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Quantitation of Abrine, an Indole Alkaloid Marker of the Toxic Glycoproteins Abrin, by Liquid Chromatography/Tandem Mass Spectrometry When Spiked into Various Beverages

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Abrine is an alkaloid chemical marker and surrogate analyte of abrin, a group of highly toxic glycoproteins. These toxins can be easily isolated from the seed of the rosary pea plant and distributed in a variety of matrices, including food. A procedure for the cleanup of abrine from various beverages, including milk, cola, juice drink, tea, and water, by C18 Strata-X solid-phase extraction (SPE) cartridges is described with comparison to a previously developed liquid−liquid extraction protocol utilizing acetonitrile and water. Analysis was by liquid chromatography/tandem mass spectrometry. Abrine quantitation was based on fragmentation of *m*/*z* 219.2 to product ion *m*/*z* 188.2. The method detection limit was 0.025 μg/mL, and the quantitation limit was 0.05 μg/mL. Fortifications of the five beverages at 0.5 and 0.05 μg/mL were recovered ranging from 88 to 111% [relative standard deviation (RSD) < 16%] by SPE and from 48 to 101% (RSD < 19%) by liquid−liquid extraction.

KEYWORDS: Abrine; abrin; liquid chromatography mass spectrometry

INTRODUCTION

The seeds of the plant *Abrus precatorius*, or the rosary pea, contain a class of five highly toxic glycoproteins, including abrin A, B, C, and D and abrus agglutinin, collectively known as "abrin". Abrus agglutinin has a molecular mass of 134.9 kDa, while abrins A−D have a molecular mass range of 63−67 kDa (*1*). Abrin is similar to ricin, a toxin from the seeds of the castor bean, although abrin is much more toxic than ricin (*2*). The potential for use as a chemical weapon stems from both high toxicity and ease of isolation from the seeds of the rosary pea at low cost using a relatively simple procedure with distribution in multiple forms, including dissolution in water or food (*3*).

The toxin abrin constitutes 0.075 (*3*) to 0.75% (*4*) by weight of the seed. In addition to abrin, the seeds of the rosary pea also contain the indole alkaloid called abrine, a secondary plant metabolite (*1*). Abrine (**Figure 1**) constitutes about 0.5 (*5*) to 1% of the dry weight of the *A. precatorius* seeds (*6*). Abrine is synthesized by other *Abrus* species and may be a useful chemical marker for plants of this genus (*7*), as is the alkaloid marker ricinine for the toxic glycoprotein ricin (*8*). Plants such as *Abrus cantoniensis*, *Abrus melanospermus*, *Abrus pulchellus*, and *A. precatorius* (rosary pea) may be used in traditional herbal remedies but are not known sources of food (*9*−*11*). Thus, abrine is a good candidate low molecular weight chemical marker in high-throughput screening methods for food matrices suspected of being poisoned with the toxic glycoproteins. Few chromatographic methods to separate and detect abrin or abrine are described in the literature, and none are quantitative (*1, 12*).

Although abrin is not known to have been used in any wars or terrorist attacks (*13*), given its high toxicity, its detection and quantitation in beverages are of interest. The estimated fatal dose for humans is 0.1−1 μg/kg body weight, although this estimate is based on unconfirmed evidence. There is some controversy over the toxicity of abrin after ingestion, given its high molecular weight and poor absorption from the intestine. The reported LD_{50} for abrin is 20 μg/kg (mouse, intraperitoneal) (*3*). A target limit of detection (LOD) for a new quantitative chromatographic method is 0.06 μg/mL. This LOD was calculated using the assumed LD_{50} value for abrin of 20 μg/kg (*3*) multiplied by 70 kg, the weight of an adult individual, with normalization by both beverage portion size (236 mL) and a sensitivity factor to account for exposure by vulnerable populations. This detection limit assumed a 1:1 ratio of abrin to abrine in the seed extract. Unlike ricinine and ricin, which have been studied extensively, there has been little information found regarding the ratios of abrin and abrine over the course of plant development or toxin extraction.

The aim was to develop a quantitative method for the analysis of abrine, as an alkaloid marker of contamination by abrin, by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis, in beverage samples as a fast "first-pass" screening method. The cleanup and extraction procedures of abrine by SPE from a variety of beverages were optimized. The extraction efficiency by optimized solid-phase extraction (SPE) cleanup was then compared to a liquid−liquid extraction cleanup

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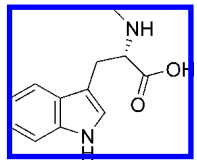

Figure 1. Structure of abrine, an indole alkaloid marker of the toxic glycoproteins, abrin.

protocol, modified from a previously published method for chemical warfare agent analysis in foods (*14*). Additionally, the stability of abrine at various temperatures over several weeks was investigated. This method allows for precise quantitative recovery of abrine from beverages by Strata-X SPE and useful qualitative screening by liquid−liquid extraction with low limits of detection for a variety of matrices.

MATERIALS AND METHODS

Chemicals and Reagents. Abrine was purchased from MP Biomedicals (Solon, OH). Ammonium hydroxide and formic acid were from Sigma-Aldrich (St. Louis, MO). LC-grade, 18 MΩ DI water filtered by a Millipore filtration system was used for all standard preparation procedures, SPE column conditioning, and for LC chromatographic mobile phase preparation. LC-grade solvents methanol and acetonitrile were from Fisher Scientific (Fair Lawn, NJ), as were all other chemicals unless specified otherwise. All beverages (bottled water, cola, juice drink, 1% low fat milk, and bottled tea) were purchased at a local grocery store.

Preparation of Standards. *Preparation of Calibration Standards.* A stock solution (2000 μg/mL) of abrine was prepared by dissolving 7.3 mg into 3.65 mL of water/methanol (50/50, v/v) and sonicating for 5 min. Calibration standards were prepared by first creating a working stock solution of 100 μg/mL in water/methanol (90/10, v/v). This working stock solution was used to prepare a high calibration standard of 10 μg/mL, which was then serially diluted to create six additional calibration standards, with the lowest standard at 0.05 μg/mL, all in water/methanol (90/10, v/v).

Preparation of Standards for Optimization of SPE Method. To optimize the SPE method, several standards were prepared at 1 μg/mL with pH adjustment to 2, 3, 4, 6, 7, and 10 by the dropwise addition of formic acid or ammonium hydroxide as appropriate. All tests of extraction procedures were conducted in triplicate.

Method Verification Experiments. For method validation experiments, a second working stock solution of 50 μg/mL was prepared in water/acetonitrile (50/50, v/v). This working stock solution was used to spike two sets of beverages at two fortification levels, 0.5 and 0.05 μg/mL, prior to the extraction by SPE (set one) and liquid−liquid extraction (set two).

Assessment of Abrine Stability. Finally, using the stock solution (2000 μg/mL) of abrine, three standards at 10 μg/mL were prepared in a solvent system of water/methanol (90/10, v/v) and stored in amber glass at 23, 4, and 0 °C.

Instrumental Conditions. A Thermo LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) was first calibrated per vendor specifications and then tuned by infusing a 61 μg/mL solution of abrine in water/methanol (50/50, v/v) and 0.1% formic acid at 10 μL/min by electrospray ionization in positive ion mode (*m/z* 219.2 $[M + H]^+$) (**Figure 2A**). Product ions were also recorded (*m/z* 188.2 and *m/z* 132.2; **Figure 2B**) and agreed with those of a previous report (*1*). The collision energy for the formation of the product ions was optimized at 36%. Settings for the mass spectrometer were as follows: spray voltage, 4.0 kV; sheath gas flow set to 27 arbitrary units; auxiliary gas set to 2.8 arbitrary units; source temperature, 300 °C; and capillary voltage, 9 V.

A Finnigan Surveyor (Thermo Fisher Scientific) LC system was utilized for chromatographic separation. The system consisted of an in-line mobile phase degasser, quaternary pump, autosampler, heated column compartment, and PDA Plus photodiode array detector. A 1 μL sample extract was injected onto a 150 mm × 2.1 mm i.d., 5 μm, Eclipse XDB C18 analytical column (Agilent Technologies, Santa Clara, CA). Solvent A was acetonitrile, and solvent B was 0.1% formic acid in water. Isocratic conditions of 20% A/80% B were used with a 200 μL/min flow rate for the duration of the program. Abrine eluted at 2.02 min.

Abrine was monitored by multiple reaction monitoring, including the transitions of *m/z* 219.2 → *m/z* 188.2 (quantitation ion) and *m/z* 219.2 → *m/z* 132.2 (confirmation ion). Example chromatograms of a standard, samples by liquid−liquid extraction and SPE, and blank are shown in **Figure 3**.

Quantitation was by linear regression with 1/X weighting from 0.05 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.

Preparation of Samples for Analysis. *SPE Cleanup.* Following optimization of the SPE protocol, abrine-spiked beverages were prepared using the Strata-X SPE cartridge columns using the following conditions. The columns were conditioned with 2 mL of methanol followed by 2 mL of water. Then, 2 mL of beverage sample (with pH adjusted to 3−6 with formic acid if pH adjustment was necessary) was loaded onto the column and allowed to elute through the column by gravity. The columns were washed with 600 μL of 95/5 water/methanol. The columns were then treated with 1 mL of methanol, and this fraction, which contained abrine, was collected into a 1.5 mL amber autosampler vial. Samples were stored at 0 °C prior to analysis LC/MS/MS.

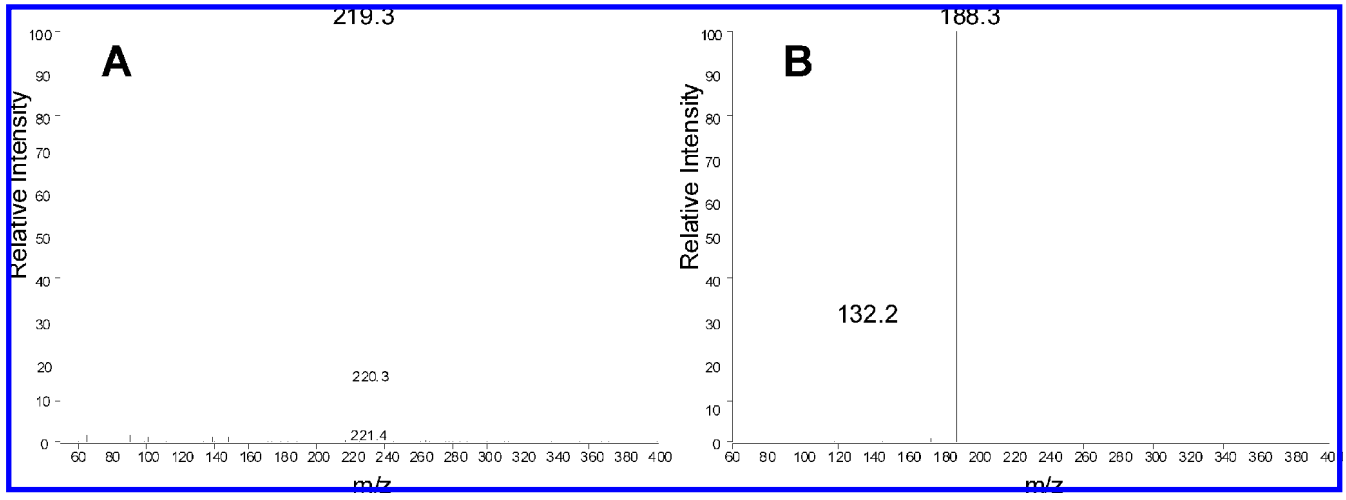

Figure 2. (**A**) Parent ion spectrum and (**B**) product ion spectrum of pure abrine (*m/z* 219.25, $[M + H]^+$) standard prepared in methanol/water (50/50, v/v).

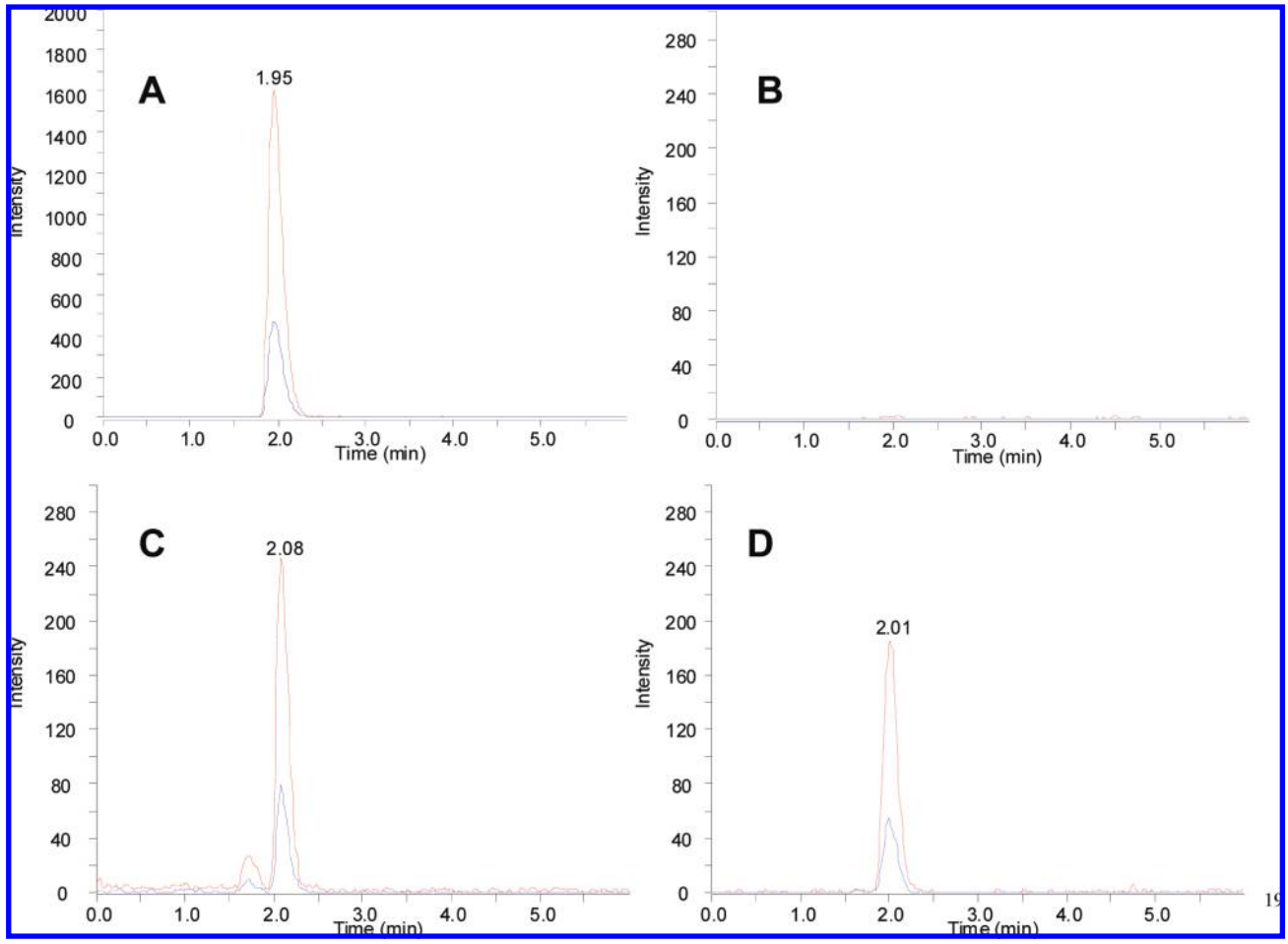

Figure 3. Example chromatograms of an abrine standard at 0.5 μg/mL (**A**), blank (**B**), bottled tea spiked at 0.05 μg/mL and cleaned up by SPE (**C**), or liquid−liquid extraction (**D**).

Liquid−Liquid Extraction. For this protocol, a method described by Kolakowski et al. (*14*) was modified. Briefly, 2 mL of beverage sample was pipetted into a 7 mL borosilicate glass scintillation vial followed by 2 mL of acetonitrile/water (75/25, v/v). The vials were capped and well mixed and allowed to equilibrate at room temperature for 10 min before centrifuging at 2000*g* for 10 min. One milliliter of the top acetonitrile layer, containing abrine, was filtered into a 1.5 mL autosampler vials using 0.25 μm PTFE (13 mm) filters. Samples were stored at 0 °C prior to analysis.

Method Verification. To verify the method, beverages were fortified with abrine from working stock solution of 50 μg/mL, at two different concentrations, 0.5 and 0.05 μg/mL. The fortification concentrations corresponded to a midpoint in the calibration curve (0.5 μg/mL), and the fortification level of 0.05 μg/mL corresponded to the target method detection limit (MDL) of 0.06 μg/mL calculated based upon toxicity values and portion size. One set of fortified samples ($n = 3$ for both fortification levels and $n = 3$ for unspiked control samples) was then extracted by Strata-X SPE. A second set of these fortified samples and unspiked control samples ($n = 5$) were then also extracted by liquid−liquid extraction as described for comparison of the two cleanup methods.

Storage Stability. The three 10 μg/mL standards (10 mL volume) were stored at 0, 4, and 23 °C in amber glass bottles. At each time point, 500 μL aliquots were analyzed, in triplicate, just after standard preparation (time, $t = 0$ days), at 1 day ($t = 1$ day), 1 week ($t = 7$ days), and 3 weeks ($t = 21$ days).

Statistical Analyses. All statistical analyses (Student's t test or single-factor analysis of variance) for determination of significance in differences between sample groups were completed using Analysis ToolPak from Microsoft Excel.

RESULTS AND DISCUSSION

Method Characteristics. The calibration curve has a linear range of 0.05 to 10 μg/mL, with an R^2 value of 0.9955 or greater. The MDL was 0.025 μg/mL, where the signal-to-noise (S/N) ratio of the *m/z* 132.2 confirmation ion was 4.47. Additionally, the ratio of the peak areas for the quantitation ion (*m/z* 188.2) vs the confirmation ion (*m/z* 132.2) was determined as an added measure of confirmation. At the 0.025 μg/mL level, this ratio had a 36% relative difference as compared to the ion ratio of the 0.05 μg/mL standard, which was the limit of quantitation (LOQ). At 0.05 μg/mL, the S/N of the *m/z* 132.2 confirmation ion was 6.0, and the S/N for the quantitation ion (*m/z* 188.2) was 11.0. The ratio of the peak areas for the two ions was 3.395 at this standard level. When samples were extracted using Strata-X SPE at the low fortification level of 0.05 μg/mL, the mean S/N was 7.87 [relative standard deviation (RSD) of 23%] for the confirmation ion, and the mean ratio of peak areas for the two ions was 3.361 (RSD of 6.7%) with a relative difference of 1% as compared to the ion ratio of the 0.05 μg/mL standard. Samples cleaned up by liquid−liquid extraction had a mean S/N of 7.00 (RSD of 14%) with a relative difference of 0.44% for the ratio of the peak areas of the two ions (mean of 3.410) as compared to the ratio of the 0.05 μg/mL standard.

The within-run and between-run variability were calculated for all standards and for the bottled tea matrix. The within-run standards were analyzed over four uninterrupted, continuous days. The RSD for all standards was 6.8% or less, and with

Table 1. Recovery of Abrine Spiked into Beverages at Two Fortification Levels and Extracted by SPE vs Liquid−Liquid Extraction[a]

	mean % recovery (RSD %)					
	0.5 μg/mL			0.05 μg/mL		
beverage	SPE	liquid−liquid extraction	$p =$	SPE	liquid−liquid extraction	$p <$
water	94 (8.4%)	77 (3.6%)	**0.0035**	94 (6.4%)	48 (14%)	**0.0001**
tea	107 (6.6%)	101 (3.1%)	0.0993	111 (4.6%)	51 (6.6%)	**0.0001**
cola	101 (9.1%)	96 (2.6%)	0.2627	107 (16%)	59 (8.8%)	**0.0001**
juice drink	107 (2.7%)	85 (6.7%)	**0.0007**	97 (2.4%)	48 (19%)	**0.0001**
milk	92 (0.5%)			88 (7.6%)		

[a] *p* values in bold text indicate significance.

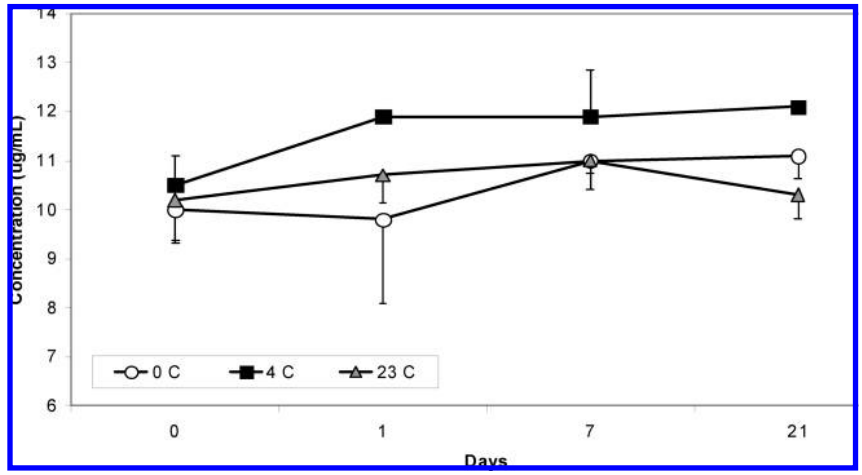

Figure 4. Effect of time and temperature on abrine stability.

one notable exception at 0.05 μg/mL, the relative percent difference of the calculated concentration from gravimetric concentrations was 9.4% or less. For the between-run samples, standards were analyzed multiple times ($n \geq 5$) over 115 days. The RSD was 9.1% or less, and the relative percent difference of the calculated concentration from gravimetric concentrations was 5.4% or less. One matrix sample, bottled tea, was also analyzed repeatedly within 1 day by both liquid−liquid extraction ($n = 5$) and SPE ($n = 3$) at the two fortification levels. The RSD (%) of the measurements was 3.1 (0.5 μg/mL spike level) and 3.9% (0.05 μg/mL spike level) for liquid−liquid extraction, respectively, and 6.6 (0.5 μg/mL spike level) and 4.6% (0.05 μg/mL spike level) for SPE, respectively. When these samples were re-extracted by liquid−liquid extraction and analyzed 10 days later, the RSD for 0.5 μg/mL was unchanged (4.2%). The RSD at 0.05 μg/mL increased to 17%.

SPE Optimization. The effects of matrix pH and the necessity of a column washing step on extraction efficiency of abrine standards were evaluated when using various SPE columns, including Oasis MCX and Oasis HLB (both 60 mg/3 mL capacity; Waters Corp., Milford, MA), BondElut C18 (100 mg/1 mL capacity; Varian, Walnut Creek, CA), and Strata-X C18 (30 mg/1 mL capacity; Phenomenex, Torrance, CA). Optimal recoveries of abrine from acidified samples were achieved using the Strata-X C18 SPE column with inclusion of a column washing step prior to elution. Recoveries of abrine standards prepared at 1 μg/mL in water, with pH adjusted to 2−6 with formic acid, ranged from 90 to 111% with a RSD of 7.3% or less.

Method Verification. *SPE.* Beverages (2 mL; $n = 3$ for each beverage at each fortification level) were spiked with abrine to obtain fortification levels of 0.5 and 0.05 μg/mL. Recovery of abrine from these samples is shown in **Table 1**. Briefly, abrine was quantitatively recovered from all five matrices (92% recovery or better) with an RSD of 9.1% or less at the 0.5 μg/mL level. At 0.05 μg/mL, the minimum recovery of abrine was 88% (low fat milk), and the maximum recovery was 111% (bottled tea). With the exception of cola, where the RSD was 16%, the RSD for all other matrices was 7.6% or less, indicating good precision.

Liquid−Liquid Extraction. At these same fortification levels (0.5 and 0.05 μg/mL), the use of the liquid−liquid extraction protocol was less robust. At 0.5 μg/mL, the recovery of abrine from water, tea, cola, and juice drink ranged from 77 to 122%, although the RSD was 6.7% or less. At 0.05 μg/mL, the recovery of abrine was poor and ranged from 48 (water and juice drink) to 60% (tea and cola) with poorer precision (RSD ranged from 6.6 to 19%).

Differences in recovery between the two extraction methods were determined using the two-sided Student's *t* test assuming equal variances. At 0.5 μg/mL, recovery of abrine by SPE was significantly higher ($P < 0.01$) for water and juice drink as compared to recovery by liquid−liquid extraction. The differences in recoveries between SPE and liquid−liquid extraction for the matrices of cola and tea were not statistically significant. At 0.05 μg/mL, the differences in recovery of abrine between methods were highly statistically significant ($P < 0.001$) for water, tea, cola, and juice drink with better abrine recoveries reported for Strata-X SPE preparation method.

Storage Stability. Abrine (10 μg/mL) solutions were stored at 0, 4, and 23 °C and analyzed at 0, 1, 7, and 21 days after sample preparation (**Figure 4**). Briefly, there was no statistically significant difference between measured concentrations of abrine at time 0 vs time 21 days for standards stored at 0 and 23 °C. At 4 °C, the difference in standard concentration at 0 and 21 days was slightly statistically significant ($P = 0.0436$). This increase in concentration over time may be due to the evaporative loss of methanol. However, the differences in measured concentration between the abrine standards stored at the three temperatures at this 21 days time point were not statistically significant.

Abrine can be easily prepared for LC/MS/MS analysis from beverages by both liquid−liquid extraction and SPE protocols, as demonstrated. The utility and effectiveness of SPE cartridges at extracting abrine from aqueous matrices were first evaluated for different sorbents and for columns from different manufacturers. Use of the Strata-X C18 SPE column resulted in optimal recovery and high precision. Using this optimized protocol for SPE preparation method and a previously published method for liquid−liquid extraction (*14*), the method was verified by extracting abrine at two fortification levels (0.5 and 0.05 μg/mL) from various beverages, including bottled water, cola, juice drink, bottled tea, and 1% low fat milk. The MDL for abrine (based upon SPE cleanup and concentration extraction protocol) in beverages was 0.025 μg/mL, and the LOQ was 0.05 μg/mL.

When abrine was added at the lower fortification level (0.05 μg/mL) to beverages and extracted using an optimized Strata-X C18 SPE protocol, recoveries ranged from 88 to 111% with an RSD of 16% or less. At this same fortification level, the recovery of abrine by liquid−liquid extraction was not quantitative (ranged from 48 to 59%, RSD of 19% or less). At the 0.5 μg/mL fortification level, the recovery of abrine by SPE ranged from 94 to 107% with an RSD of 9.1% or less. When using liquid−liquid extraction at this same fortification level of 0.5 μg/mL, abrine recovery ranged from 77 to 101%, with an RSD of 6.6% or less. Thus, the SPE method is a good method for quantitation at low levels (at 0.05 μg/mL) of abrine, and the liquid−liquid extraction method is equivalently good for screening for abrine at higher levels of ≥0.5 μg/mL.

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