN tetramine and LLNL tetramine were prepared by weighing out approximately two mg of crystalline tetramine and dissolving in acetone. Appropriate dilutions of all these two stock solutions were made to prepare a 20 μg/mL solution. One μL of this 20 μg/mL solution was injected into a 250 °C injector port in splitless

mode (0.75 min purge delay) and chromatographed on a DB-5 column (30 m x 0.25 mm i.d. x 0.25 μm; J & W Scientific, Folsom, CA) using a general temperature program. Helium was used as a carrier gas at a flow rate of 0.8 mL/min at a constant linear velocity of 32 cm/s. The initial temperature was 40 °C (hold 1 min) and ramped at 8 °C/min to 300 °C (hold for 5 min), for a total run time of 38.5 min. The MS scan range was *m/z* 50 to 550. The source was maintained at 230 °C, the quadrupoles at 150 °C, and the transfer line heater at 280 °C.

Using this general temperature program, the tetramine eluted at 22.24 minutes and the higher molecular weight derivative of tetramine (*20*) [4,10,13-trithia-1,3,5,7,9,11-hexaazatetracyclo(5.5.1.$1^{3,11}$.$1^{5,9}$)pentadecane 4,10,13-tris(dioxide)] (**Figure 1B**), hereafter referred to as the 'tetramine dimer,' eluted at 35.27 min. The LLNL tetramine source contained the tetramine dimer at 27% and the CFSAN tetramine contained < 1% dimer. The ions and abundances for the three sources were as follows: CFSAN tetramine (70 eV, ten ions above *m/z* 30): 30 (9), 42 (100), 76 (9), 92 (24), 94 (7), 121 (20), 132 (21), 149 (5), 212 (76), and 240 (40); for LLNL tetramine (70 eV, ten ions above *m/z* 30): 30 (7), 42 (100), 76 (9), 92 (26), 94 (7), 121 (19), 132 (21), 149 (5), 212 (76), and 240 (42); and finally, for the LLNL tetramine dimer (70 eV, twelve ions above *m/z* 30): 30 (14), 42 (100), 44 (27), 92 (31), 121 (27), 132 (19), 148 (50), 176 (11), 212 (30), 268 (6), 360 (50), and 362 (9).

Preparation of Calibration Standards

CFSAN tetramine (2.37 mg; > 98% purity) was dissolved in 25 mL acetone to prepare *stock solution I* with a concentration of 94.8 μg/mL. A high level calibration standard (10 μg/mL) was prepared in ethyl acetate from *stock solution I.* This high calibration standard was serially diluted with ethyl acetate to prepare eight additional standards such that the nine calibration standards ranged from 0.01 µg/mL to 10 µg/mL for both LC/MS/MS and GC/MS analysis.

Preparation of Standards for Stability Study

Standards of concentration 1 μg/mL were prepared in water (pH 7) from *stock solution I*. Sets of amber glass autosampler vials containing one-mL aliquots were stored at 0 °C, 4 °C, and 23 °C and analyzed in triplicate over a 35 day (d) period by LC/MS/MS. A second standard with concentration of 5 μg/mL was prepared in acetone and stored at room temperature over a 21 day period and analyzed by GC/MS.

Sample Preparation and Clean-Up

Liquid-Liquid Extraction

Samples were prepared by allowing the beverage sample (2 mL in borosilicate glass) to sit at room temperature for 10 min before addition of 2 mL ethyl acetate (with the exception of milk samples, to which 4 mL ethyl acetate was added). The samples were capped and mixed vigorously for 1 minute. Sodium chloride (1 g) was then added and the samples were again vigorously mixed before being centrifuged at 3000 x *g* for 10 minutes (Fisher AccuSpin 400 centrifuge, Fisher Scientific, Fair Lawn, NJ). An aliquot (~1 mL) of sample was removed by a disposable glass pipette and dried over 150 mg anhydrous sodium sulfate in amber glass vials. Samples were stored at 0 °C until analysis prior to analysis by GC/MS. A second small aliquot (100 μL) was transferred to a poly-spring insert for analysis by LC/MS/MS.

Solid Phase Extraction

C8 Clean-Extract SPE columns (200 mg/4 mL; Alltech, Deerfield, IL) were conditioned with 5 mL methanol followed by 5 mL water. The beverage sample (1 mL) was loaded and allowed to drip through by gravity. Tetramine was eluted with 1 mL GC-grade ethyl acetate into an amber autosampler vial. Samples were stored at 0 °C until analysis by LC/MS/MS.

Instrumental Conditions

GC/MS

Tetramine was analyzed by an Agilent GC/MS controlled by ChemStation software. Briefly, one μL of standard or sample was injected into a 250 °C injector port in splitless mode (0.75 min purge delay) and chromatographed on a DB-5 column (30 m x 0.25 mm i.d. x 0.25 μm; J & W Scientific, Folsom, CA) using a general temperature program. Helium was used as a carrier gas at 0.8 mL/min with a constant linear velocity of 32 cm/s. The initial temperature was 40 °C (hold 3 min) and ramped at 8 °C/min to 300 °C (hold for 3 min) for a total run time of 38.5 min. Tetramine was detected using selected-ion monitoring (SIM) method for ions *m/z* 212, 240, and 360 (data rate at 20 Hz) after a 3 min solvent delay. The source was maintained at 230 °C, the quadrupoles at 150 °C, and the transfer line heater at 280 °C. Tetramine eluted at 22.03 min and the tetramine dimer eluted at 35.16 min (**Figure 2**).

Quantitation was by linear regression with no weighting from 0.0 μg/mL (blank) to 10 μg/mL. A standard curve was prepared at the beginning and of each sequence run and a mid-point calibration standard of 2.5 μg/mL was included throughout the sequence list after every six matrix samples. The responses of these 'through-run' standards were also included in the standard curve preparation. Quantitation was based upon the *m/z* 212 ion with the *m/z* 240 ion serving as a confirmation ion and *m/z* 360 ion serving as a confirmation ion for the tetramine dimer.

Evaluation of Hypothesized Electrospray Ionization Mechanism

To our knowledge, no report of tetramine analysis by LC/MS or LC/MS/MS has been reported in the literature. A Waters Micromass Quattro *micro* API triple quadrupole mass spectrometer (Waters Corporation, Milford, MA) was tuned by infusing a 94.8 μg/mL solution of CFSAN tetramine prepared in 50/50 water/acetonitrile (v/v) + 0.1% formic acid at 20 μL/min by external

Harvard Syringe pump, model 22 (Harvard Apparatus, Holliston, MA). Ionization in negative ion-mode electrospray ionization (ESI) resulted in better response. The hypothesized ionization mechanism is shown in **Figure 3**. Briefly, in the presence of heat and acid, tetramine readily rearranges to form the tetramine dimer (**Figure 1B**) given that there is little stability because of the N-C-N-C-N sequences (*20*). Cursory Hückel charge calculations (Chem3D Pro, v. 11, Cambridge Software, Cambridge, MA) indicated that two of the six nitrogen atoms carry a negative charge (-0.414 and -0.434). It was hypothesized that one of these nitrogen atoms became protonated in the presence of heat and acid before loss of a methylene bridge to form *m/z* 347 ($[M + H - CH_2]^-$). Product ions of the *m/z* 347 parent ion were: *m/z* 268, *m/z* 227, *m/*z 175 m*/z* 148, and *m/z* 134. Previous work by Kang et al. (*20*) reported the fragment ions (abundances, %) *m/z* 268 (5%) and *m/z* 148 (29%) for the tetramine dimer when analyzed by electron-impact GC/MS. These ions and abundances were confirmed by GC/MS analysis as just described for assessment of standard purity.

To evaluate this hypothesized ionization mechanism, a standard of CFSAN tetramine (> 98% purity) at 94.8 μg/mL in acetone + 0.1% formic acid was mixed with pinacolyl methylphosphonic acid (*m/*z 179.08359, $[M - H]^-$) and bromadiolone (*m/z* 525.07003, $[M-H]^-$), each at 100 μg/mL, and infused at 10 μL/min and ionized by ESI in negative ion mode with analysis by an Orbitrap FT mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The Orbitrap MS was operated in the FTMS mode at a resolution of 30,000 at FWHM for a scan range of *m/z* 50 to 500. The 'lock mass' function was enabled and the ions for pinacolyl methylphosphonic acid and bromadiolone were monitored. Settings were as follows: capillary voltage at 3.5 kV, sheath gas at 15, auxiliary gas at 10, sweep gas at 10, capillary temperature at

270 °C, capillary voltage at -45 V, and tube lens at -76 V. Both full scan and product ion spectra (collision energy at 35%) were collected.

LC/MS/MS

The Waters Quattro *micro* API triple quadrupole mass spectrometer described above was optimized for the tetramine ionization and analysis from beverage samples. The instrument was first calibrated using a NaI/CsI solution per manufacturer's specifications. After tuning the instrument with a 94.8 μg/mL solution of CFSAN tetramine in acetone + 0.1% formic acid, optimized settings were as follows: negative ion mode ESI with capillary voltage at 3.0 kV, cone voltage at 35 V for *m/z* 347 ion and 60 V for *m/z* 268 ion, extractor voltage at 2 V, RF lens at 0.2V, source temperature at 120 °C, desolvation temperature at 300 °C, desolvation gas at 300 L/h, and cone gas at 25 L/h. The low mass and high mass resolution 1 settings were both set at 14.5, ion energy 1 at 1.0, entrance energy at -1 eV, collision energy setting at 20 eV for *m/z* 347 → *m/z* 268 and 30 eV for *m/z* 347 → *m/z* 148, and exit energy at 2 eV. The low mass and high mass resolution 2 settings were both at 15.0 and ion energy 2 was set at 1.5. The multiplier was set at 650. The inter-channel delay was set to 0.02 s, the inter-scan delay to 0.1 s, repeats at 1, and the span at 0 Da. Dwell time was at 0.1 s for all transitions.

A Waters 2795 LC system consisting of a quaternary pump, in-line mobile phase degasser, temperature-controlled autosampler (maintained at 4 °C) and column compartments was utilized for chromatography. Mobile phase A consisted of water + 0.1% formic acid and mobile phase B consisted of acetonitrile + 0.1% formic acid. Twenty μL were injected onto a 150 mm x 2.1 mm i.d, 5 μm, Symmetry300 C4 analytical column (Waters Corp., Milford, MA). The column was maintained at 30 °C throughout the chromatographic run with a 2 min column equilibration time between samples. The gradient mobile phase conditions were as follows: 95% A at 0 min (hold

for 1 min) to 5% A within 2 min (hold for 5 min), return to 95% A in 2 min (hold for 4 min) for a total run time of 14 min with a 0.2 mL/min flow rate maintained throughout. Tetramine eluted at 5.54 min.

Quantitation was by linear regression with 1/x weighting from 0.25 μg/mL to 10 μg/mL with n $\geq$ 2 measurements per standard. A standard curve was prepared at the beginning and end of each sequence run and individual standards were included throughout the sequence list after every six matrix samples. The responses of these 'through-run' standards were also included in the standard curve preparation. Tetramine was analyzed using multiple reaction monitoring (MRM) and the following transitions were monitored: *m/z* 347 → *m/z* 268 (quantitation ion) and *m/z* 347 → *m/z* 148 (confirmation ion) (**Figure 4**).

Analyte Stability

In a pilot study, a 5 μg/mL standard of tetramine was prepared in acetone and stored at 23 °C. Over a period of 21 days, the standard was analyzed by GC/MS. This was done to assess stability of the standard during the following: i) optimization experiments for clean-up protocols; ii) method verification experiments, and iii) storage in the autosampler while awaiting analysis.

The stability of tetramine at various temperatures and in matrix was then also assessed. Standards of tetramine (1 μg/mL) were prepared in water (pH 7) and stored at 4 °C in amber-glass vials and analyzed by LC/MS/MS in triplicate just after standard preparation (t = 0 d), at 1 day (t = 1 d), at one week (t = 7 d) and at 5 weeks (t = 35 d).

Tetramine was also spiked in triplicate into 2 mL aliquots of the six beverages at a concentration of 2.5 μg/mL and stored for 14 days at 4 °C to determine the effect of matrix on stability. The samples were extracted using ethyl acetate (2 mL of solvent was added to all

beverages with the exception of milk, to which 4 mL of ethyl acetate were added) for analysis by GC/MS.

Method Verification

Two sets of beverage samples were used in the method verification procedures. Aliquots (2 mL) of each beverage were spiked at two levels to obtain both 2.5 μg/mL and 0.25 μg/mL concentrations (n = 3 per spike level per beverage sample) and were also left un-fortified (n = 3) to include control samples. The first set of beverage samples was extracted with 2 mL ethyl acetate and sodium chloride as described above in *Liquid-Liquid Extraction* with analysis by both GC/MS and LC/MS/MS. For an additional LC/MS/MS sample preparation, tetramine spiked beverage samples was extracted using the C8 SPE procedure as described above.

Statistical Analyses

All statistical analyses, including two-tailed Student's *t*-test assuming equal variances and single-factor analysis of variance, were completed using Analysis ToolPak from Microsoft Excel.

RESULTS AND DISCUSSION

Evaluation of Hypothesized Electrospray Ionization Mechanism

The hypothesized ionization by ESI is shown in **Figure 3**. The parent ion, which was the hypothesized tetramine dimer adduct (*m/z* 347, $[M + H - CH_2]^-$), had a theoretical mass of 36.9902 amu with the following isotopic cluster: 346.9902 amu (100%), 348.9860 amu (13.6%), and 347.9936 (5.4%). The actual masses, as shown in **Figure 3**, were 346.9907 amu (100%), 348.9866 amu (12.2%), and 347.9951 amu (3%). The software suggested composition was $C_5H_{11}N_6O_6S_3^-$ (mass accuracy of -0.15 ppm), which matched the hypothesized composition

(**Figure 3**). The theoretically- and experimentally-derived masses of the product ions (*m/z* 268, *m/z* 227, *m/z* 175, m*/z* 148, and *m/z* 134) compared very well (**Figure 3**). The mass accuracy between the hypothesized compositions versus experimentally-derived composition was between -1.23 ppm and -3.72 ppm for the five product ions. This provided convincing evidence to support the hypothesized ESI mechanism to allow for further LC/MS/MS method development.

Method Characteristics

GC/MS

The linear calibration curve (with no weighting) had a linear range of 0.0 μg/mL (blank) to 10 μg/mL, with an R^2 value of 0.9999. The MDL was determined by calculating peak-to-peak signal to noise (S/N) of the confirmation ion (*m/z* 240) for all standards (n = 6) and then graphing these S/N values versus concentration. For the lowest standard, 0.25 μg/mL, the S/N was 46.2. The LOD (S/N = 3) was determined to be 0.05 μg/mL by extrapolation. The limit of quantitation (LOQ; = MDL × 3) was calculated to be 0.15 μg/mL. Finally, using the calibration curve, the concentrations of the standards were calculated to ensure that they were within 10% of their reported amount.

To determine within-run and between-run variability, a mid-point calibration standard at 2.5 μg/mL was analyzed repeatedly (n = 4) and the within-run relative standard deviation (RSD) was 8.2%. This same mid-point calibration standard of 2.5 μg/mL was also analyzed over a 3 day period and the between-run variability was 17.3%. Milk samples were spiked in triplicate at 2.5 μg/mL and analyzed by GC/MS after liquid-liquid extraction. Within-run RSD was 13.2% (mean recovery of 73%). A second set of milk samples spiked in triplicate were extracted after 14 days of storage at 4 °C. The between-run variability for the six samples over the 2 weeks was 13.9% (mean recovery of 68%).

LC/MS/MS

Tetramine analyzed by LC/MS/MS was quantified using a quadratic calibration curve with 1/x weighting from 0.10 μg/mL to 10 μg/mL, though the linear range was from 0.25 μg/mL to 5 μg/mL. The calibration curves had a minimum R^2 value of 0.9900. The MDL was determined by calculating the peak-to-peak S/N of the confirmation ion (*m/z* 148) for a series of low standards. At the LOQ of 0.10 μg/mL, this S/N for the confirmation ion was 6.09 and the S/N for the quantitation ion was 21.7. The ratio of the peak areas for each ion was also determined for a low standard (0.25 μg/mL) and the samples fortified at the low spike level. The ratio of the peak areas for the spiked matrices was 0.485 (RSD of 7.2%), which compared well to the ratio of the peak areas for this low standard (0.490, RSD of 9.4%, n = 5).

To determine within-run and between-run variability, a mid-point calibration standard at 2.5 μg/mL was analyzed repeatedly (n = 3). The within-run RSD was 3.9%. This same standard was analyzed over a 5 day period (n = 12) and the between-run RSD was 7.5%. The within-run variability for milk spiked at 2.5 μg/mL with preparation by SPE was 17%. This same sample set was analyzed 4 days later to determine the between-run variability, which was determined to be 15%.

Method Verification

The six beverages included in this study were fortified at 2.5 μg/mL and 0.25 μg/mL, with this latter level below the target method detection limit of 0.3 μg/mL. When these samples were prepared for GC/MS analysis at the 2.5 μg/mL level, the recoveries ranged from 73% (milk) to 125% (orange juice) (**Table 1**). The high fat content of the milk samples complicated the extraction of tetramine. The RSD was 2.6% or less, with the exception of the milk samples,

which had an RSD of 13%. At the 0.25 μg/mL fortification level, the recoveries of tetramine ranged from 81% (milk) to 128% (orange juice). The RSD was 11% or less.

These same samples that were prepared by liquid-liquid extraction and analyzed by GC/MS were also analyzed by LC/MS/MS (**Table 1**). When analyzed by LC/MS/MS, the recoveries ranged from 10 to 94% at the 2.5 μg/mL fortification level and from below the LOQ to 101% at the 0.25 μg/mL fortification level. RSD (%) were 16% or lower for the 2.5 μg/mL level and 23% or lower at the 0.25 μg/mL level. A Bland-Altman difference plot (*21*) was prepared to assess agreement between the two instrumental methods (**Figure 5A**), though the matrices tea and orange juice were not included in this sample set because of the poor recovery of tetramine from these beverages. As shown in **Figure 5A**, the recovery of tetramine from beverages analyzed by LC/MS/MS is 9.8% lower than the recoveries determined by GC/MS. Fifty-four % of the samples analyzed by both methods were within 1 standard deviation of the mean difference between the two instrumental methods; and all of the samples were within 2 standard deviations of the mean difference. The water and cola samples (at both fortification levels) had higher recoveries by LC/MS/MS versus GC/MS, whereas milk and juice drink samples at the 0.25 μg/mL level had lower recoveries by LC/MS/MS versus GC/MS.

Samples prepared by C8 SPE for LC/MS/MS analysis at the 2.5 μg/mL level had recoveries ranging from 13% (tea) to 95% (cola), with RSD of 17% or less (**Table 1**). With the exception of the tetramine recoveries from tea and orange juice, from which tetramine was poorly recovered, there was no statistically significant difference between extraction methods when analyzed by LC/MS/MS at the 2.5 μg/mL fortification level. At the 0.25 μg/mL fortification level, the recoveries of tetramine were much poorer when prepared by SPE. The recoveries of tetramine from water, cola, and juice drink were not statistically different between the two

extraction protocols when analyzed by LC/MS/MS. Recoveries of tetramine from whole milk were significantly lower when prepared by SPE versus liquid-liquid extraction. Tetramine was not quantitatively recovered from tea or orange juice. Tea and orange juice are both rich in vitamins, alkaloids, and phenolics, which may bind to tetramine and interfere with its extraction and clean-up from beverage matrices. Alternatively, these compounds, or some other matrix component, may cause ion suppression during ESI. Further investigation of the effect of these matrices on analysis of tetramine by LC/MS/MS is one aim of future investigation.

The agreement between the two extraction protocols followed by LC/MS/MS analysis is shown in **Figure 5B**. The mean difference between the two extraction protocols was 0.3%, indicating that there is a high level of agreement across the recovery range (10 to 101% by liquid-liquid extraction and 13 to 96% by SPE). Ninety-seven % of the samples prepared by these two protocols were within 2 standard deviations of the mean and 67% were within one standard deviation of the mean. With one exception, the beverages spiked at the 0.25 μg/mL fortification level and cleaned up by SPE tended to be in the 1 to 2 standard deviation range outside the mean.

Optimization of Clean-Up and Extraction Protocols

The optimization of the clean-up and extraction protocols for both SPE and liquid-liquid extraction were evaluated extensively. For samples prepared for GC/MS analysis, the solvents methylene chloride, ethyl acetate, and acetone were evaluated for their suitability in the liquid-liquid extraction protocol. Overall, extraction of tetramine using either methylene chloride or ethyl acetate was favorable with a recovery of > 91% for a 2.5 μg/mL spike added to reagent water. The difference in recovery between these two solvents was not statistically significant (*P*

= 0.4799). However, ethyl acetate was chosen as the extraction solvent because the chromatograms had less noise in the baseline compared to methylene chloride.

Solid phase extraction cartridges, including C8 and CN, were evaluated in triplicate for tetramine extraction from reagent water. For the C8 columns, the elution solvents of acetonitrile, acetone, acetonitrile/acetone (50/50, v/v), acetone/ethyl acetate (90/10, v/v), and ethyl acetate were evaluated. Use of the acetone/ethyl acetate resulted in good recovery (83%). However, ethyl acetate as the elution solvent resulted in the highest recoveries (98%) with an RSD of 11% (n = 3). For the CN columns, the elution solvents acetonitrile, acetone, acetonitrile/acetone (50/50, v/v), and water were evaluated. Use of water as an elution solvent on the CN columns resulted in quantitative recovery (110%) of tetramine with an RSD of 0% (n = 3). However, when the CN columns were employed to extract tetramine spiked into the beverages included in this study, the resulting recoveries were very poor (< 50%). Thus, the C8 columns were selected for tetramine clean-up from foods. The optimized protocol was as follows: condition column with 5 mL methanol followed by 5 mL water, load sample (1 mL) and elute with 1 mL ethyl acetate.

Stability study

Tetramine is a relatively persistent environmental contaminant. Early investigators confirmed that toxicity of aqueous tetramine solutions had not attenuated six weeks after being prepared (*4*). To our knowledge, no reports of tetramine breakdown products have been identified. Only the higher molecular weight derivative of tetramine, 4,10,13-trithia-1,3,5,7,9,11-hexaazatetracyclo[5.5.1.$1^{3,11}$.$1^{5,9}$]pentadecane 4,10,13-tris(dioxide) (**Figure 1B**), which forms in the presence of heat and acid, has been identified (*20*). Other adamantane compounds that are

similar in structure to tetramine, including a class of antiviral drugs, retain full biological activity for decades when stored at room or refrigeration temperature (*22*).

In a pilot study, the stability of an LLNL tetramine standard in acetone at 5 μg/mL and stored at 23 °C was evaluated over 21 days by GC/MS. The relative standard deviation of the area for the *m/z* 212 quantitation ion was less than 11% and the RSD of the ratio of areas of the *m/z* 212 (tetramine quantitation) to *m/z* 360 (tetramine dimer) was less than 7%. Thus, tetramine is stable when stored at room temperature in acetone. Additionally, the ratio of peak areas for *m/z* 240 (confirmation ion) to *m/z* 212 (quantitation ion) was monitored. At the 5 μg/mL level, the ratio of the peak areas for the two ions was 1.75 (RSD of 2.4%).

Standards were also prepared in water (pH 7) and stored at various temperatures over a 35 d period with analysis by LC/MS/MS. The *m/z* 347 → *m/z* 268 and *m/z* 347 → *m/z* 148 transitions were monitored over this period. Temperature had a significant effect on tetramine stability in water (**Figure 6**). When stored at 23 °C, only 2.9% of the *m/z* 347 adduct remained after 1 day. Within one week, the ions were non-detectable. At 4 °C, 76.7% of the original *m/z* 347 adduct remained after 1 day. After 1 week, only 13% of the original adduct remained. However, when the aqueous tetramine standards were stored at 0 °C, there was no significant decrease in concentration until 35 days after standard preparation (91.8% remaining; $P = 0.037$ by Student's *t*-test). Tetramine has limited solubility in water (approximately 250 μg/mL) and of these sample sets (set 1 at 0 °C, set 2 at 4 °C, and set 3 at 23 °C), only sample set 1 was well-mixed each time before analysis to aid in the thawing of the sample prior to analysis. One aim for future work is to determine the factors that influence the stability of the tetramine dimer adduct (*m/z* 347) when stored in water.

Finally, CFSAN tetramine was spiked into the beverage matrices included in the study and stored at 4 °C for two weeks before undergoing liquid-liquid extraction for GC/MS analysis to assess tetramine stability in matrix. There was no excess formation of the tetramine dimer after two week storage in acidic matrix above baseline levels. Tetramine was recovered with minimum 60% efficiency (bottled water, RSD 4.8%), but was recovered at approximately 70-75% for the remaining five matrices (juice drink, cola, tea, orange juice, and milk). Possible tetramine losses include sorption onto the unsilanized glass surface of the storage vial or particulates within the beverage matrix itself.

As we have shown, tetramine can be analyzed by both GC/MS and LC/MS/MS with comparable results as shown by Bland-Altman difference plots (**Figure 5A** and **5B**). Tetramine can be extracted from beverages with reasonable efficiency by liquid-liquid extraction for all matrices included in the study (> 73% recovery), while the sample preparation by SPE is less robust at the lower fortification level. The liquid-liquid extraction protocol allows for rapid extraction of tetramine from beverages, whereas the solid phase extraction protocol is more time-intensive. For the majority of beverages, however, there was no statistically significant difference between recoveries for the two extraction methods at the 2.5 μg/mL fortification level with the exceptions of tea and orange juice. Tetramine is reasonably stable in beverages with approximately 75% recovery after storage for 2 weeks at 4 °C. The stability of tetramine in water, however, requires further investigation, especially when analyzed by LC/MS/MS and is one aim for future work. Tetramine can be analyzed with good sensitivity by GC/MS, and for the first time, we have shown that it can be easily analyzed by LC/MS/MS. The required method

detection limit for tetramine in beverages (0.3 μg/mL) is easily achievable using either extraction protocol or instrumental method.

SAFETY

Tetramethylenedisulfotetramine is an extremely hazardous chemical (human oral LD_{50} = 0.1 mg/kg) and is a persistent environmental contaminant. Proper personal protective equipment should be used at all times. All solid and liquid waste containing tetramethylenedisulfotetramine should be treated as extremely hazardous.

ACKNOWLEDGEMENTS

We thank Karen Wolnik, Frederick Fricke, Bryan Gamble, and Kevin Mulligan of the FDA Forensic Chemistry Center for their assistance and insightful discussions; Lowrie De Jager of the FDA's Center for Food Safety and Applied Nutrition for a source of tetramine; and Phil Pagoria of LLNL for the gift of reagents to synthesize tetramine. Funding from the U.S. Food and Drug Administration is gratefully acknowledged. This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

LITERATURE CITED

1. Whitlow, K. S.; Belson, M.; Barrueto, F.; Nelson, L.; Henderson, A. K., Tetramethylenedisulfotetramine: old agent and new terror. *Ann Emerg Med* **2004**, *45*, 609-613.

2. Wu, Y.-Q.; Sun, C.-Y., Poison control services in China. *Toxicol* **2004**, *198*, 279-84.

3. O'Neil, M. J.; Smith, A.; Heckelman, P. E., The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals; 13th Ed. Merck & Co., Inc.: Whitehouse Station, NJ, 2001.

4. Croddy, E., Rat poison and food security in the People's Republic of China: focus on tetramethylene disulfotetramine (tetramine). *Arch Toxicol* **2004**, *78*, 1-6.

5. Ratra, G. S.; Kamita, S. G.; Casida, J. E., Role of human $GABA_A$ receptor B3 subunit in insecticide toxicity. *Toxicol Appl Pharmacol* **2001**, *172*, 233-240.

6. Poon, W. T.; Chan, K.; Lo, M. H.; Yip, K. K.; Lee, T.; Chan, A. Y. W., A case of tetramine poisoning: a lethal rodenticide. *Hong Kong J Med* **2005**, *11*, 507-9.

7. Chau, C. M.; Leung, A. K. H.; Tan, I. K. S., Tetramine poisoning. *Hong Kong J Med* **2005**, *11*, 511-4.

8. Barrueto, F.; Nelson, L. S.; Hoffman, R. S.; Heller, M. B.; Furdyna, P. M.; Hoffman, R. J.; Whitlow, K. S.; Belson, M. G.; Henderson, A. K., Poisoning by an illegally imported Chinese rodenticide containing tetramethylene-disulfotetramine--New York City, 2002. *MMWR* **2003**, *52*, 199-201.

9. Xiang, P.; Shen, M.; Bu, J.; Huang, Z., The stability of tetramine, morphine, and meperidine in formalin solution. *Foren Sci Intl* **2001**, *122*, 159-62.

10. Liu, J.; Fan, C.; Wu, G., Determination of tetramethylenedisulfotetramine in blood by gas chromatography. [Letter]. *Clin Chem* **1993**, *39*, 173-4.

11. Guan, F.-Y.; Liu, Y.-T.; Hu, X.-Y.; Liu, F.; Li, Q.-Y.; Kang, Z.-W., GC/MS identification of tetramine in samples from human alimentary intoxication and evaluation of artificial carbonic kidneys for the treatment of victims. *J Anal Toxicol* **1993**, *17*, 199-201.

12. Luan, T.; Li, G.; Zhao, M.; Zhang, Z., Rapid detection of tetramethylenedisulfotetramine in human blood by solid-phase microextraction/gas chromatography. *Anal Chim Acta* **2000**, *404*, 329-334.

13. Zeng, D.; Chen, B.; Yao, S.; Ying, J., Determination of tetramethylenedisulfotetramine in human urine with gas chromatograph-flame thermionic detection coupling with direct immersed solid phase micro-extraction. *Foren Sci Intl* **2006**, *159*, 168-74.

14. De Jager, L. S.; Perfetti, G. A.; Diachenko, G. W., Analysis of tetramethylene disulfotetramine in foods using solid-phase microextraction–gas chromatography–mass spectrometry. *J Chromatogr A* **2008**, *1192*, 36-40.

15. Almeida, A. A.; Campos, D. R.; Bernasconi, G.; Calafatti, S.; Barros, F. A. P.; Eberlin, M. N.; Meurer, E. C.; Paris, E. G.; Pedrazzoli, J., Determination of memantine in human plasma by liquid chromatography-electrospray tandem mass spectrometry: Application to a bioequivalence study. *J Chromatogr B* **2007**, *848*, 311-316.

16. Arndt, T.; Guessregen, B.; Hohl, A.; Reis, J., Determination of serum amantadine by liquid chromatography-tandem mass spectrometry. *Clin Chim Acta* **2005**, *359*, 125-131.

17. Wang, P.; Liang, Y.-Z.; Chen, B.-M.; Zhou, N.; Yi, L.-Z.; Yu, Y.; Yi, Z.-B., Quantitative determination of amantadine in human plasma by liquid chromatography-mass spectrometry and the application in a bioequivalence study. *J Pharmaceut Sci Biomed Anal* **2007**, *43*, 1519-1525.

18. Esser, T.; Karu, A. E.; Toia, R. F.; Casida, J. E., Recognition of tetramethylenedisulfotetramine and related sulfamides by the brain GABA-gated chloride channel and a cyclodiene-sensitive monoclonal antibody. *Chem Res Toxicol* **1991**, *4*, 162-167.

19. Thyagarajan, B. S.; Kang, J.-B., Acid catalyzed rearrangements of thia and aza analogs of adamantanes: A new derivative of sulfamide, formaldehyde, and ammonia. *J Heterocycl Chem* **1974**, *11*, 681-5.

20. Kang, J.-B.; Thyagarajan, B. S.; Gilbert, E. E.; Siele, V., A new condensation product from sulfamide and paraformaldehyde. *Intl J Sulfur Chem A* **1971**, *1*, 261-8.

21. Bland, J. M.; Altman, D. G., Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1986**, *1*, 307-310.

22. Suckow, R. F., Review: Separation methods for tricyclic antiviral drugs. *J Chromatogr B* **2001**, *764*, 313-325.

FIGURE CAPTIONS

Figure 1: Chemical structures of (A) tetramethylenedisulfotetramine, or 'tetramine': 2,6-dithia-1,3,5,7-tetraazatricyclo[3.3.1.$1^{3,7}$]decane 2,2,6,6-tetraoxide and (B) 'tetramine dimer': 4,10,13-trithia-1,3,5,7,9,11-hexaazatetracyclo[5.5.1.$1^{3,11}$.$1^{5,9}$]pentadecane 4,10,13-tris(dioxide).

Figure 2: GC/MS chromatograms of (A) CFSAN tetramine standard at 2.5 μg/mL and (B) juice drink spiked with CFSAN tetramine with ions *m/z* 212, *m/z* 240 (tetramine), and *m/z* 360 (tetramine dimer) indicated.

Figure 3: Hypothesized ionization mechanism of tetramine by negative ion mode electrospray ionization. In the presence of heat and acid, tetramine forms the tetramine dimer, which in turn becomes protonated before losing water to form ion *m/z* 347. Proposed product ion structures (*m/z* 268, *m/z* 227, *m/z* 148, and *m/z* 134) are also indicated.

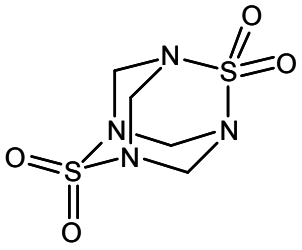
Figure 4: LC/MS/MS chromatograms of (A) CFSAN tetramine standard at 2.5 μg/mL, (B) blank and (C) cola spiked with CFSAN tetramine and cleaned up by SPE.

Figure 5: Bland-Altman difference plots illustrating (A) agreement between samples cleaned up by liquid-liquid extraction with analysis by LC/MS/MS versus GC/MS analysis and (B) agreement between samples cleaned up by C8 SPE versus liquid-liquid extraction with analysis by LC/MS/MS only.

Figure 6: Temperature stability of tetramine standard in water stored at various temperatures over a period of 35 days with analysis by LC/MS/MS.

Figure 1

A



B

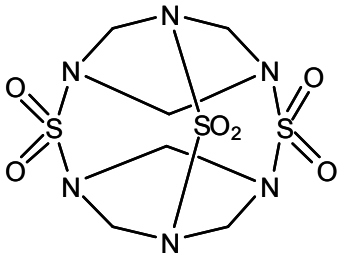


Figure 2

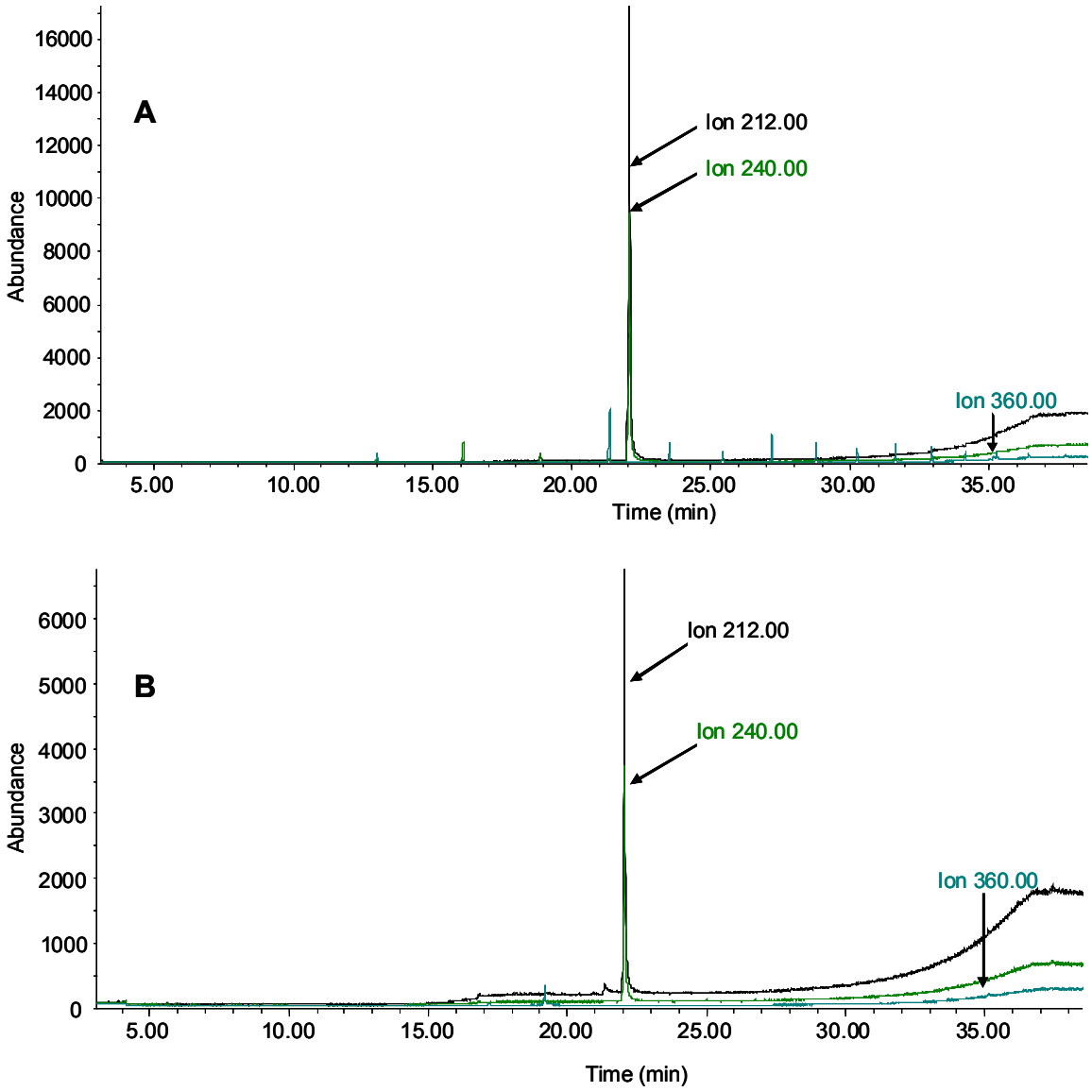

A
Ion 212.00
Ion 240.00
Ion 360.00
Abundance
Time (min)
B
Ion 212.00
Ion 240.00
Ion 360.00
Abundance
Time (min)

Figure 3

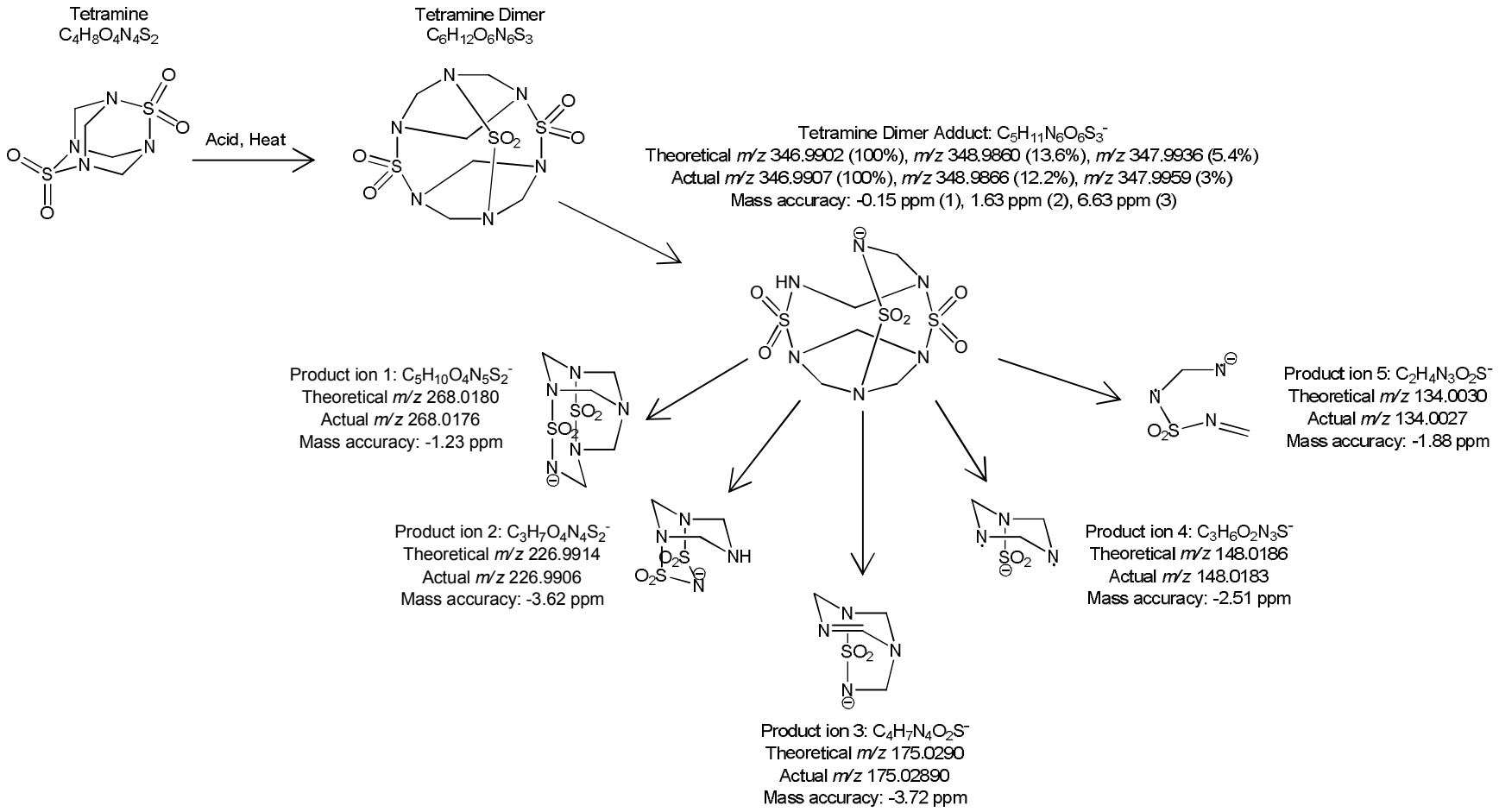

Tetramine
C4H8O4N4S2
Acid, Heat
Tetramine Dimer
C6H12O6N6S3
Tetramine Dimer Adduct: C5H11N6O6S3-
Theoretical m/z 346.9902 (100%), m/z 348.9860 (13.6%), m/z 347.9936 (5.4%)
Actual m/z 346.9907 (100%), m/z 348.9866 (12.2%), m/z 347.9959 (3%)
Mass accuracy: -0.15 ppm (1), 1.63 ppm (2), 6.63 ppm (3)
Product ion 1: C5H10O4N5S2-
Theoretical m/z 268.0180
Actual m/z 268.0176
Mass accuracy: -1.23 ppm
Product ion 2: C3H7O4N4S2-
Theoretical m/z 226.9914
Actual m/z 226.9906
Mass accuracy: -3.62 ppm
Product ion 3: C4H7N4O2S-
Theoretical m/z 175.0290
Actual m/z 175.02890
Mass accuracy: -3.72 ppm
Product ion 4: C3H6O2N3S-
Theoretical m/z 148.0186
Actual m/z 148.0183
Mass accuracy: -2.51 ppm
Product ion 5: C2H4N3O2S-
Theoretical m/z 134.0030
Actual m/z 134.0027
Mass accuracy: -1.88 ppm

Figure 4

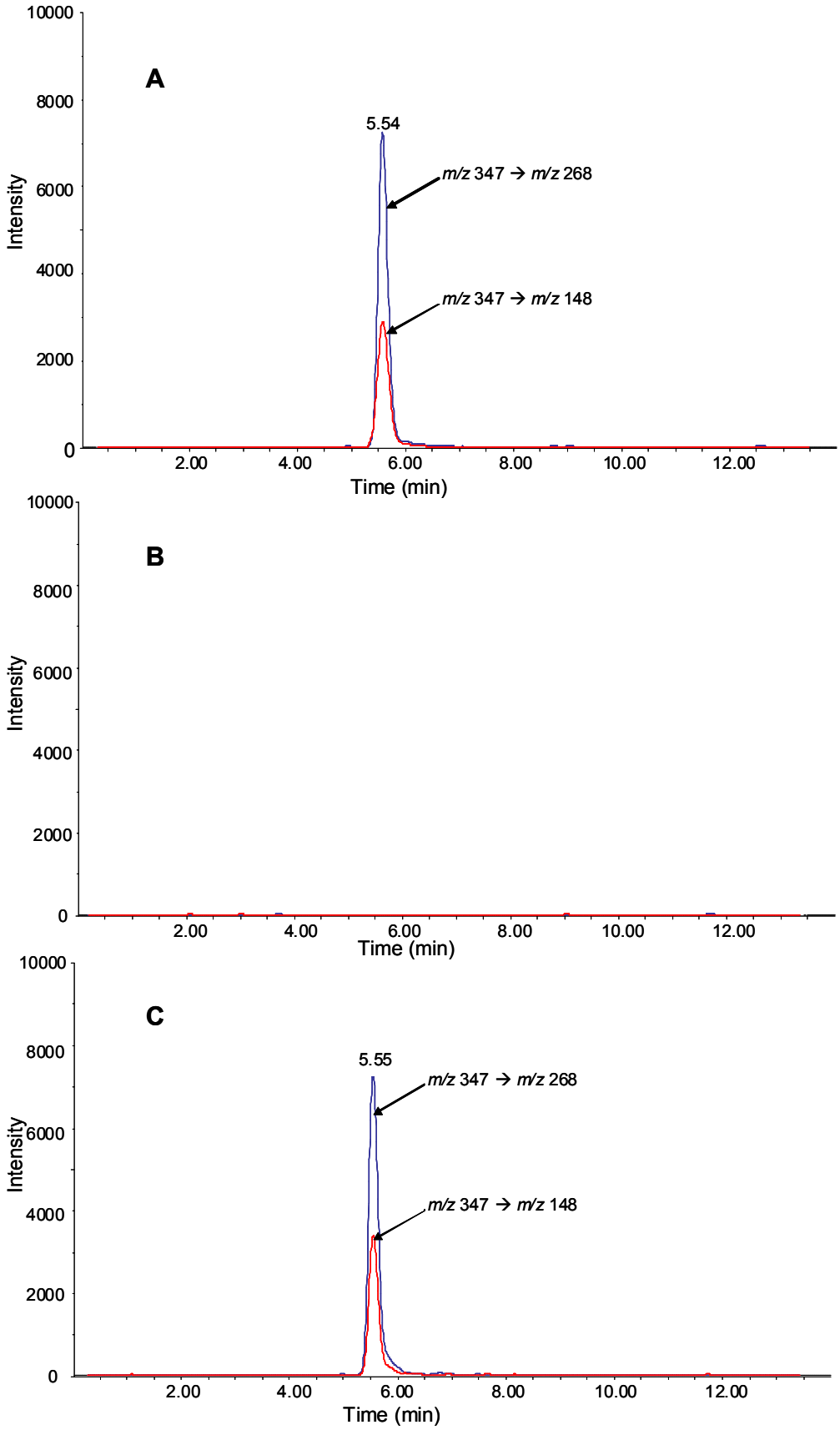


Figure 5

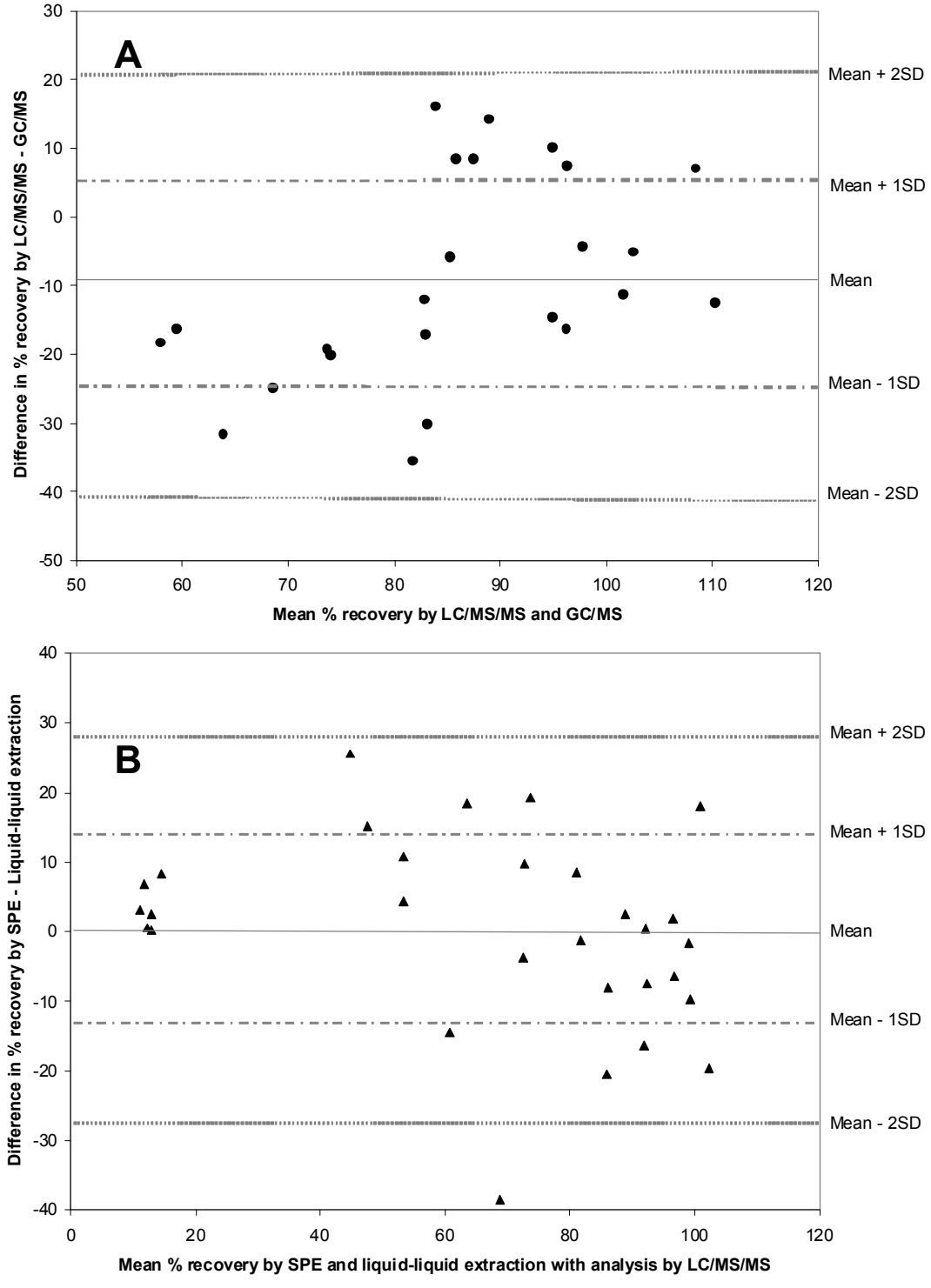

A
Mean + 2SD
Mean + 1SD
Mean
Mean - 1SD
Mean - 2SD
Difference in % recovery by LC/MS/MS - GC/MS
Mean % recovery by LC/MS/MS and GC/MS
B
Mean + 2SD
Mean + 1SD
Mean
Mean - 1SD
Mean - 2SD
Difference in % recovery by SPE - Liquid-liquid extraction
Mean % recovery by SPE and liquid-liquid extraction with analysis by LC/MS/MS

Figure 6

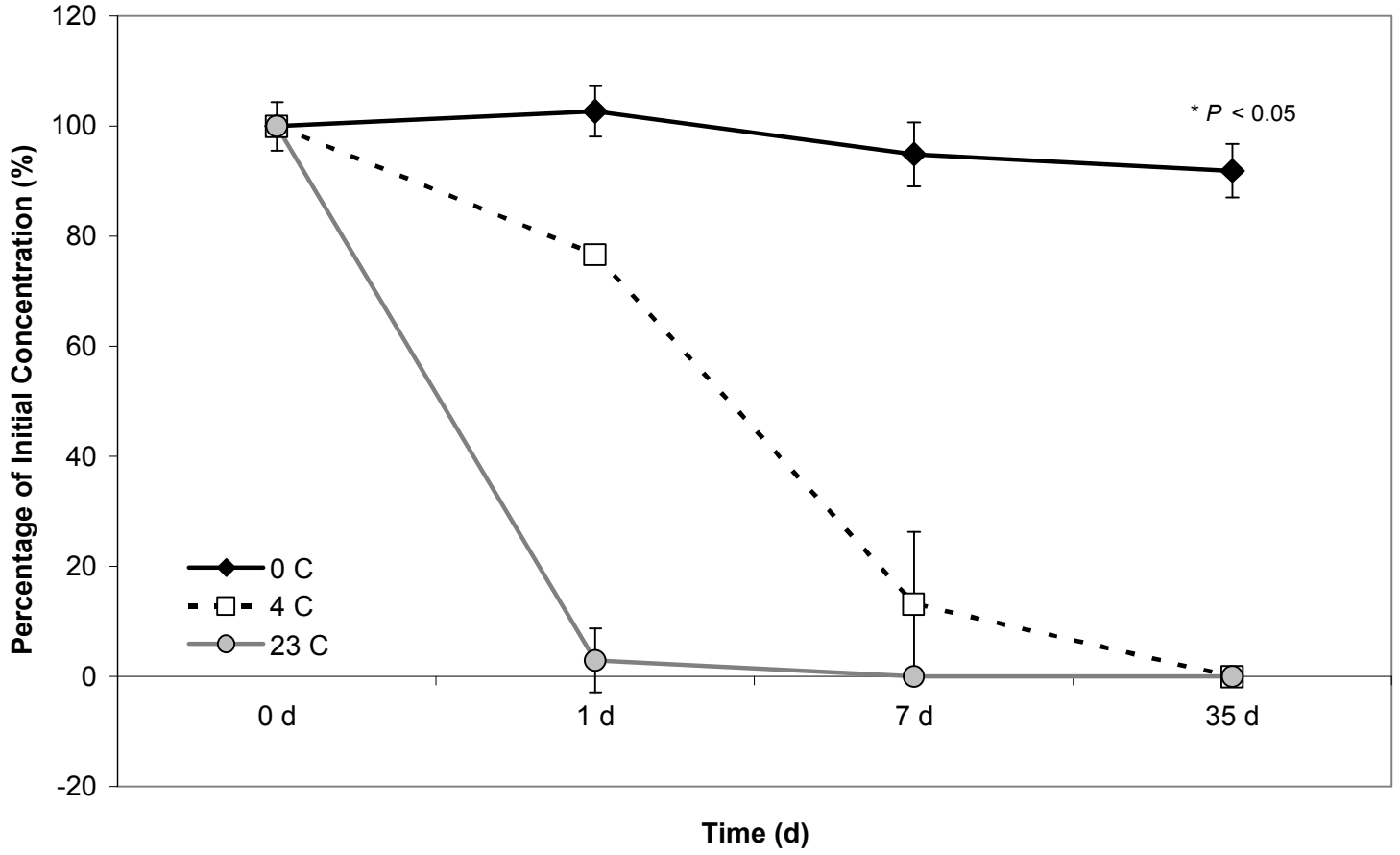

120
100
80
60
40
20
0
-20
Percentage of Initial Concentration (%)
* P < 0.05
0 C
4 C
23 C
0 d
1 d
7 d
35 d
Time (d)

Table 1: Mean % Recovery of Tetramine Spiked into Beverages at Two Levels and Prepared for Instrumental Analysis by Liquid-Liquid Extraction or Solid Phase Extraction

	mean % recovery (RSD, %)				
matrix spiked at 2.5 μg/mL	liquid-liquid extraction with GC/MS analysis	liquid-liquid extraction with LC/MS/MS analysis	P^a	SPE with LC/MS/MS analysis	P^b
water	82 (1.1)	93 (3.4)	**0.0050**	88 (6.8)	0.2288
cola	102 (2.5)	94 (6.7)	0.1118	95 (4.7)	0.8532
tea	98 (2.6)	12 (15)	**< 0.0001**	13 (2.3)	0.2478
orange juice	125 (1.3)	10 (16)	**< 0.0001**	16 (15)	**0.0232**
juice drink	89 (2.0)	78 (5)	**0.0111**	79 (9.6)	0.8115
whole milk	73 (13)	58 (15)	0.1201	69 (17)	0.2702

	mean % recovery (RSD, %)				
matrix spiked at 0.25 μg/mL	liquid-liquid extraction with GC/MS analysis	liquid-liquid extraction with LC/MS/MS analysis	P^a	SPE with LC/MS/MS analysis	P^b
water	86 (11)	97 (3.4)	0.1243	96 (14)	0.8538
cola	109 (6.4)	101 (12)	0.4245	79 (32)	0.2372
tea	106 (7.1)	Below LOQ	-	Below LOQ	-
orange juice	128 (2.9)	Below LOQ	-	Below LOQ	-
juice drink	103 (6.2)	76 (23)	0.0710	71 (22)	0.7181
whole milk	81 (2.2)	40 (20)	**0.0010**	57 (3.2)	**0.0222**

[a] Student's *t*-test assuming equal variances for determination of significant differences in recovery between samples prepared by liquid-liquid extraction and analyzed by GC/MS versus LC/MS/MS. Bold values indicate significance at $P < 0.05$.
[b] Student's *t*-test assuming equal variances for determination of significant differences in recovery between liquid-liquid extraction by LC/MS/MS and SPE with LC/MS/MS. Bold values indicate significance at $P < 0.05$.