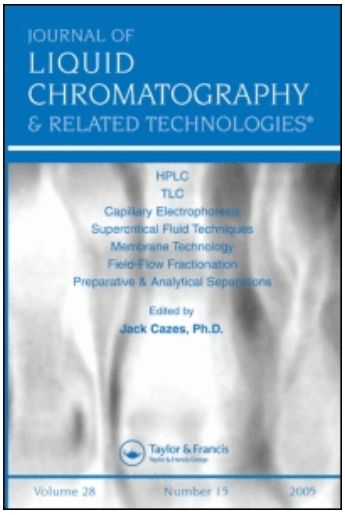


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# EVALUATION OF HPLC SYSTEMS FOR THE SEPARATION AND QUANTIFICATION OF ARSENIC COMPOUNDS FROM APPLE EXTRACTS

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# EVALUATION OF HPLC SYSTEMS FOR THE SEPARATION AND QUANTIFICATION OF ARSENIC COMPOUNDS FROM APPLE EXTRACTS

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## ABSTRACT

Several different high performance liquid chromatography (HPLC) systems were evaluated for the applicability for the determination of trace level arsenic compounds from extracts of freeze-dried apple samples. Various gradients using ion-pair reversed-phase HPLC were explored. Gradient anion-exchange HPLC, as well as a simple isocratic system, were also evaluated in this study. Two extraction procedures were used. Simple sonication by a common laboratory ultrasonic cleaner using a 50/50 (v/v) water/methanol solvent mixture and a two step procedure using initial overnight treatment with α-amylase enzyme followed by sonication with a 60/40 (v/v) acetonitrile/water solvent mixture were evaluated.

Inductively Coupled Plasma-Mass Spectrometric (ICP-MS) detection was used to measure the arsenic compounds contained

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within the apple extracts. The three most abundant arsenic compounds found in the extracts were arsenite (As III), arsenate (As V) and dimethylarsinic acid (DMAA).

## INTRODUCTION

Arsenic is present in the environment and is of concern because of pollution from industrial sources as well as its use in agriculture. Lead arsenate was widely used for many years as an insecticide and herbicide; lead arsenite was used as an insecticide. Arsenic is of interest because of the possible accumulation in food. The identification and quantification of individual arsenic compounds is of significant importance because of the differences in toxicity of arsenic compounds, which is well documented (1,2). The inorganic forms of arsenic, arsenite (As III) and arsenate (As V), are highly toxic (3), while the organic forms have varying degrees of toxicity. Monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA) exhibit a toxicity factor of one in four hundred of that of the inorganic forms (1). The inorganic compounds of arsenic have also been classified as carcinogenic (4), and MMAA and DMAA have been identified as possible cancer promoters (5). Arsenobetaine, on the other hand, is virtually non-toxic (6,7). Total arsenic concentration is, therefore, not an adequate measurement for estimating toxicity for food levels or environmental impacts (8,9).

Elemental speciation is defined among spectroscopists as the identification and quantification of the actual chemical form of an element. The identification and quantification of the chemical forms of an element is the desired goal and presents several challenges. The separation steps must be considered carefully in order to maintain the integrity of the chemical species. The extraction conditions used must be mild chemically but efficient to remove the compounds from the food stuff matrix. High sensitivity of detection is another important requirement for a project of this nature. High performance liquid chromatography (HPLC) is an efficient and straight forward technique and readily adaptable to many forms of detection. Inductively coupled plasma-mass spectrometry (ICP-MS) has been used extensively in the literature for many years as an HPLC detector for the low level determination of arsenic compounds (10,11). Therefore, HPLC/ICP-MS was chosen for this project for the same reasons.

Arsenic compounds needed to be separated efficiently from the freeze-dried apple samples and low detection levels were required. Arsenic speciation analysis using ion-pair reversed-phase HPLC has been reported previously by this research group (12,13). In the current study, gradient ion-pair reversed-phase HPLC was investigated. Also, the Hamilton PRP-X100 anion-exchange column used to separate arsenic compounds has been relatively well documented in the





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literature (14–22). In a previous apple extract study, phosphate/nitrate buffer was used in the mobile phase of the Hamilton PRP-X100 column (23). Nonvolatile inorganic buffers, especially phosphates, leave deposits on the sampler/skimmer cones and on the focusing lenses of the mass spectrometer (24). Such deposits can cause signal loss over time. Ammonium carbonate buffer was chosen for the current study to effect the separation of arsenic compounds, while leaving little residue on the mass spectrometer interface (18).

## EXPERIMENTAL

### Instrumentation

#### ICP-MS

All detection of arsenic was performed using a Perkin-Elmer (Norwalk, CT) Elan 6000 inductively coupled plasma-mass spectrometer (ICP-MS). Tuning of the ICP-MS was performed daily with multi-element solutions purchased from Perkin-Elmer. A quartz double pass spray chamber, standard quartz torch and Gem-Tip cross flow nebulizer were used. The dwell time was 200 ms, and the nebulizer gas flow was approximately 0.9 L/min and was optimized using the Perkin-Elmer software daily.

#### HPLC/ICP-MS

A Spectra-Physics (San Jose, CA) Model SP8800 or a Dionex (Sunnyvale, CA) Advanced Gradient HPLC pumping system were used to produce the chromatograms for this study. A Rheodyne (Coatati, CA, USA) Model 7010 valve was used for sample injections.

##### *Gradient Ion-Pair HPLC Conditions*

A 150 mm × 4.6 mm (id) Alltech (Deerfield, IL) Alltima 5 μm C18 column was used in all the gradient ion-pairing experiments. Two mobile phases systems were studied. The first was 10 mM in hexane sulfonic acid sodium salt and 40 mM in citric acid aqueous buffer (apparent pH 2.3). Organic content started a 2% (by volume) for mobile phase A and increased to 12% (by volume) for mobile phase B. The second system used 10 mM heptane sulfonic acid sodium salt and 40 mM citric acid aqueous buffer (apparent pH 2.3). Mobile phase A was 4% (by volume) methanol and mobile phase B was 14% (by volume) methanol. Both

used linear gradients over a thirty minute period. Flow rates were constant and were varied between 0.8 to 1.0 mL/min to check the ability to separate arsenic compounds. A 50 μL loop was used with the Rheodyne injector.

*Gradient Anion-Exchange HPLC Conditions*

A 150 mm × 4.1 mm (id) Hamilton (Reno, NV) PRP-X100 anion-exchange column was used to separate the arsenic compounds running a buffer concentration gradient. Mobile phase A was of 12.5 mM ammonium carbonate and mobile phase B was 50 mM ammonium carbonate; both mobile phases having been adjusted to pH 8.5 by the addition of dilute ammonium hydroxide. The gradient program consisted of an initial hold at 100% mobile phase A for the first eight minutes, then a linear ramp from 100% A to 100% mobile phase B during a twelve minute period and a final hold at 100% mobile phase B. Constant flow rates of 0.7 to 1.0 mL/min was used during this evaluation. A 200 μL loop was used with the Rheodyne injector.

*Isocratic Anion-Exchange HPLC System*

A 150 mm × 4.1 mm (id) Hamilton PRP-X100 anion-exchange column was used to separate the arsenic compounds running 30 mM ammonium carbonate buffer with an adjusted pH of 8.5. A flow rate of 1.0 mL/min was used, and a 200 μL loop was used with the Rheodyne injector when actual apple extract sample solutions were chromatographed.

## Reagents

All arsenic standard compounds were provided by the U.S. Food and Drug Administration, which included As (III), As (V), dimethylarsinic acid (DMAA), monomethylarsonic acid (MMAA), arsenobetaine and arsenocholine.

Doubly de-ionized water (18 mΩ-cm) was obtained from a Barnstead NanoPure treatment system (Boston, MA). Doubly de-ionized waster was used to prepare all solutions and the HPLC mobile phase. Methanol and acetonitrile were of HPLC grade (J. T. Baker Chemical Co., Phillipsburg, NJ). Glacial acetic acid, ammonium carbonate, ammonium hydroxide (J. T. Baker), and citric acid (Sigma Chemical Co., St. Louis, MO) were all ACS reagent grade. The ion-pair reagents, hexane sulfonic acid sodium salt and heptane sulfonic acid sodium salt, were HPLC reagent grade (Fisher Chemical, Fairlawn, NJ).

Ground freeze-dried apple samples were provided by the U.S. Food and Drug Administration. The samples had been cored prior to being freeze-dried and ground. Three batches were provided and identified as sample batches A, B, and C for this publication. The total arsenic levels had been previously determined by nitric acid digestion. Apple batch A had 80.9 ppb (μg/kg) arsenic, batch B had 8.2 ppb arsenic, and batch C had 21.5 ppb arsenic (23). The variety of apple or the original source were not disclosed for this paper.

## Extractions

### Sonication Extractions

The extraction procedures have been described by Caruso *et al*. (23), and will be repeated in detail here. A Branson Model 3510 Ultrasonic cleaner (Danbury, CT) was used for all sonication experiments. Accurately weighed 1.0 g samples of the freeze-dried apples were placed in 50 mL plastic centrifuge tubes. A 20 mL aliquot of the 50/50 methanol/water solvent was added to the sample and sonicated for six hours. After sonication, the tubes were centrifuged at 2500 rpm for five minutes, the extract solution removed, and the solid washed three times with approximately 5 mL of 50/50 methanol/water. The extract solution and the washes for each sample were combined and then evaporated by a Labconco Centrivap Concentrator (Labconco Corp., Kansas City, MO). The dried extracts were dissolved and diluted to 5.0 mL with the appropriate mobile phase for arsenic speciation using HPLC/ICP-MS.

Each HPLC sample solution was divided into two aliquots and diluted with either an equal volume of mobile phase to make the final sample solution, or an equal volume of an arsenic standard solution prepared in mobile phase to make the standard addition sample. All solutions were filtered with 0.2 micron nylon filters before being used in either the ICP-MS or the HPLC/ICP-MS. Blank samples were identically prepared to verify no arsenic was being introduced from an outside source.

#### *Amylase/Sonication Extraction*

Accurately weighed 0.5 g samples of the freeze-dried samples were treated with 30 mg of α-amylase (Sigma) in 10 mL of a 0.1 M solution of ammonium carbonate (J. T. Baker) with pH adjusted to approximately 7.2 by the addition of some 6M acetic acid. This solution was allowed to react overnight while being swirled at constant temperature of 37°C. [The temperature was maintained by placing a flask swirler in a controlled temperature room]. The second phase of





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extraction was done by adding 8.0 mL of acetonitrile and 2.0 mL of water and then sonicating each sample for six hours. After sonication, the solutions were transferred to centrifuge tubes. The extract solution was removed after centrifugation, and the solid residue was washed three times with the 40/60 (v/v) acetonitrile/water extraction solvent. The extraction and wash solutions were combined for each sample and the solvents were removed in the Labconco Centrivap Concentrator. The solid residues were diluted initially to 5.0 mL with mobile phase for the chromatographic solutions. Each HPLC sample solution was divided into two aliquots and diluted with either an equal volume of mobile phase to make the final sample solution or an equal volume of an arsenic standard solution, prepared in mobile phase, to make the standard addition sample. All solutions were filtered with nylon filters before being used in either ICP-MS or HPLC/ICP-MS. Blank samples were prepared identically without freeze-dried apple sample to verify that arsenic was not introduced from an outside source.



### Data Acquisition

All chromatograms used for quantification in this paper were collected using the Perkin-Elmer Elan 6000 software. The files were converted to ASCII format and analyzed by Galactic (Salem, NH) Grams/32 software. Microsoft (Seattle, WA) Excel was used to produce the accompanying chromatograms used in the figures of this paper.

### Calculations

The limits of detection for the isocratic anion-exchange chromatography were calculated by the standard way method: D.L. $= 3\sigma/\mathrm{m}$, where $\sigma$ is the standard deviation of the response noise of 100 data points in a chromatogram. The slope, m, was calculated using least squares linear regression from the peak height response of a series of standard solution injections. The standards used for the calibration curve were at the expected concentration range of the chromatographic samples and ranged from 0.5 to 10 ppb (mg/kg).

## RESULTS AND DISCUSSION

### Chromatographic Evaluation

The ion-pair reversed-phase chromatography proved to not be quite adequate for the separation of arsenic compounds. All the ion-pair systems



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evaluated had difficulty in separating arsenite (As III), arsenate (As V), and monomethylarsonic acid (MMAA). The best separation employed hexane sulfonic acid sodium salt as the ion pair reagent and a flow rate of 0.8 mL/min. min. A chromatogram of a solution containing standards using these conditions is shown in Figure 1. Arsenite (As III) and MMAA are not resolved from each other in this chromatogram. The other arsenic compounds are well separated and elute within the 30 minute gradient run. It should be noted that organic gradients are usually avoided when using ICP-MS detection; the change in organic content over the run changes the background signal due to extra load on the inductively coupled plasma. In these experiments, a shallow gradient, with only a 10% change in organic content, caused only minor drift in the baseline of the chromatograms. Also, the 12% maximum level of methanol in the mobile phase was still low enough not to cause any plasma instability. All peaks could be

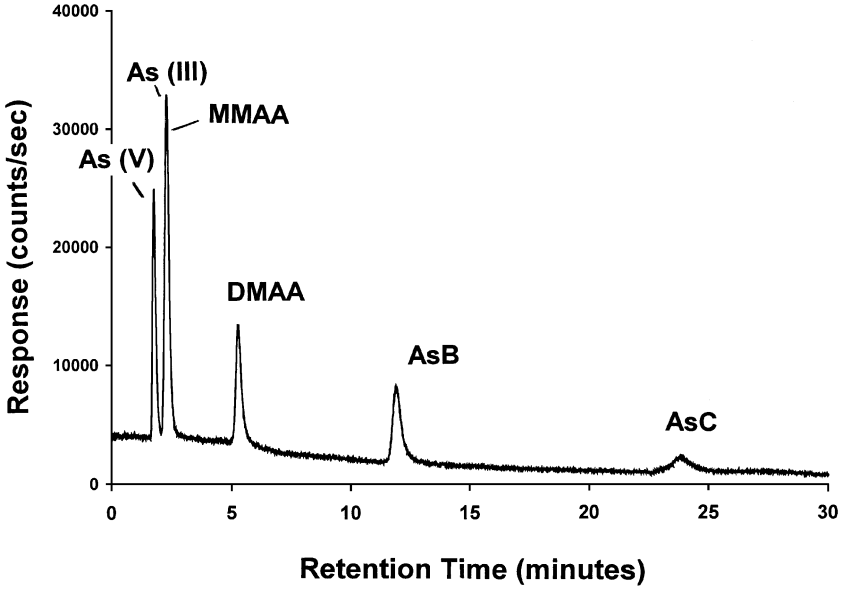


***Figure 1.*** Chromatogram of a solution containing 5 ppb ( μg/kg) each As (V), As (III), monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), arsenobetaine (AsB) and arsenocholine (AsC) using the gradient ion-pair system. Conditions: Column, Alltima C18; flow rate, 0.8 mL/min; mobile phase A, 2/98 (v/v) methanol/water 10 mM hexane sulfonic acid and 40 mM citric acid, pH 2.3; mobile phase B 12/88 (v/v) methanol/water 10 mM hexane sulfonic acid and 40 mM citric acid, pH 2.3; 30 minute linear gradient 100% A to 100% B.





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readily integrated and were not distorted by the shallow long term baseline drift. Another problem with this ion-pair chromatographic system was with the apple sample matrix. The concentration of the extracts was high to detect the low levels of arsenic compounds, which lead to rapid column performance degradation. High back pressure was noticed after a few sample injections. A guard column would not solve this problem. The samples had high levels of residual sugars, starches, etc., and an adequate sample cleanup could not be devised.

The anion-exchange gradient conditions resolved all arsenic compounds with the exception of arsenocholine and arsenobetaine. Figure 2 shows a typical chromatogram of standard solutions chromatographed at a flow rate of 0.8 mL/min. Arsenocholine and arsenobetaine are usually difficult to resolve in isocratic anion-exchange systems, but they have only been partially separated by the gradient system used here. Also, this buffer concentration gradient shows some baseline drift, and a gradient generated rise occurred near the elution time

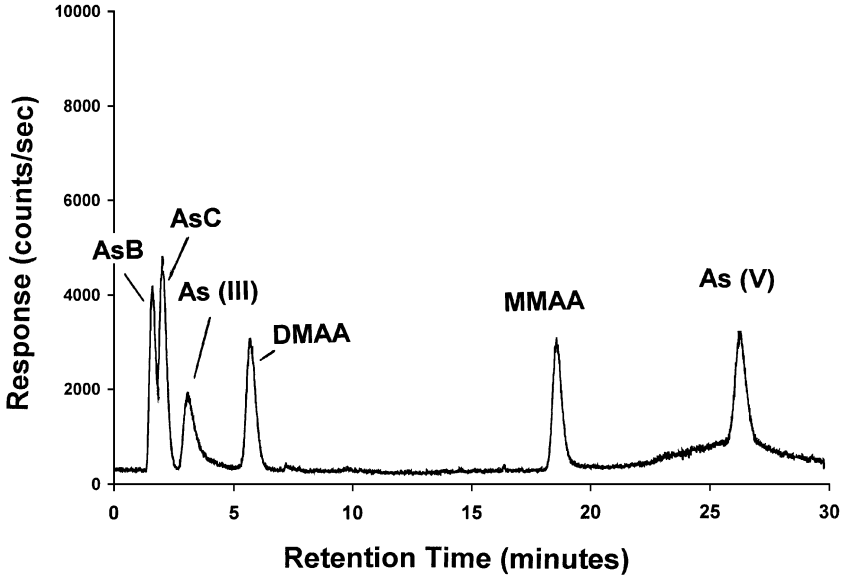


***Figure 2.*** Chromatogram of a solution containing 2 ppb (μg/kg) each arsenocholine (AsC), arsenobetaine (AsB), As (III), dimethylarsinic acid (DMAA), monomethylarsonic acid (MMAA), and As (V) using the gradient anion-exchange chromatographic procedure. Conditions: Column, Hamilton PRP-X100 anion-exchange; flow rate 0.8 mL/min; mobile phase A, 12.5 mM ammonium carbonate, pH 8.5; mobile phase B, 50 mM ammonium carbonate, pH 8.5; gradient, isocratic for 8 minutes at 100% A, 12 minute linear gradient 100% A to 100% B, then isocratic at 100% B.

for arsenate (As V). This baseline shift near arsenate was observed to be more pronounced in older and used columns. Hamilton recommends the use of a 50/49/1 methanol/water/6 N $HNO_3$ solution to regenerate their PRP-X100 columns. After using this treatment, the used columns generally had a worse baseline shift at the elution time of As (V). Retention of low levels of arsenic compounds could be a drawback with the Hamilton PRP-X100 column when using gradient elution.

In Figure 2, this baseline rise is still not significant to cause integration problems with the arsenic peak at the 2 ppb level and was reproducible in blank gradient chromatograms. Other arsenic compound gradients have had greater problems reported in the literature. A gradient system used by Branch and his co-workers (19) showed significant baseline drift from buffer incompatibility with ICP-MS. Pardo-Martinez *et al*. (25) could not obtain the level of resolution for arsenobetaine, DMAA, and MMAA, in a sodium sulfate buffer gradient system, as was demonstrated by the ammonium carbonate buffered system studied in the current work.

The ammonium carbonate gradient system was superior in separating capability as compared to the isocratic system, but as a gradient system, it had the disadvantage of requiring more run time for each sample injection. Because of the extra run time consideration, the slight baseline shift problem, and the fact that none of the apple extracts contained arsenocholine or arsenobetaine, all apple sample extracts were run for quantitative results using the isocratic system.

The isocratic anion-exchange procedure proved to be useful for apple extract analysis. A chromatogram of a standard solution containing As (III), DMAA, MMAA, and As (V) is shown in Figure 3. A typical apple sample chromatogram is displayed in Figure 4. All apple samples in this study showed only the presence of As (III), DMAA, and As (V). Peaks were identified by spiked sample solutions. Sample matrix problems did slightly affect the chromatographic performance of the anion-exchange system; the retention time of As (V) was one to two minutes shorter in sample chromatograms versus standard solutions. Therefore, all apple sample solutions were chromatographed along with a corresponding spiked solution to verify all arsenic peaks for identification. No other problems were encountered with the isocratic system, but other chromatographic issues need to be discussed.

The ammonium carbonate buffered anion-exchange systems evaluated showed little signal drift after prolonged use, and no excessive residue was noticed on the sampler/skimmer cones after fifteen hour run intervals. Ammonium carbonate buffer has been reported as a low residue buffer for ICP-MS detection (18), and was found to require minimal MS interface maintenance during this apple sample extract study. Detection limits were also calculated for the isocratic system. The detection limits were 0.089 ppb (mg/kg) for As (III), 0.034 ppb for DMAA, 0.063 for MMAA, and 0.19 ppb for As (V).





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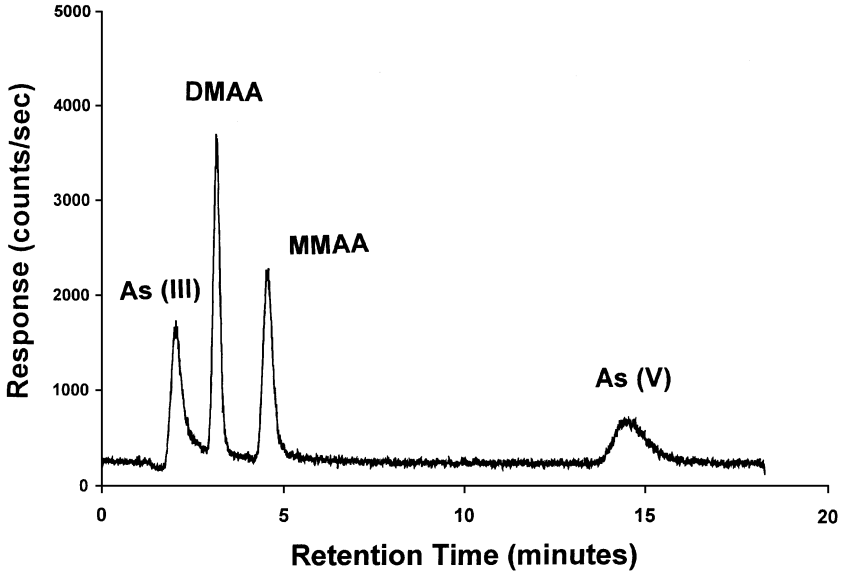


***Figure 3.*** Chromatogram of a solution containing 2 ppb (μg/kg) each As (III), dimethylarsinic acid (DMAA), monomethylarsonic acid (MMAA), and As (V) using the isocratic anion-exchange procedure. Conditions: Column, Hamilton PRP-X100 anion-exchange; flow rate 1.0 mL/min; mobile phase 30 mM ammonium carbonate, pH 8.5.

These values represent solution concentrations and were adequate to quantify the levels of arsenic compounds found in the apple sample extracts.

## Apple Extract Evaluation

The results of an isocratic anion-exchange chromatographic study of sonication extracts and amylase/sonication extracts are shown in Tables 1 and 2. The apple batches all show that the major component of arsenic compounds extracted and identified are As (III) and As (V) and some DMAA. Recovery of total arsenic, defined in this case as the sum total of the three components, was between 54 and 67 percent for the sonication only treatment samples. The sonication only extraction procedure using 50/50 methanol/water had shown in a previous extraction recovery study low 70 percent recoveries when measuring total arsenic (23).

Since only one sample weight was chromatographed for each apple batch, this data should be considered somewhat preliminary. Also, the Caruso *et al*. (23)

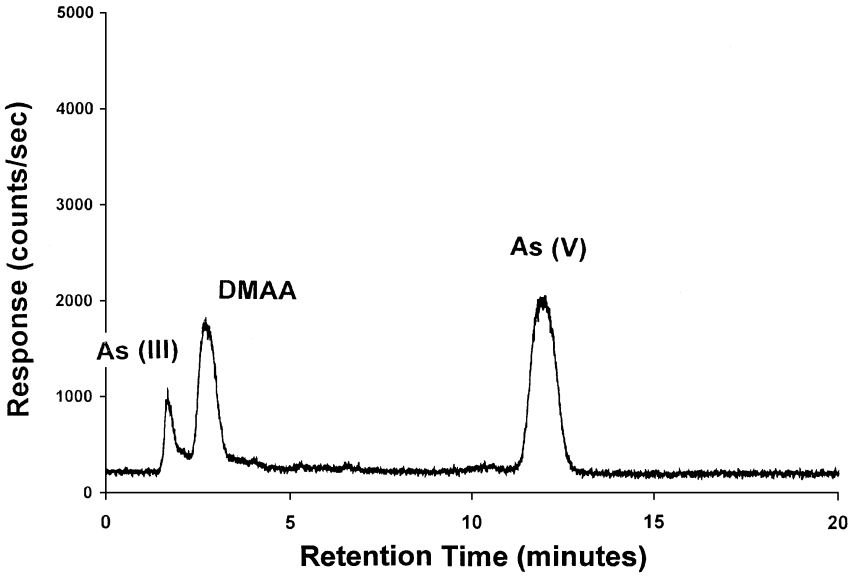


***Figure 4.*** Chromatogram of an amylase/sonication extract solution of apple batch A. As (III), dimethylarsinic acid (DMAA) and As (V) are the only arsenic compounds present at any significant level. The chromatogram was obtained using the isocratic anion-exchange procedure described in Figure 3.

***Table 1.*** Speciation of Sonication Extracts (50/50 Methanol/Water) Using Isocratic Anion-Exchange HPCL on Three Batches of Apples

| Apple Batch | Arsenic (III) (ppb) | DMAA (ppb) | Arsenic (V) (ppb) | Total of Three Main Arsenic Compounds (ppb, summation) | Percent Chromatographic Recovery of Arsenic* |
|---|---|---|---|---|---|
| A | 27.7 | 15.8 | 7.2 | 50.7 | 63 |
| B | 2.7 | 1.9 | 0.9 | 5.5 | 67 |
| C | 5.4 | 1.5 | 4.7 | 11.6 | 54 |

*Percent chrom. recovery = 100*[As (III) + DMAA + As (V)]/[total arsenic level of the apple batch]. The total arsenic level = 80.9 ppb (mg/kg) for apple batch A, 8.2 ppb for batch B, and 21.5 ppb for batch C. The total arsenic level for each batch was determined by nitric acid digestion.





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***Table 2.*** Speciation of Amylase/Sonication Extracts (40/60 Acetonitrile/Water) Using Isocratic Anion-Exchange HPLC on Three Batches of Apples

| Apple Batch | Arsenic (III) (ppb) | DMAA (ppb) | Arsenic (V) (ppb) | Total of Three Main Arsenic Compounds (ppb, summation) | Percent Chromatographic Recovery of Arsenic* |
|---|---|---|---|---|---|
| A** | 23.6 | 17.8 | 26.1 | 67.5 | 83 |
| B | 2.1 | 1.8 | 3.5 | 7.4 | 90 |
| C | 10.0 | 1.8 | 8.8 | 20.6 | 96 |

*Percent chrom. recovery = 100*[As (III) + DMAA + As (V)]/[total arsenic level of the apple batch]. The total arsenic level = 80.9 ppb (mg/kg) for apple batch A, 8.2 ppb for batch B, and 21.5 ppb for batch C. The total arsenic level for each batch was determined by nitric acid digestion.

**Results are the average of two sample weights for this batch, other batches are the result of one sample weight.



study showed much better recoveries with the amylase/sonication extraction procedure, so any further study or consideration was not necessary. Looking at the amylase/sonication arsenic extraction data using 40/60 (v/v) acetonitrile/water as the solvent system (see Table 2), much better recoveries were obtained. The chromatographic recovery of the sum total of the three detected compounds of arsenic was 83 to 96 percent. This represents an improvement over the 79 to 117 percent recovery reported by Caruso *et al*. (23) using the phosphate/nitrate anion-exchange chromatography. Also, total arsenic extraction efficiency for the amylase/sonication procedure had been found to be 87.5% for apple batch A (23). The 83% chromatographic recovery for that same batch is within very good agreement with that value. Chromatographic recovery of arsenic representing 90 and 96% for apple batches B and C is also very good.

In any arsenic compound extraction study of this nature, it is important to demonstrate that individual compounds are not altered during the extraction or chromatographic process. A reference material with certified levels of individual arsenic compounds of interest would be ideal for this; however, currently, no such reference material exists. Caruso *et al*. (23) demonstrated, by use of spiked recoveries, that the amylase/sonication extraction procedure displayed no evidence of species conversion for the organoarsenic compounds. Oxidation of As (III) to As (V) was noticed in the past study. Even if oxidation reduction of the inorganic arsenic occurred, in consideration of toxicity; this is a minor issue. Both inorganic forms of arsenic are highly toxic and any interchange of the chemical oxidation states would not make any difference in evaluating the toxicity of an apple sample.



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Also, the other issue on toxicity is in the total levels of arsenic compounds found. The arsenic levels in these apples, both organic and inorganic arsenic, are extremely low. The arsenic values reported are based on freeze-dried weights of the apples. Apple batch A contained 80.9 ppb (μg/kg) total arsenic based on the dry sample weight. Apple batches B and C were lower, 8.2 and 21.5 ppb, respectively. Since the edible part of an apple is 84 percent water by weight (26), total arsenic levels of all compounds in these batches of apples would be in the 1.3 to 13 ppb range as a food stuff. This would represent a low threat as far as the human diet is concerned, and also considering that arsenic is found at much higher levels in other foods, especially seafood (27,28).

## CONCLUSIONS

Three HPLC systems were evaluated in this study; a gradient ion-pair reversed-phase system, a gradient buffer anion-exchange system, and an isocratic anion-exchange chromatographic system. The isocratic anion-exchange system using ammonium carbonate buffer, was found to be the most practical to easily quantify trace arsenic compounds typically found in the apple samples. The buffer system had a low residue for the mass spectrometer interface and detection limits were acceptable for the trace levels of arsenic compounds found in the apple samples. Three arsenic compounds were observed in all the batches of freeze-dried apple studied. They were arsenite (As III), arsenate (As V), and dimethylarsinic acid (DMAA). Other arsenic compounds may be present at extremely low levels and were beyond the quantification abilities of the chromatography used in this study. The level of total arsenic in the three batches of apples studied was extremely low and the contribution of other arsenic compounds would be relatively insignificant in view of the high chromatographic recoveries obtained, which matched extraction recoveries well.

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