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ARTICLE

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

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Tetramethylenedisulfotetramine, commonly known as tetramine, is a highly neurotoxic rodenticide (human oral LD_{50} = 0.1 mg/kg) used in hundreds of deliberate and accidental food poisoning events in China. This paper describes a method for the quantitation of tetramine spiked into beverages, including milk, juice, tea, cola, and water, with cleanup 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 selected ion monitoring 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 and 0.25 μg/mL were recovered ranging from 73 to 128% by liquid−liquid extraction for GC-MS analysis, from 13 to 96% by SPE, and from 10 to 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 sale of tetramine, also known under such names as Dushuqiang, Meishuming, and Shanbudao(*1*), along with its production and use, has been forbidden since the 1970s (*2*). In 2000, the National Poison Control Center of China surveyed rodenticides from free markets in China and found that nearly 50% of the 116 rodenticides contained both tetramine and fluoroacetamide [also known as 1081 (*3*)], another highly toxic rodenticide, thus indicating the wide availability of tetramine (*2*).

It has been estimated that there have been thousands of tetramine poisonings through food in China, resulting in hundreds of human deaths from 1977 through 2002 (*2*, *4*). A 7−10 mg primary dose of tetramine is considered to be lethal for humans (*1*), although 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 noncompetitively 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 United States, 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 and accidental food poisoning events in China with this toxic rodenticide (*2*, *4*), the development of quantitative methods for cleanup 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 microextraction (*14*), stir-bar sorptive extraction (*15*), and membrane-assisted solvent extraction (*16*) was optimized for a variety of food matrices, including juice, peanut butter, and potato chips.

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 been reported (*17*−*19*). The objective of this work was to develop methods for the quantitative

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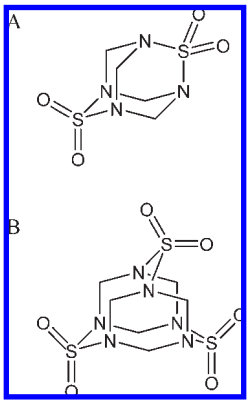

Figure 1. Chemical structures of (**A**) tetramethylenedisulfotetramine (tetramine), 2,6-dithia-1,3,5,7-tetraazatricyclo[3.3.1.1^{3,7}]decane 2,2,6,6-tetraoxide, and (**B**) tetramine dimer, 4,10,13-trithia-1,3,5,7,9,11-hexaazatetracyclo[5.5.1.1^{3,11}.1^{5,9}]pentadecane 4,10,13-tris(dioxide).

extraction of tetramine from various beverage samples utilizing both liquid−liquid extraction and solid-phase extraction (SPE) 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 Lawrence Livermore National Laboratory (LLNL; 77.3% 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; ≥99% purity) using the protocol described by Esser et al. (*20*). 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 (*21*). For all method development, verification, and stability experiments described, only the CFSAN tetramine was utilized.

Formic acid, sodium chloride, and anhydrous sodium sulfate were from Sigma-Aldrich. 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 <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 a 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 2 mg of crystalline tetramine and dissolving in acetone. Appropriate dilutions of these two stock solutions were made to prepare a 20 μg/mL solution. One microliter 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 × 0.25 mm i.d. × 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 for 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 50−550. The source was maintained at 230 °C, the quadrupoles were maintained at 150 °C, and the transfer line heater was maintained at 280 °C.

Using this general temperature program, the tetramine eluted at 22.24 min and the higher molecular weight derivative of tetramine (*22*), 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 22.7%, and the CFSAN tetramine contained <1% dimer. The mass spectra for tetramine and tetramine dimer are shown in **Figure 2A**. The mass spectra of tetramine and the tetramine dimer between the two sources were nearly identical.

Preparation of Calibration Standards. CFSAN tetramine (2.37 mg; > 98% purity) was dissolved in 25 mL of 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 to 10 μg/mL for both LC-MS/MS and GC-MS analyses.

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 1 mL aliquots were stored at 0, 4, and 23 °C and analyzed in triplicate over a 35 day period by LC-MS/MS. A second standard with a 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 Cleanup. *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 the addition of 2 mL of ethyl acetate (with the exception of milk samples, to which 4 mL of ethyl acetate was added). The samples were capped and mixed vigorously for 1 min. Sodium chloride (1 g) was then added, and the samples were again vigorously mixed before being centrifuged at 3000*g* for 10 min (Fisher AccuSpin 400 centrifuge, Fisher Scientific). An aliquot (~1 mL) of sample was removed by a disposable glass pipet and dried over 150 mg of anhydrous sodium sulfate in amber glass vials. Samples were stored at 0 °C until 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 of methanol followed by 5 mL of water. The beverage sample (1 mL) was loaded and allowed to drip through by gravity. Tetramine was eluted with 1 mL of 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 as described above under Preparation of Standards with slight modifications to the temperature program. The initial temperature was 40 °C (hold for 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. Tetramine eluted at 22.03 min, and the tetramine dimer eluted at 35.16 min (**Figure 2B**).

Quantitation was by linear regression with no weighting from 0.25 to 10 μg/mL. A standard curve was prepared at the beginning and end of each sequence run, and a midpoint calibration standard of 2.5 μg/mL was included throughout the sequence list after every six matrix samples. The responses of these check calibration standards were also included in the standard curve preparation. Quantitation for both tetramine and tetramine dimer was based upon the m/z 212 ion with the m/z 240 ion serving as the tetramine confirmation ion and the 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 Corp., Milford, MA) was tuned by infusing a 94.8 μg/mL solution of tetramine prepared in 50:50 water/acetonitrile (v/v) + 0.1% formic acid at 20 μL/min by external Harvard Syringe pump,

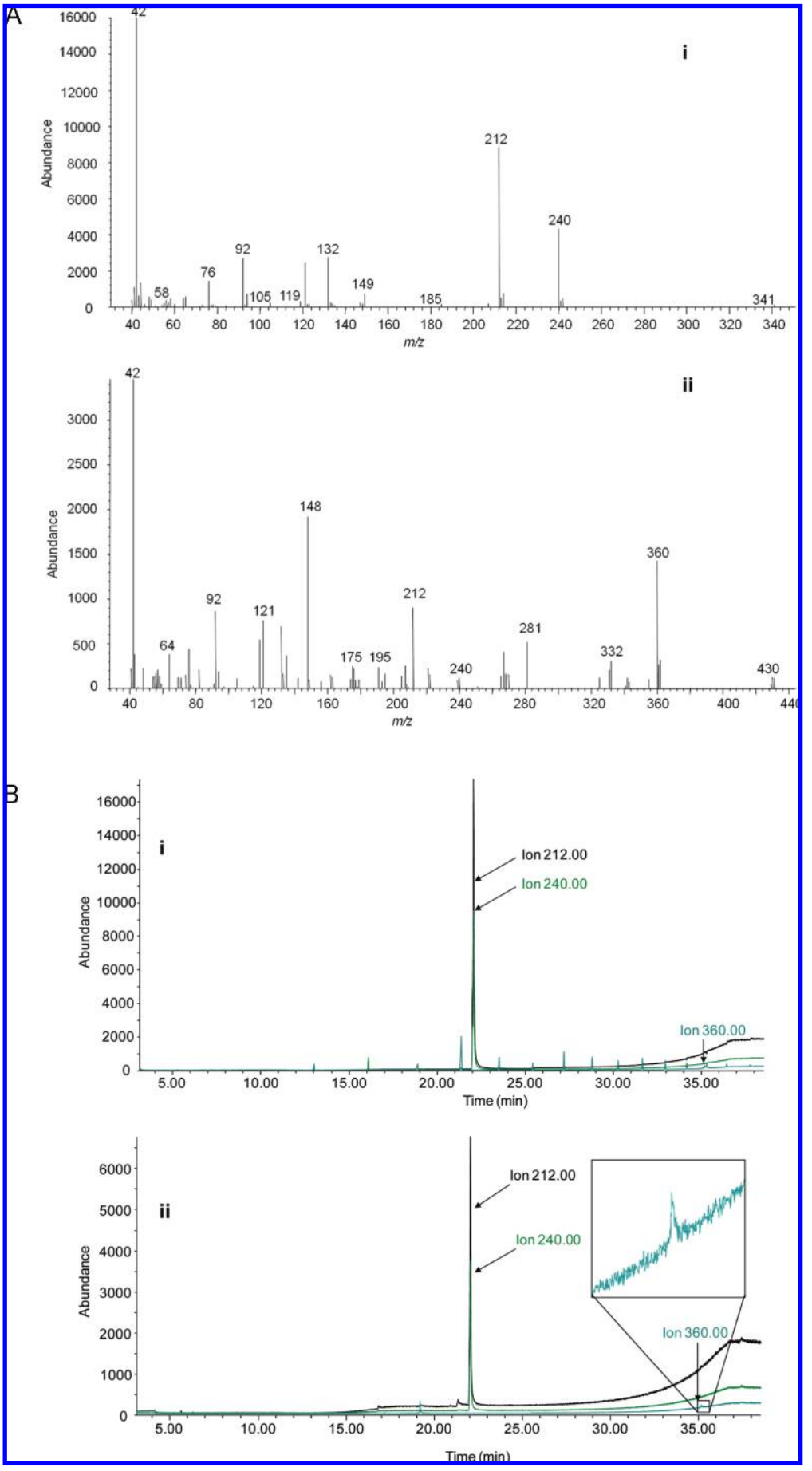

Figure 2. (**A**) Mass spectrum of tetramine (i) and tetramine dimer (ii) and (**B**) GC-MS chromatograms of (i) CFSAN tetramine standard at 2.5 μg/mL and (ii) juice drink spiked with CFSAN tetramine with ions *m*/*z* 212, 240 (tetramine), and 360 (tetramine dimer) indicated.

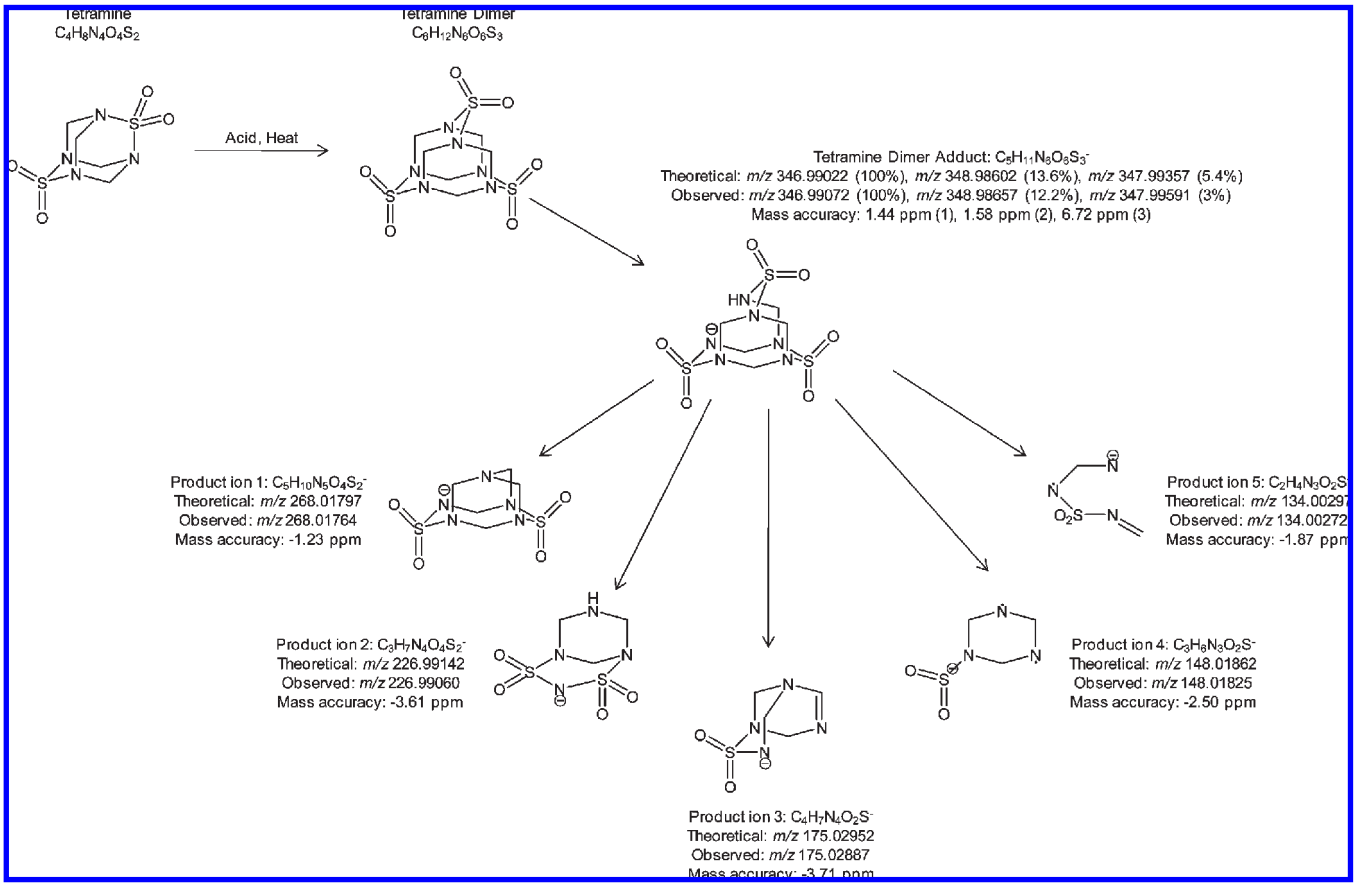

Figure 3. Hypothesized ionization mechanism of tetramine by negative ion mode electrospray ionization. In the presence of heat and acid, tetramine forms the tetramine dimer, which in turn becomes protonated before losing methylene to form ion *m/z* 347. Proposed product ion structures (*m/z* 268, 227,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

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**.

To evaluate this hypothesized ionization mechanism, a standard of 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 30000 at fwhm for a scan range of *m/z* 50−600. 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 Micromass 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 the manufacturer's specifications. After the instrument had been tuned 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 at 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 at 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 interchannel delay was set to 0.02 s and the interscan delay to 0.1 s; repeats were set at 1, and the span was set 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 microliters was injected onto a 150 mm × 2.1 mm i.d, 5 μm, Symmetry300 C4 analytical column (Waters Corp.). 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) and 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 quadratic regression with $1/x$ weighting from 0.10 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 check calibration 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 the stability of the standard during the following: (i) optimization experiments for cleanup 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 0, 4, and 23 °C in amber glass vials and

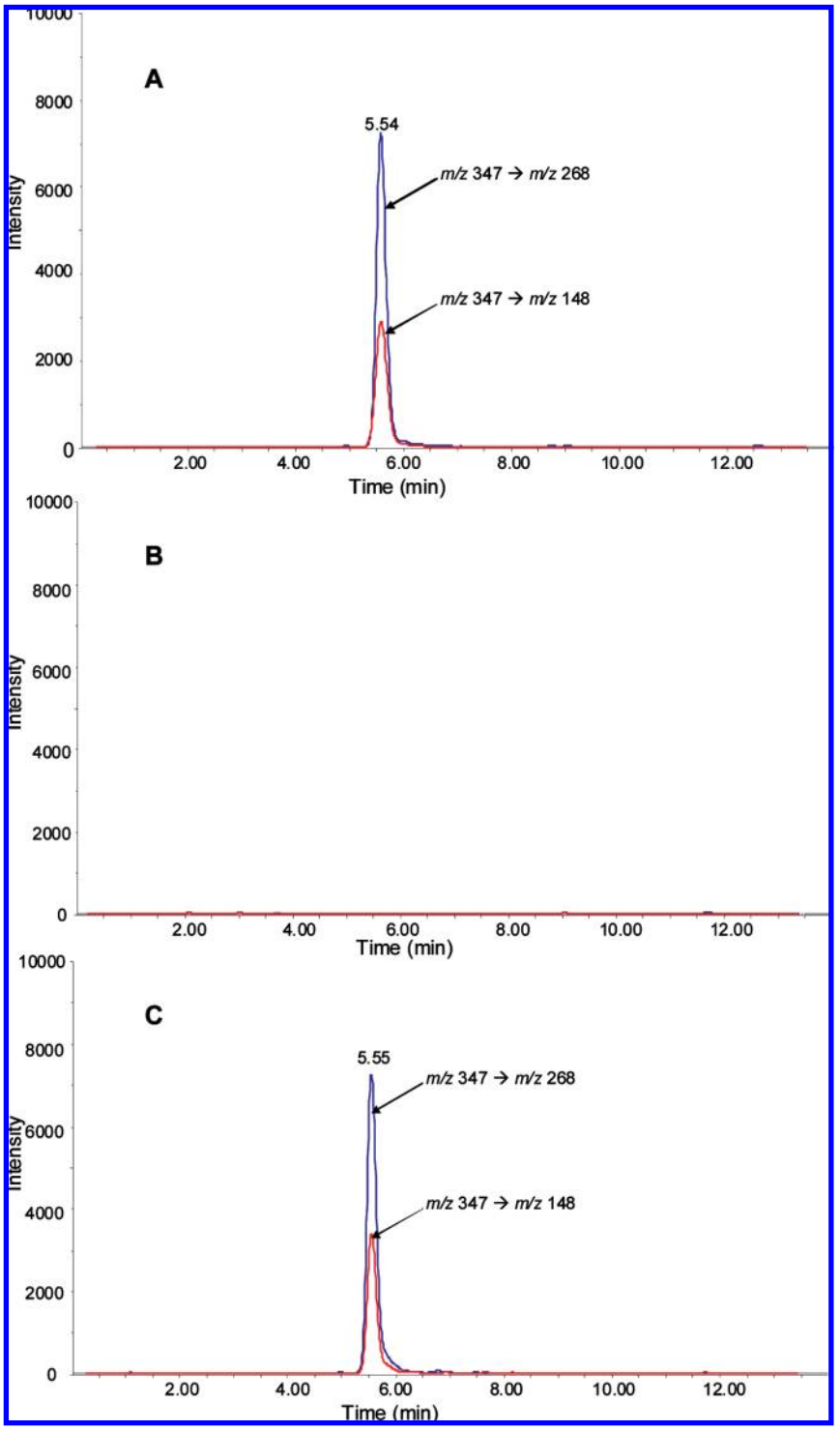

Figure 4. LC-MS/MS chromatograms of (**A**) CFSAN tetramine standard at 2.5 μg/mL, (**B**) blank, and (**C**) cola spiked with CFSAN tetramine and cleaned up by SPE.

analyzed by LC-MS/MS in triplicate just after standard preparation (t = 0 d), at 1 day (t = 1 day), at 1 week (t = 7 days), and at 5 weeks (t = 35 days).

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 was 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 and 0.25 μg/mL concentrations (n = 3 per spike level per beverage sample) and were also left unfortified (n = 3) as "control" or matrix blank samples. The first set of beverage samples was extracted with 2 mL of ethyl acetate and sodium chloride as described under 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**. 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 (*22*). 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, 227, 17, 148, and 134. Previous work by Kang et al. (*22*) reported the fragment ions (percent abundances) *m*/*z* 268 (5%) and 148 (29%) for the tetramine dimer when analyzed by electron-impact GC-MS. These ions and abundances were confirmed by GC-MS analysis as previously described for assessment of standard purity.

The parent ion, which was the hypothesized tetramine dimer adduct (*m*/*z* 347, $[M + H - CH_2]^-$), had a theoretical *m*/*z* 346.99022 with the following isotopic cluster: *m*/*z* 346.99022 (100%), 348.98602 (13.6%), and 347.99357 (5.4%). The observed ions, as shown in **Figure 3**, were *m*/*z* 346.99072 (100%), 348.98657 (12.2%), and 347.99591 (3%). The software results suggested the composition was $C_5H_{11}N_6O_6S_3^-$ (mass accuracy of 1.44 ppm), which matched the hypothesized composition (**Figure 3**). The theoretically and experimentally derived masses of the product ions (*m*/*z* 268, 227, 175, 148, and 134) compared very well (**Figure 3**). The mass accuracy between the hypothesized compositions versus experimentally derived composition was between −3.72 and 6.72 ppm for the product ions.

To provide one more additional piece of evidence, two approximate 10 μg/mL solutions of tetramine (one prepared from CFSAN stock standard and one from LLNL stock standard) were analyzed on the Waters LC-MS/MS system. In both cases, only one peak was visible in the resulting chromatograms. After purity and slight differences in concentration had been accounted for, the area of the LLNL tetramine peak was determined to be 23.1% higher than the area of the CFSAN tetramine peak. The LLNL tetramine stock standard was determined to contain 77.3% tetramine and 22.7% tetramine dimer when analyzed by GC-MS for assessment of standard purity, as described above. The experiment was repeated with 5 and 2.5 μg/mL solutions each of LLNL tetramine and CFSAN tetramine. On average, the LLNL tetramine peaks had an area that was 20.4% higher than the CFSAN tetramine (RSD of < 20%). 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 0.25−10 μg/mL, with an R^2 value of 0.9948. The instrument detection limits (IDL) 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 IDL (S/N = 3) was determined to be 0.05 μg/mL by extrapolation. The limit of quantitation (LOQ; = IDL × 3.3 or S/N = 10) 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 variabilities, a midpoint calibration standard at 2.5 μg/mL was analyzed repeatedly ($n = 4$). The within-run relative standard deviation (RSD) was 8.2%. This same midpoint 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 was 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 to 10 μg/mL, although the linear range was from 0.25 to 5 μg/mL. The calibration curves had a minimum R^2 value of 0.9900. The IDL 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 variabilities, a midpoint 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, the most complex sample, 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 and 0.25 μg/mL, with the 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 RSD was 2.6% or less, with the exception of the milk samples, which had an RSD of 13%. The high fat content of the milk samples complicated the extraction of tetramine. 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. RSDs 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 (*23*) was prepared to assess agreement between the two instrumental methods (**Figure 5A**), although the matrices tea and orange juice were not included in this sample set because of the matrix effects of these beverages when analyzed by LC-MS/MS. 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 percent 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.

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

	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
		Matrix Spiked at 2.5 μg/mL			
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
		Matrix Spiked at 0.25 μg/mL			
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 $P < 0.05$.

Owing to matrix effects seen in samples prepared by liquid−liquid extraction with analysis by LC-MS/MS, samples were then prepared by C8 SPE. When spiked at the 2.5 μg/mL level, recoveries ranged from 13% (tea) to 95% (cola), with RSDs of 17% or less (**Table 1**). Thus, the matrix effects on ionization of tetramine from tea and orange juice were not eliminated with SPE cleanup. However, there was no statistically significant difference between extraction methods analyzed by LC-MS/MS at the 2.5 μg/mL fortification level for matrices other than tea and orange juice. The recoveries of tetramine at the 0.25 μg/mL fortification level 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 higher when prepared by SPE versus liquid−liquid extraction.

As previously indicated, tetramine was quantitatively recovered from tea or orange juice as shown by GC-MS results, although these matrices experienced significant ion suppression effects during ESI for LC-MS/MS analysis. Further investigation of the effect of these matrices on analysis of tetramine by LC-MS/MS is one aim of future investigation. Use of matrix-matched standards or an isotopically labeled internal standard would aid in quantitation and recovery experiments.

The agreement between the two extraction protocols with analysis by LC-MS/MS is shown in **Figure 5B**. The mean difference between the two extraction protocols was 0.3%, indicating that there was a high level of agreement across the recovery range (10−101% by liquid−liquid extraction and 13−96% by SPE). Ninety-seven percent 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 1−2 standard deviation range outside the mean.

Optimization of Cleanup and Extraction Protocols. The optimizations of the cleanup and extraction protocols for both SPE and liquid−liquid extraction were evaluated extensively. Initial attempts for sample preparation for LC-MS/MS included a "dilute and shoot" approach. Tetramine is not soluble in methanol or ethanol, so the solvents acetonitrile, acetonitrile/acetone (75:25, v/v), and acetonitrile/ethyl acetate (75:25, v/v) were individually evaluated. In these experiments, 2 mL of reagent water was spiked with tetramine to prepare a 1 μg/mL solution. Two milliliters of one of the above solvent systems was added to the 2 mL of reagent water before being shaken vigorously, centrifuged to settle any particulates, and filtered through 0.2 μm PTFE filters. Reasonable recoveries (~50%, as expected) were achieved for this 1× dilution. The dilute and shoot approach (using acetonitrile) was then evaluated with food samples, including orange juice, bottled tea, bottled water, cola, and whole milk. Again, 2 mL of the beverage sample was diluted with 2 mL of acetonitrile, shaken vigorously, centrifuged, and filtered through PTFE filters. In all cases, including bottled water, tetramine was not detected at the 1 μg/mL spike level. Given the target limit of detection of 0.25 μg/mL, the dilute and shoot method was abandoned for methods that would provide cleaner sample extracts to avoid matrix effects that could cause signal suppression during ESI-LC-MS/MS analysis. Additionally, although only the preparation of beverage samples is presented here, robust methods that provide clean sample extracts of more complex matrices, such as peanut butter or processed foods, would be required. Thus, there was motivation to optimize sample preparation methods by liquid−liquid extraction and SPE.

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 those with methylene chloride.

SPE cartridges, including C8, CN, strata-X, and Oasis mixed-mode cation exchange (MCX), were evaluated in triplicate for tetramine extraction from reagent water. Use of strata-X or MCX columns with various elution solvents for the cleanup of tetramine was not quantitative and thus not evaluated further. 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%) or matrix effects were significant. Thus, the C8 columns were selected for tetramine cleanup from foods. The optimized protocol was as follows: condition column with 5 mL of methanol followed by 5 mL of water, load sample (1 mL), and elute with 1 mL of ethyl acetate.

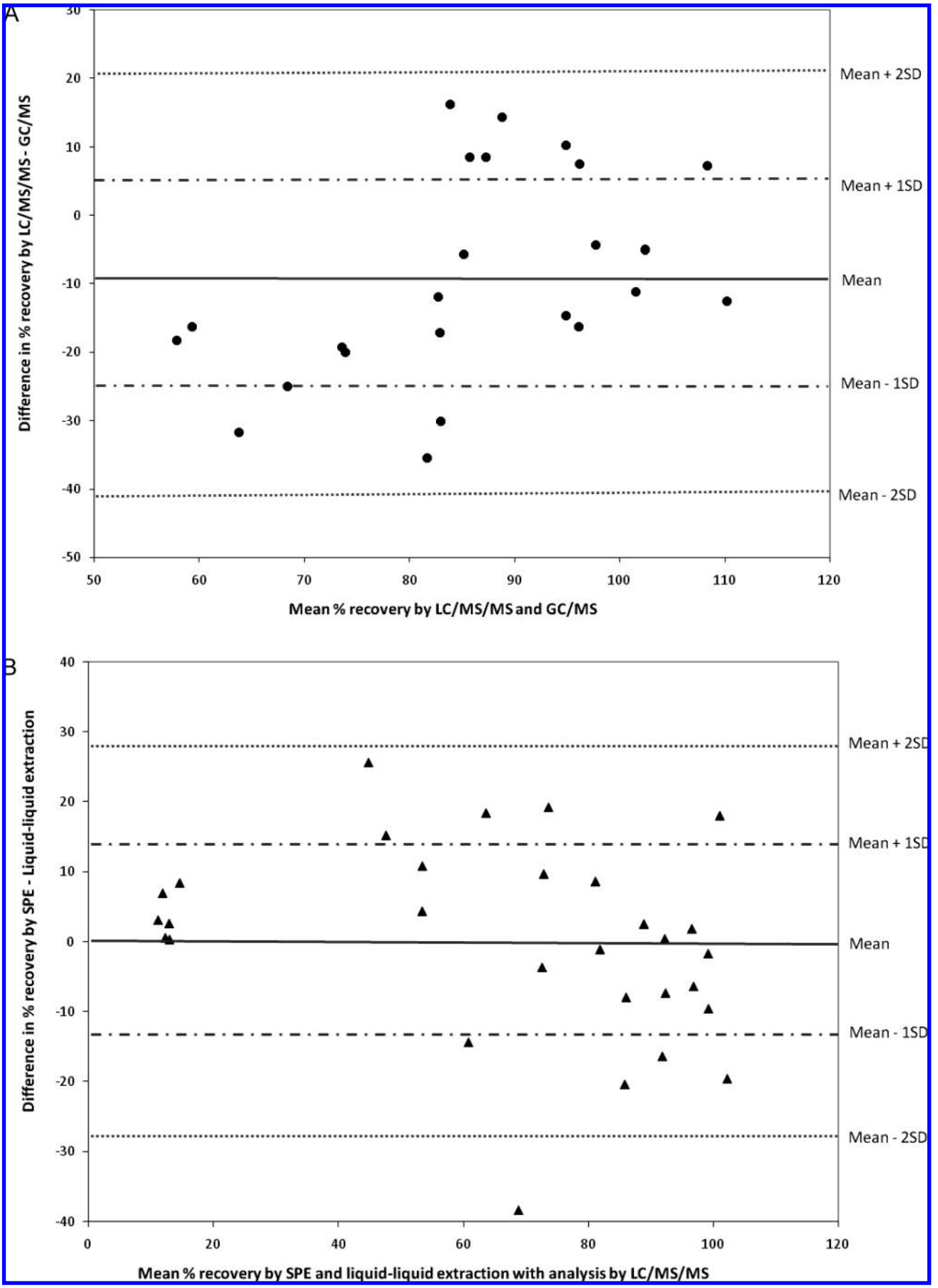

Figure 5. Bland−Altman difference plots illustrating (**A**) agreement between samples cleaned up by liquid−liquid extraction with analysis by LC-MS/MS versus GC-MS analysis and (**B**) agreement between samples cleaned up by C8 SPE versus liquid−liquid extraction with analysis by LC-MS/MS only.

Stability Study. Tetramine is a relatively persistent environmental contaminant. Early investigators confirmed that toxicity of aqueous tetramine solutions had not attenuated 6 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 (*22*). 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 (*24*).

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 < 11% and the RSD of the ratio of areas of the *m*/*z* 212 (tetramine quantitation) to 360 (tetramine dimer) was < 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 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 day 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 the ability of tetramine to form the tetramine adduct (*m*/*z* 347). When stored at 23 °C, only 2.9% of the tetramine remained to form *m*/*z* 347 adduct after 1 day. Within 1 week, the tetramine adduct was nondetectable by LC-MS/MS. At 4 °C, 76.7% of the tetramine formed *m*/*z* 347 adduct after 1 day. After 1 week, only 13% of the tetramine remained. However, when the aqueous tetramine standards were stored at 0 °C, there was no significant decrease in adduct formation for the first few weeks of storage. When analyzed at 35 days after standard preparation, 91.8% of the original standard remained (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 ability of the tetramine dimer adduct (*m*/*z* 347) to form during ionization when tetramine is stored in water at various temperatures.

Finally, tetramine was spiked into the beverage matrices included in the study and stored at 4 °C for 2 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 2 weeks of storage in acidic matrix above baseline levels. Tetramine was recovered with minimum 60% efficiency (bottled water, RSD of 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 5**). Tetramine can be extracted from beverages with reasonable efficiency by liquid−liquid extraction for all matrices included in the study (> 73% recovery), whereas 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 SPE protocol is more time-intensive. There seems to be little effect of matrix on analysis of tetramine when analyzed by GC-MS, although for certain matrices, including tea and orange juice, matrix ion suppression significantly affects sensitivity for analysis by LC-MS/MS. 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 also be 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 with analysis by GC-MS for all matrices evaluated. Improving the sensitivity of LC-MS/MS for complex matrices is one aim for future work.

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.

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