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Quantitative Analysis of Chemical Warfare Agent Degradation Products in Beverages by Liquid Chromatography Tandem Mass Spectrometry

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Though chemical warfare agents (CWAs) have been banned by the Chemical Weapons Convention, the threat that such chemicals may be used, including their deliberate addition to food, remains. In such matrixes, CWAs may hydrolyze to phosphonic acids, which are good surrogate markers of CWA contamination. The method described here details the extraction of five CWA degradation products, including methylphosphonic acid (MPA), ethyl-MPA, isopropyl-MPA, cyclohexyl-MPA, and pinacolyl-MPA, from five different beverages by strata-X solid phase extraction cartridges. Samples were analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) with multiple reaction monitoring. The limit of quantitation ranged from 0.05 to 0.5 ng on-column, and the limit of detection was >0.02 ng on-column. Beverages were fortified with the five phosphonic acids at 1 μg/mL and 0.25 μg/mL and quantitated using both an internally standardized method and matrix-matched standards. Reasonable recoveries (>50%) were achieved for ethyl, isopropyl, cyclohexyl, and pinacolyl-MPA for most matrixes.

KEYWORDS: Phosphonic acids; chemical warfare agents; liquid chromatography; mass spectrometry; food

INTRODUCTION

The highly toxic organophosphate nerve agents, including G and V agents, are considered to be the most lethal of the classical chemical warfare agents (CWAs) (*1*). These CWAs bind irreversibly to acetylcholinesterase in the central and peripheral nervous system, resulting in toxic accumulation of acetylcholine (*1*, *2*). The threat remains that such chemicals will be used, including their deliberate addition to food (*3*), despite being banned since 1997 by the Chemical Weapons Convention. Because of this concern, reliable analytical methods for the detection and quantitation of CWAs and their hydrolytic degradation products, namely, alkyl methylphosphonic acids, are required for complex matrixes. Such protocols may be of interest to diverse analytical laboratories for application in multiple matrixes.

These phosphonic acid hydrolytic degradation products are relatively persistent and significantly less toxic than the parent CWA and as such, are good surrogate marker compounds of CWA contamination. The hydrolysis reactions are catalyzed by a variety of different chemicals. Hydrolysis rates are dependent on the effects of temperature, pH, and cation and anion concentrations, though the hydrolysis of CWAs is generally fairly rapid. Hydrolysis reactions may even be accelerated because of the greater availability of a wider range of catalysts (*4*) in heterogeneous matrixes, which may be the case for foods. Analysis of such degradation products would be important for identifying foods contaminated by CWAs because levels may have fallen below the CWA method detection limits by the time contaminated foods are identified and analyzed. The contamination of foods by CWAs presents an analytical challenge as there are few methods that have been described and validated for either CWAs or their phosphonic acid degradation products in these complex matrixes (*3*, *5*).

Previous methods for the analysis of CWAs and phosphonic acids from matrixes such as water, soil, and urine with analysis by predominantly gas chromatography/mass spectrometry and tandem mass spectrometry (GC/MS and GC/MS/MS) with various interfaces (*6*−*9*) have been described. While GC/MS is the most common analytical technique for the analysis of CWAs, the low-volatility phosphonic acid degradation products require extensive extraction and derivatization prior to analysis with this technique. However, more rapid and high-throughput procedures for sample preparation have been recently developed for GC/MS analysis (*16*). Liquid chromatography mass spectrometry and tandem mass spectrometry (LC/MS and LC/MS/MS) systems have also been utilized with a variety of interfaces, including atmospheric pressure chemical ionization (*10*−*12*), electrospray ionization (ESI) (*13*−*15*), flow injection high field asymmetric ion mobility (*5*), and inductively coupled plasma (*3*).

A common preparation technique for food samples is solid phase extraction (SPE). To our knowledge, there are no descriptions of SPE protocols for the cleanup of phosphonic acids from foods and beverages. Previous SPE protocols described in the literature (*7*, *8*, *13*, *17*, *18*) for quantitative recovery of phosphonic acids have met with varying success. Thus, the objectives of this work were to develop a quantitative SPE protocol for the

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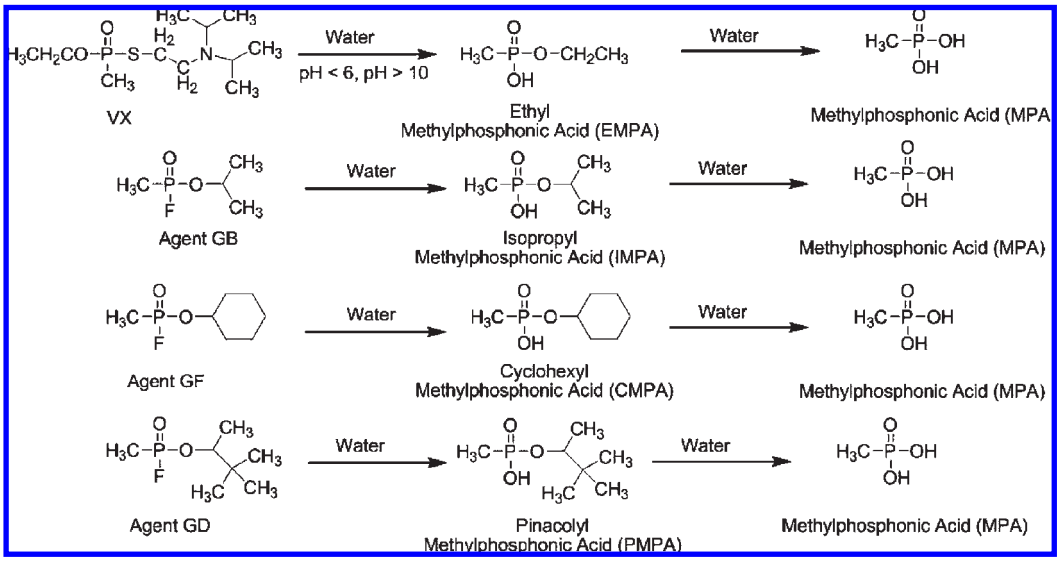


Figure 1. Hydrolysis degradation products, including ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (IMPA), cyclohexyl methylphosphonic acid (CMPA), and pinacolyl methylphosphonic acid (PMPA), of nerve agents VX, GB (sarin), GF (cyclosarin), and GD (soman). The four alkyl methylphosphonic acids may then further degrade to methylphosphonic acid (MPA) (*6*).

Table 1. Target Method Detection Limits (MDLs) for Various Nerve Agents

name	LD_{50} (μg/kg)	route	species	solubility (μg/mL)	MDL (μg/mL) for beverages
VX	100	oral	rat	30000	0.30
sarin, GB	550	oral	rat	miscible	1.6
cyclosarin, GF	350	dermal[a]	human	4.2	1.0
soman, GD	400	oral	rat	21000	1.2

[a] No oral LD_{50} values are reported for any species.

extraction of phosphonic acid degradation products (**Figure 1**) (*6*) of classical CWAs, including GB (sarin), GD (soman), GF (cyclosarin), and VX, from various beverages with analysis by LC/MS/MS at sub-ppm levels. The target method detection limits (MDL) were calculated using oral LD_{50} values of the parent CWA compounds, an assumed consumer body weight of 70 kg, beverage portion size of 236 mL, and sensitivity factor of 100 to account for exposure by vulnerable populations [thus, MDL = (LD_{50} × body weight)/(portion size × sensitivity factor)]. The calculated target MDLs are included in **Table 1** and range from 0.30 μg/mL to 1.6 μg/mL.

MATERIALS AND METHODS

Chemicals and Reagents. Methylphosphonic acid (MPA; 98% purity), ethyl methylphosphonic acid (EMPA; 98% purity), pinacolyl methylphosphonic acid (PMPA; 97% purity), and GC-grade ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO). Individual analytical standards (99% purity, prepared in methanol) of isopropyl methylphosphonic acid (IMPA) and cyclohexyl methylphosphonic acid (CMPA), both at 1000 ± 31 μg/mL, and the isotopically labeled internal standards methylphosphonic acid-D_3 (MPA-D_3) and pinacolyl methylphosphonic acid-$^{13}C_6$ (PMPA-$^{13}C_6$), both at 100 μg/mL, were from Cerilliant (Round Rock, TX). HPLC-grade water (18 MΩ) was prepared using a Barnstead Nanopure filtration system. HPLC-grade solvents including methanol and acetonitrile were from Fisher Scientific, as were all other chemicals unless specified otherwise.

Beverages, including apple juice, bottled water, cola, whole milk, and juice drink (containing less than 15% juice), were purchased from a local store and stored at 4 °C until use.

Preparation of Standards. Individual stock solutions of MPA, EMPA, and PMPA were prepared by weighing approximately 10 mg and dissolving in 25 mL of methanol. Standards of IMPA and CMPA were purchased at concentrations of 1000 μg/mL in methanol, and the internal standards (IS) of MPA-D_3 and PMPA-$^{13}C_6$ were purchased at concentrations of 100 μg/mL in methanol. Composite stock solution I containing 50 μg/mL each MPA, EMPA, IMPA, CMPA, and PMPA was prepared in methanol. Composite stock solution II containing 10 μg/mL each MPA, EMPA, IMPA, CMPA, and PMPA was also prepared in methanol. The IS mix solution was prepared in methanol for a final concentration of 25 μg/mL for both MPA-D_3 and PMPA-$^{13}C_6$.

Assessment of Standard Purity. Calibration standards of MPA ranging from 0.01 μg/mL to 10 μg/mL were prepared and analyzed by LC/MS/MS. Individual analytical standards at 1 μg/mL of EMPA, IMPA, CMPA, and PMPA were prepared and analyzed by LC/MS/MS and the amount of MPA, the primary breakdown product of the alkyl methylphosphonic acids (**Figure 1**), was quantified for each standard. Only the PMPA standard was found to contain MPA.

Preparation of Calibration Standards. Calibration standards containing MPA, EMPA, IMPA, CMPA, and PMPA were prepared by serially diluting composite stock solution II with 20/50/30 acetonitrile/methanol/water (v/v/v) containing 0.1% formic acid. Concentrations of the resulting calibration standards ranged from 0.005 μg/mL to 5 μg/mL. Fifteen microliters of the IS mix solution was then added to each calibration standard such that each IS had a final concentration of 0.25 μg/mL for an analyte/IS ratio of 0.02 to 20. The standards were stored at 0 °C until analysis.

Matrix-matched calibration standards containing MPA, EMPA, IMPA, CMPA, and PMPA were also prepared from 0.01 μg/mL to 1 μg/mL for each of the matrixes included in this study (bottled water, apple juice, juice drink, cola, and whole milk) that were cleaned up using the strata-X SPE system described below. The standards were stored at 0 °C until analysis.

Analyte Stability. Standards of 0.5 μg/mL were prepared in 20/50/30 acetonitrile/methanol/water (v/v/v) containing 0.1% formic acid from composite stock solution II and stored at 0 °C, 4 °C, and 23 °C for 1, 7, 14, and 28 days before being analyzed in triplicate by LC/MS/MS to assess general standard stability. To assess the stability of the phosphonic acids in matrix, apple juice samples containing 1 μg/mL of the phosphonic acid mix from composite stock solution II were stored at 4 °C and analyzed at

1, 7, and 14 days by LC/MS/MS after strata-X SPE cleanup (described below).

Sample Preparation by SPE. The phosphonic acids were extracted from beverage and drinking water samples using two strata-X SPE cartridges (33 mg/1 mL; Phenomenex, Torrance, CA) connected in series. Both cartridges were conditioned with 2 mL of methanol followed by 2 mL of water containing 1.0% formic acid. The sample (0.5 mL, with the exception of milk to which an additional 0.5 mL of reagent water + 0.1% formic acid was added) was acidified with formic acid such that the final acid concentration was 10% formic acid (v/v). The acidified sample was loaded onto the top cartridge and allowed to drip through by gravity to the second cartridge connected below. The top cartridge was dried by applying positive pressure using a 5-mL plastic syringe that was fitted with a latex rubber gasket to form a good seal between the syringe and the flanges of the cartridge. Any remaining sample in the hold-up volume of the top cartridge was hence pushed to the bottom cartridge. This bottom cartridge was dried as well by first removing the top cartridge and then applying positive pressure with the syringe to the flanges of the bottom cartridge.

To elute the phosphonic acids, the top cartridge was replaced above the bottom cartridge, and 0.5 mL of water containing 0.1% formic acid was added to the top cartridge. Positive pressure was applied to both cartridges in turn, and the eluent was collected into a 1.5-mL amber glass autosampler vial. Then, 0.5 mL of 50/50 methanol/acetonitrile containing 0.1% formic acid was applied to the top cartridge. Positive pressure was applied, and the eluent was collected before going through the second column. Finally, a 0.5 mL aliquot of methanol containing 0.1% formic acid was added and collected into the autosampler vial containing the two previous fractions after positive pressure was applied after going through both columns. The IS mix solution containing MPA-D_3 and PMPA-$^{13}C_6$ was added before the vials were capped for a final IS concentration of 0.25 μg/mL. The vials were stored at 0 °C until analysis.

Instrumental Conditions. A Waters Alliance HT 2795 liquid chromatograph with a Waters Micromass Quattro Micro *api* triple quadrupole mass spectrometer (Waters Corporation, Milford, MA) was used for analysis of phosphonic acids. The Waters 2795 HPLC consisted of a quaternary pump, in-line mobile phase degasser, temperature-controlled autosampler (maintained at 15 °C), and column compartment (maintained at 30 °C). Ten microliters of phosphonic acid standard mix or prepared sample was injected onto a 150 × 2.1 mm i.d., 3.5 μm particle Atlantis C18 analytical column (Waters Corp.). Mobile phase A was water + 0.1% formic acid, and mobile phase B was acetonitrile + 0.1% formic acid. The gradient program was as follows: at 0 min, 100% A (hold for 4 min) to 55% A at 5 min (hold for 4 min) to 0% A at 10 min (hold for 3 min), returning to 100% A within 1 min, and holding for 6 min for a total run time of 20 min. A constant flow rate of 0.2 mL/min was maintained throughout. Column equilibration time between samples was 2 min. MPA and MPA-D_3 eluted at 2.49 min, EMPA at 2.96 min, IMPA at 8.80 min, CMPA at 10.10 min, and PMPA and PMPA-$^{13}C_6$ at 10.68 min (**Figure 2A** and **B**).

The Micromass Quattro Micro *api* mass spectrometer was first calibrated using a solution of sodium iodide/cesium iodide (both purchased from Sigma-Aldrich) per manufacturer's specifications. Tune parameters for each analyte and IS were established by infusing an approximately 100 μg/mL solution of each analyte or IS prepared in acetonitrile/water (50/50, v/v) containing 0.1% formic acid at 20 μL/min. The instrument was operated in both positive and negative ion modes. Ion transitions listed in **Table 2** were monitored for each analyte and IS as listed. Settings were as follows: capillary was at 3.0 kV, cone voltages were variable (**Table 2**), extractor was at 2 V, RF lens was at 0.2 V, source temperature was at 120 °C, desolvation temperature was at 300 °C, desolvation gas flow was at 500 L/h, cone gas flow was at 25 L/h, low mass and high mass resolution were at 14.5, ion energy 1 was at 0.5, entrance energy was at −1 eV, collision energies were variable (**Table 2**), exit energy was at 2 eV, low mass and high mass resolution 2 were at 15.0, ion energy 2 was at 1.5, and the multiplier was set to 650 arbitrary units. The dwell time was 0.1 ms, interchannel delay was 0.1 s, interscan delay was 0.3 s, repeats were at 1, and the span was set to 0 Da.

Quantitation was by quadratic regression with $1/x$ weighting from 0.05 μg/mL to 5 μg/mL with $n \geq 4$ measurements per standard. MPA-D_3 served as the internal standard for MPA, whereas PMPA-$^{13}C_6$ was the internal standard for EMPA, IMPA, CMPA, and PMPA. A standard curve was prepared at the beginning of each sequence run, and individual standards were included throughout the sequence list after every six matrix samples. The responses of these the check calibration standards were also included in the standard curve preparation. The phosphonic acids were analyzed using multiple reaction monitoring (MRM), and the transitions listed in **Table 2** were monitored. Example chromatograms of a standard at 0.25 μg/mL, solvent blank, and cola spiked at 1 μg/mL are shown (**Figure 2A** and **B**) when analyzed in negative ion mode. Additionally, samples were quantitated using matrix-matched standards (0.1 μg/mL to 1 μg/mL). No internal standards were utilized in this quantitation method.

Method Verification. Three sets of beverage samples, where each matrix was included in triplicate, were used to verify the method. The first set of beverage samples were fortified with 50 μL from composite stock solution II at 10 μg/mL to obtain a 1 μg/mL concentration for all phosphonic acids. The second set of samples was fortified with 12.5 μL from composite stock solution II for a final concentration of 0.25 μg/mL for all phosphonic acids. The third set of samples was not fortified and used as a control set. All sample sets were allowed to equilibrate at 23 °C for 10 min before being acidified with 50 μL of formic acid such that the final concentration of formic acid was approximately 10% (v/v), and the pH was approximately 1. For whole milk samples, an additional 0.5 mL of water + 0.1% formic acid was added. The samples were then prepared for analysis using the two-tiered strata-X SPE method described above. After cleanup, 15 μL of the IS solution mix was added to the 1.5 mL of pooled solvent fractions for a final concentration of 0.25 μg/mL. The samples were stored at 0 °C prior to analysis.

Statistical Analyses. All statistical analyses were completed using Analysis ToolPak from Microsoft Excel. Such analyses included a two-tailed Student's *t*-test assuming equal variances and single-factor analysis of variance (ANOVA).

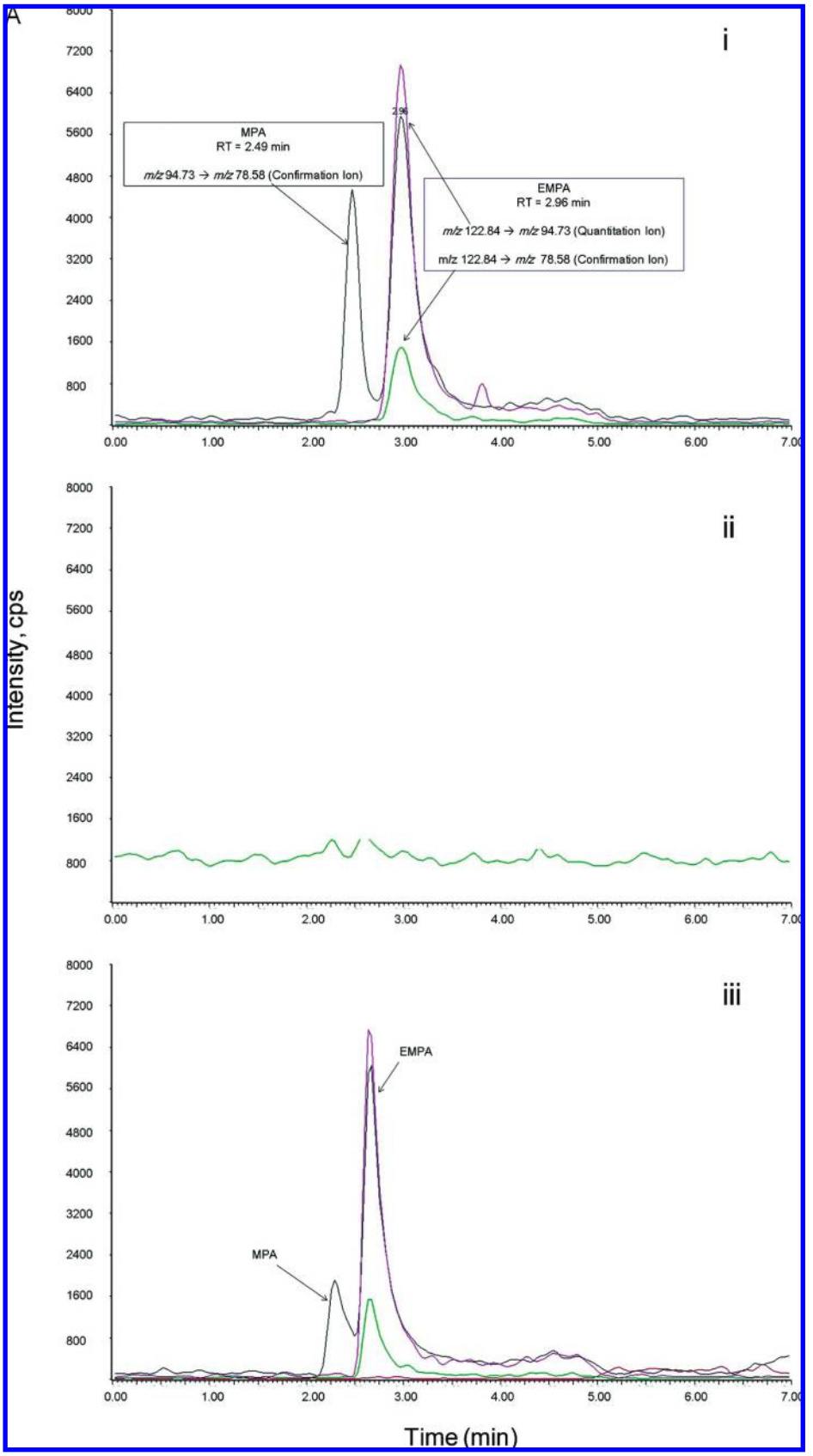
RESULTS AND DISCUSSION

Method Characteristics. The quadratic regression of the internally standardized calibration curves (with $1/x$ weighting) had a minimum R^2 value of 0.9900 (**Table 3**). The standards were analyzed over a five day period, and all were included in the standard curve preparation. The limits of detection (LODs) were determined by calculating peak-to-peak signal-to-noise ($S/N = 3$) of the confirmation ion (**Table 2**) for all standards and the limits of quantitation (LOQs) were determined by calculating the peak-to-peak S/N (where $S/N = 10$) of the quantitation ion.

To determine within-run and between-run variability, calibration standards at 0.25 μg/mL were analyzed repeatedly (**Table 3**). The within-run variability was 2.8% or less. These same calibration standards were also analyzed over a 14 day period, and the between-run variability is also shown in **Table 3** (relative standard deviation (RSD) of 7.8% or less). Apple juice samples were spiked in triplicate at 1 μg/mL with the phosphonic acid mix and analyzed after cleanup. Within-run RSD was 5.3% or less (**Table 3**). A second set of apple juice samples spiked in triplicate were extracted at 14 days of storage at 4 °C. The between-run variability for the six samples over the 14 days was 15.9% or less (**Table 3**).

Optimization of SPE Protocol. Initially, a dilute and shoot approach using a 1× dilution with acetonitrile was evaluated. Though CMPA and PMPA were unaffected by ion suppression or other matrix effects, the responses of IMPA, EMPA, and MPA were affected. Though only the preparation of beverage samples is presented here, a robust method for the preparation of more complex food matrixes is desired, thus motivating the SPE protocol development.

Riches et al. developed an SPE protocol using Oasis HLB columns (Waters Corp.), which have a polymeric sorbent phase, for cleanup of alkyl methylphosphonic acids from urine. In their method, recoveries were good (> 85%) for all analytes examined with the exception of EMPA, which was recovered at 24% (*7*). Given this earlier success with new polymeric sorbent phases, the optimization of the method presented here began by using a



similar phase found in the strata-X SPE columns (33 mg/1 mL; Phenomenex). The elution solvent was first optimized. Initially, the extraction efficiency of seven different solvents, including acetonitrile, acetonitrile containing 0.1% formic acid, 90/10 acetonitrile/ethyl acetate (v/v), methanol, methanol containing 0.1% formic acid, 90/10 methanol/ethyl acetate (v/v), and ethyl acetate were all evaluated. The various solvents were evaluated in a previous work by Mawhinney et al. and were found to have improved S/N and sensitivity with aprotic solvents, including ethyl acetate and acetonitrile, for alkyl methylphosphonic acids (*19*).

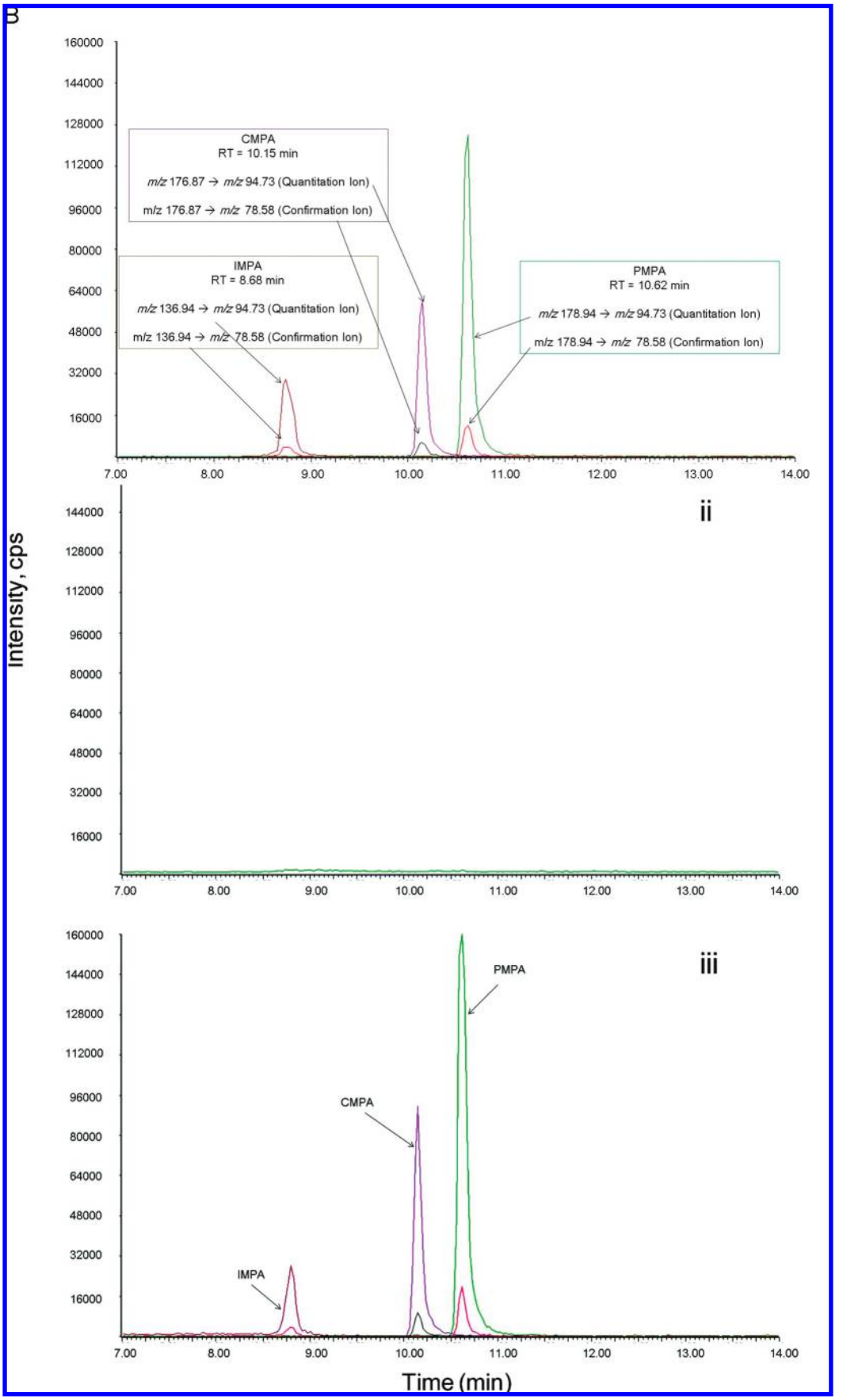


Figure 2. Example chromatograms of MPA, EMPA, IMPA, CMPA, and PMPA in a standard at 0.25 μg/mL (i), solvent blank (ii), and spiked at 1 μg/mL in cola (iii) with analysis by LC/MS/MS with multiple reaction monitoring. Ions *m*/*z* 94.7 → *m*/*z* 78.6 (MPA), *m*/*z* 122.8 → *m*/*z* 94.7 (EMPA), *m*/*z* 136.9 → *m*/*z* 94.7 (IMPA), *m*/*z* 176.9 → *m*/*z* 94.7 (CMPA), and *m*/*z* 178.9 → *m*/*z* 94.7 (PMPA) were monitored for confirmation (MPA) or quantitation by negative ion mode ESI, as indicated.

Table 2. Mass Spectrometer Settings for Phosphonic Acid Analytes and Isotopically-Labeled Internal Standards by Electrospray Ionization

	quantitation ion			confirmation ion		
analyte or IS	(polarity) transition	cone (V)	collision (eV)	(polarity) transition	cone (V)	collision (eV)
MPA	(+) *m/z* 96.7 → *m/z* 78.6	35	14	(−) *m/z* 94.7 → *m/z* 78.6	35	15
EMPA	(−) *m/z* 122.8 → *m/z* 94.7	25	10	(−) *m/z* 122.8 → *m/z* 78.6	25	18
IMPA	(−) *m/z* 136.9 → *m/z* 94.7	18	9	(−) *m/z* 136.9 → *m/z* 78.6	18	13
PMPA	(−) *m/z* 178.9 → *m/z* 94.7	25	7	(−) *m/z* 178.9 → *m/z* 78.6	25	20
CMPA	(−) *m/z* 176.9 → *m/z* 94.7	30	8	(−) *m/z* 176.9 → *m/z* 78.6	30	20
MPA-D_3 (IS)	(+) *m/z* 99.8 → *m/z* 81.7	45	15			
PMPA-$^{13}C_6$ (IS)	(−) *m/z* 185.1 → *m/z* 94.7	35	18			

Table 3. Method Characteristics for the Analysis of CWA Degradation Products by ESI-LC/MS/MS

analyte (ESI polarity)	R^2	LOD (ng on-column)	LOQ (ng on-column)	within-run variability for standard; *n* = 1 day (RSD, %)	between-run variability for standard; *n* = 14 days (RSD, %)	within-run variability for apple juice; *n* = 1 day (RSD, %)	between-run variability for apple juice; *n* = 14 days (RSD, %)
MPA (+)	0.9966	0.1	0.25	2.8	7.8	2.6	9.7
EMPA (−)	0.9925	0.25	0.5	1.2	7.6	4.4	15.9
IMPA (−)	0.9935	0.1	0.25	2.7	7.0	3.6	2.5
CMPA (−)	0.9974	0.1	0.25	2.5	4.7	2.9	2.7
PMPA (−)	0.9971	0.02	0.05	1.6	1.7	5.3	4.0

Use of acetonitrile or acetonitrile solvent systems resulted in recoveries ranging from approximately 40 to 100%. However, the RSD was generally greater than 20%, indicating poor precision. Use of ethyl acetate as an elution solvent resulted in poor recoveries for the more polar analytes (MPA and EMPA) and was not evaluated further. Methanol and acidified methanol resulted in recoveries ranging from approximately 40 to 125% recovery with a RSD of 8% or less for all analytes.

While the precision improved when methanol was used as an elution solvent compared to the use of acetonitrile, the recoveries of the very polar analytes, MPA and EMPA, were still not quantitative. Additionally, methanol adversely affected the peak shape of EMPA by causing a broadening of the peak. Therefore, it was hypothesized that adoption of an SPE method that used an ordered addition of various elution solvents containing acidified water, methanol, and acetonitrile through two SPE columns would result in quantitative recoveries of the more polar analytes with improved peak shape. Various permutations of the tiered SPE method with ordered addition of elution solvents were evaluated for optimal recovery of all analytes. In early trials, the recoveries of MPA and EMPA could be dramatically improved (>80%), though the recoveries of the more nonpolar analytes, such as CMPA and PMPA, would drop to <60% with a RSD of 20% or greater.

The optimized method of using the tiered SPE columns resulted in an elution order in which acidified water was added to both columns, 50/50 methanol/acetonitrile (+ 0.1% formic acid) was added only to the top column, and finally, acidified methanol was added to both columns as the final elution step. With this method of using the tiered SPE cartridges and ordered addition of elution solvents, the recoveries of the analytes were 52% (MPA), 84% (EMPA), 91% (IMPA), 92% (CMPA), 94% (PMPA) with a RSD of 19% or less ($n = 3$ replicates) when using reagent−water as a matrix.

Method Verification. *EMPA*. EMPA, the degradation product of VX, was recovered with good precision (RSD of 11.4% or less) when using the solvent standards containing IS for quantitation (**Figure 3**). The recoveries of EMPA ranged from 25.2% (whole milk at 1.0 μg/mL) to 79.2% (bottled water at 0.25 μg/mL) with a mean recovery of 52.9% at 1.0 μg/mL and 58.5% at 0.25 μg/mL ($n = 15$ for both levels) with $P = 0.1163$. When using the matrix-matched standards, the recoveries of EMPA ranged from 22.1% (whole milk at 1.0 μg/mL) to 88.8% (juice drink at 0.25 μg/mL). The mean recovery of EMPA when using the matrix-matched standards was 50.7% at the 1.0 μg/mL level and 63.6% at the 0.25 μg/mL level ($P = 0.1500$).

IMPA. IMPA, the degradation product of GB or sarin, was recovered with reasonable precision (RSD of 22.1% or less; **Figure 3**) with recoveries that ranged from 38.1% (apple juice at 1.0 μg/mL) to 75.6% (juice drink at 1.0 μg/mL) when using solvent standards containing IS. The mean recovery of IMPA at the 1.0 μg/mL fortification level was 52.9% and 51.6% at the 0.25 μg/mL fortification level ($P = 0.7590$). When using the matrix-matched standards for quantitation, the mean recovery of IMPA from the five beverage matrixes was 100.8% at the 1.0 μg/mL fortification level and the 105.8% at the 0.25 μg/mL level ($P = 0.5487$). The recovery of IMPA from beverages was statistically significantly higher when using matrix-matched standards at both fortification levels, with the exception of cola at 1.0 μg/mL (**Figure 3**).

CMPA. CMPA, the degradation product of cyclosarin or GF, was quantitatively recovered with good precision (RSD of 9.4% or less, **Figure 3**). Recoveries ranged from 74.4% (whole milk at 0.25 μg/mL) to 103.6% (apple juice at 0.25 μg/mL) when the solvent standards containing IS were used for quantitation. The mean recoveries of CMPA at the two fortification levels for the five matrixes were 91.5% (1.0 μg/mL) and 91.8% (0.25 μg/mL) with $P = 0.9530$. When using the matrix-matched standards for quantitation, recoveries of CMPA from the five beverages ranged from 85.0% (cola at 1.0 μg/mL) to 120.9% (apple juice at 1.0 μg/mL) with mean recoveries of 100.9% at the 1.0 μg/mL level and 103.6% at the 0.25 μg/mL level ($P = 0.5093$ with $n = 15$ for each level). The recoveries when using the matrix-matched standards were significantly higher for both fortification levels ($P = 0.0168$ for 1.0 μg/mL and $P = 0.0066$ for 0.25 μg/mL) compared to the recoveries obtained when using solvent standards containing IS.

PMPA. PMPA, the degradation product of soman or GD, was quantitatively recovered from the five beverage matrixes evaluated with good precision (RSD of 9.7% or less; **Figure 3**). When using solvent standards containing IS for quantitation, the recoveries of PMPA ranged from 89.6% (whole milk at 0.25 μg/mL) to 110.4% (apple juice at 0.25 μg/mL). The mean recovery of PMPA for the five matrixes at 1.0 μg/mL was 96.4% (RSD of 4.8%) and 98.2% (RSD of 9.9%) at the 0.25 μg/mL. When using matrix-matched standards, the recoveries ranged from 87.2% (cola at 1.0 μg/mL) to 116.1% (apple juice at 1.0 μg/mL) with

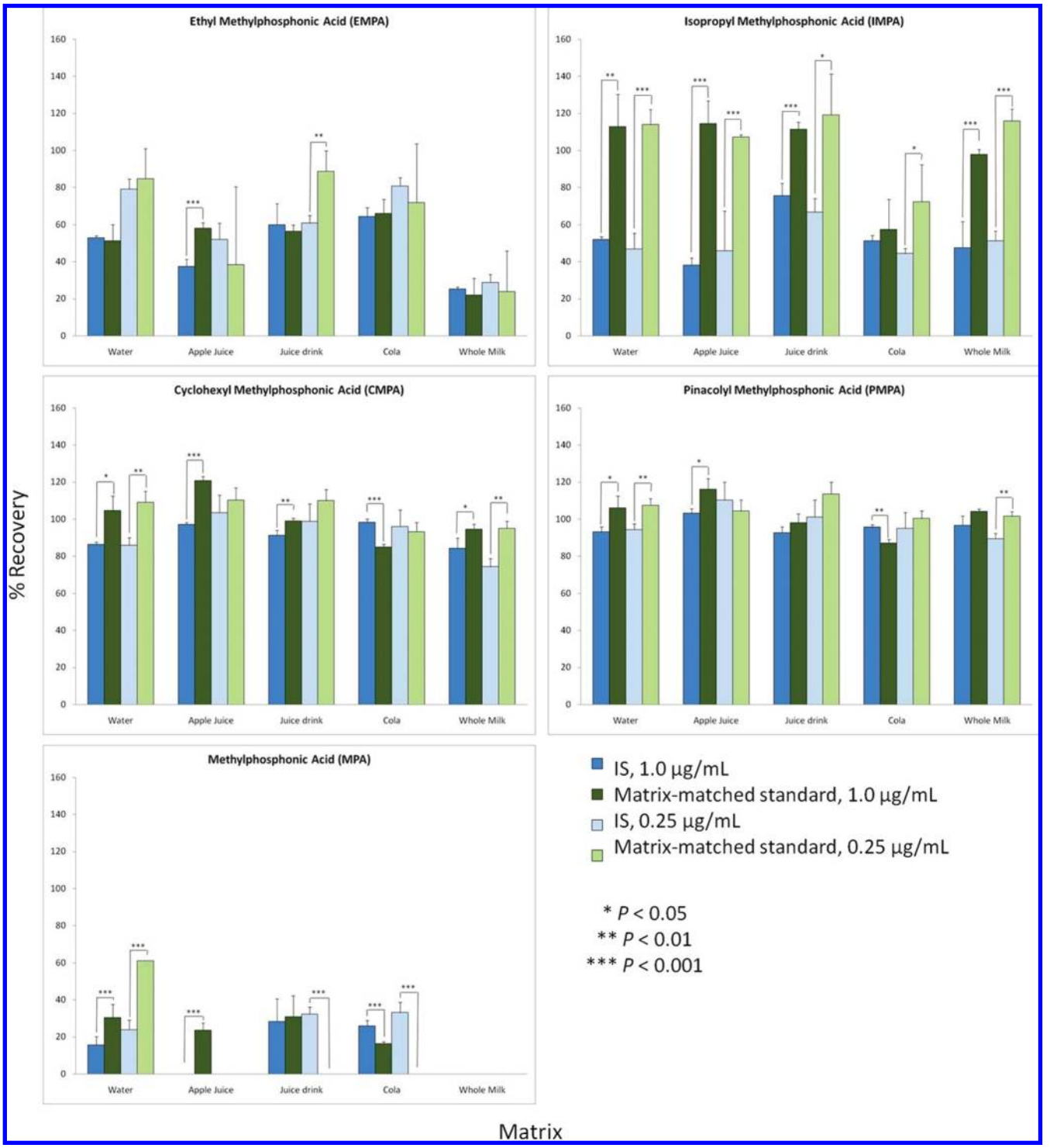


Figure 3. Recoveries of EMPA, IMPA, CMPA, PMPA, and MPA from various beverage matrixes at fortification levels of 1 μg/mL and 0.25 μg/mL.

mean recoveries of 102.4% (RSD of 10.5%) at the 1.0 μg/mL level and 105.5% (RSD of 6.2%) at the 0.25 μg/mL fortification level (n = 15 for both levels). At the 1.0 μg/mL level, the difference in recovery between the quantitation methods relying upon solvent standards containing IS versus matrix-matched standards approached statistical significance (P = 0.0555). At the 0.25 μg/mL level, the difference in recovery between the two quantitation methods was statistically significant (P = 0.0218).

MPA. In this method, MPA was poorly recovered from all matrixes, though the precision was reasonable (RSD of 12.3% or less). At the 1.0 μg/mL and 0.25 μg/mL fortification levels, MPA was recovered only from bottled water, juice drink, and cola (15.8 to 33.2% recovery) when using the solvent standards containing the IS for quantitation. When using the matrix-matched standards for quantitation, MPA was recovered from all matrixes except whole milk at the 1.0 μg/mL level (16.4% to 31.0%). At the 0.25 μg/mL level, MPA was recovered only in bottled water (61.2%). MPA levels in other matrixes were either not detectable or below the limit of quantitation at this fortification level. The use of matrix-matched standards resulted in significantly higher recovery for bottled water and apple juice, and significantly lower recovery for juice drink and cola.

Table 4. *P*-Values by Student's *t*-Test for the Determination of the Effect of Storage Time on Standard Analyte Stability when Stored in Solvent

temperature	MPA	EMPA	IMPA	CMPA	PMPA
0 °C	0.0152 (D1 vs D7)[a]	NS[b]	NS	NS	NS
	0.0130 (D7 vs D14)				
	0.0185 (D7 vs D28)				
	0.0446 (D14 vs D28)				
4 °C	0.0504 (D1 vs D14)	NS	0.0110 (D7 vs D28)	NS	0.0190 (D1 vs D28)
	0.0573 (D7 vs D14)				0.0160 (D7 vs D28)
23 °C	0.0307 (D7 vs D14)	NS	NS	NS	0.0472 (D1 vs D28)
	0.0455 (D14 vs D28)				0.0121 (D7 vs D28)

[a] *P*-values <0.05 as determined by Student's *t*-test. [b] Not significant as determined by single-factor ANOVA.

The quantitation of MPA was known to be difficult because it is a low molecular weight compound (MW = 96 g/mol) with only one product ion. Additionally, it elutes early from the analytical column (RT = 2.46 min), near the void volume, along with food matrix components that interfere with its detection. Adoption of other HPLC columns, such as HILIC phases, may be able to improve the capacity factor and resolution from food components, and is one area for future investigation. Additionally, many of these hydrolysis products, such as EMPA, IMPA, CMPA, and PMPA, originate from one specific parent CWA. Methylphosphonic acid may have come from many different parent CWAs (**Figure 1**) (*3*) after slow secondary hydrolysis reactions. Thus, MPA might not be a reliable surrogate compound of CWA contamination because of its nonspecificity and low abundance.

Comparison of Quantitation Methods. In most cases, the use of the matrix-matched standards resulted in higher recoveries for the analytes at both fortification levels for most matrixes, with the exception of EMPA. When analyzing the pooled results of EMPA recoveries, there was no statistically significant difference in recovery between the two quantitation methods (solvent standards containing IS versus matrix-matched standards) described here. Because the recoveries between the two quantitation methods were generally in good agreement, the recovery of EMPA when using strata-X SPE is matrix-dependent and is not reasonably quantitative. No ion suppression was noted for this analyte in the various matrixes analyzed. An isotopically labeled EMPA internal standard may be able to correct for the deficiencies in recoveries from more complex matrixes. However, the use of the two-tiered SPE method did improve the recovery of EMPA compared to that in the previous work of Riches et al., who recovered EMPA from acidified urine at 24% when using one the Oasis HLB SPE cartridge (*7*).

For other analytes, including IMPA, CMPA, and PMPA, the use of the matrix-matched standards for quantitation resulted in a significant increase in analyte recoveries. For certain matrixes, these analytes suffer from ion suppression that can be corrected by the use of matrix-matched standards.

Stability Study. Standards containing 0.5 μg/mL of the phosphonic acids were prepared in 20/50/30 acetonitrile/methanol/water (v/v/v) containing 0.1% formic acid and stored at 0 °C, 4 °C, and 23 °C for 1, 7, 14, and 28 days before being analyzed in triplicate by LC/MS/MS to assess general standard stability. For four of the five phosphonic acids, including EMPA, IMPA, CMPA, and PMPA, storage temperature had no significant effect on analyte stability (single-factor ANOVA, $P \geq 0.1369$) when stored in the acetonitrile/methanol/water solvent system. There was no significant effect of temperature on MPA stability between days 1, 7, and 14. At day 28, the MPA concentration in the standard stored at 23 °C was significantly lower than that when stored at 4 °C ($P = 0.0474$), though this effect was borderline significant.

Additionally, the phosphonic acid mix was spiked into apple juice at 1 μg/mL and stored at 4 °C with analysis at 1, 7, and 14 days to assess analyte stability in the matrix. The only temperature that was evaluated was 4 °C because the juice sample was opened in order to spike in the analyte, and we wished to avoid the complications of mold or bacterial growth that occurs when opened food samples are stored at room temperature over a two week time span. Additionally, the stability of these analytes was only evaluated up to 2 weeks, which is a reasonable shelf life for an opened container of juice.

EMPA. There was no statistically significant effect of temperature on EMPA stability over the 28 day period when analyzed by single-factor ANOVA ($P = 0.0833$; **Table 4**). Relative standard deviations were 16.1% or less.

EMPA concentrations in apple juice at day 14 were significantly lower than concentrations at day 1 ($P = 0.0009$) or at day 7 ($P = 0.0158$). The concentrations of EMPA measured at day 1 versus day 7 were not statistically significant by Student's *t*-test.

IMPA. There were no statistically significant differences in standard IMPA concentrations over the 28 day time period for two of the three storage temperatures. When stored at 4 °C, the determined mean peak area at day 7 was significantly lower than the mean peak area at day 28 ($P = 0.0110$ by two-tailed Student's *t*-test; **Table 4**). Relative standard deviation was 16.8% or less.

IMPA concentrations in apple juice at days 7 and 14 were significantly lower than concentrations on day 1 ($P = 0.0005$ by single-factor ANOVA), and concentrations on day 14 were significantly lower than concentrations of IMPA measured on day 7 ($P = 0.0132$ by Student's *t*-test).

CMPA. There were no statistically significant differences in CMPA concentrations in the analyzed standards over the 28 day time period (single factor ANOVA; $P \geq 0.0822$; **Table 4**). Relative standard deviation was 16.4% or less.

CMPA concentrations in apple juice measured at day 14 were significantly lower than concentrations of day 1 ($P = 0.0001$ by Student's *t*-test). There was no significant difference between day 1 and day 7 concentrations ($P = 0.5345$).

PMPA. There were no statistically significant differences in PMPA concentrations in the analyzed standards over the 28 day time period when stored at 0 °C. When stored at 4 and 23 °C, PMPA concentrations at day 1 and day 7 were statistically significantly higher than concentrations determined at day 28 by Student's *t*-test ($P \leq 0.0472$; **Table 4**). Relative standard deviation was 15.6% or less.

PMPA concentrations in apple juice were significantly lower in apple juice at day 14 compared to day 1 concentrations ($P = 0.0004$). There was no significant difference between day 1 and day 7 concentrations ($P = 0.1236$).

MPA. The differences in peak areas of MPA were not statistically significant over a 7 day time period for the three storage temperatures. At 0 °C, day 14 concentrations were significantly higher than concentrations of MPA determined on

day 1 ($P = 0.0152$), day 7 ($P = 0.0130$), and day 28 ($P = 0.0446$). Additionally, day 28 concentrations were significantly higher than concentrations at day 7 ($P = 0.0185$). These trends were repeated when MPA was stored at 4 and 23 °C (**Table 4**). MPA is the secondary breakdown product of CWAs and the primary breakdown product of EMPA, IMPA, CMPA, and PMPA. Any decrease in concentration of these analytes over the 28 day period would be reflected in increasing concentrations of MPA. Relative standard deviation was 25.6% or less.

Methyl alkylphosphonic acids, including EMPA, IMPA, CMPA, and PMPA, can be reasonably recovered from various beverage matrixes using a tiered SPE method with good precision and with good limits of detection and quantitation. Additionally, the use of matrix-matched standards can mitigate the effects of ion suppression on quantitation, especially for IMPA. MPA, EMPA, IMPA, CMPA, and PMPA are reasonably stable in a solvent standard over at least 28 days. However, all analytes suffered from stability problems when stored in apple juice over a two-week period. One aim for future work is to improve the quantitation of EMPA in various matrixes, perhaps with the use of an isotopically labeled EMPA standard. Additionally, adoption of HILIC or other column stationary phases may be important for improving the detection and quantitation of MPA, which currently elutes near the void volume.

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