APPLICATION NOTE

HPLC/ICP-MS

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Accurate and Rapid Determination of Arsenic Speciation in Apple Juice

Introduction

In the past several years, concern about the presence of arsenic (As) in apple juice has grown greatly due to its publicity in the popular media. Arsenic can enter apple juice either naturally through environmental uptake by the apple trees or anthropogenically through the use of pesticides and/or contamination during processing. Regardless of how it enters the juice, the presence of arsenic is a concern, especially since apple juice is commonly consumed by children.

In 2013, the U.S. FDA proposed an action level of 10 µg/L inorganic arsenic in apple juice, following the U.S. EPA regulated limit of 10 µg/L As in drinking water. If samples read at or above this level, they should undergo speciation analysis to determine which forms of the arsenic are present. It is possible that in the future, the action level for arsenic in apple juice will decrease due to the susceptibility of children.

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Arsenic can be divided into two classes: inorganic and organic. While inorganic arsenic is toxic, the organic forms typically found in apple juice are considerably less toxic. Therefore, it is important to distinguish and measure the various forms of arsenic in apple juice as opposed to just monitoring the total arsenic concentration.

This work builds on our previous study of arsenic species in apple juice[1] by incorporating several improvements to the methodology and exploring the analysis more deeply.

Experimental

Sample Preparation

Seven apple juice samples were purchased at local grocery stores and filtered through 0.45 µm filters prior to analysis. Analyses were performed on undiluted samples.

All quantitative measurements were carried out against external calibration curves ranging from 0.1 – 15 µg/L. This range was chosen to both give accurate results at low concentrations, yet also include the action level (10 µg/L). The following reagents were used for preparation of standards: As3 1000 ppm in 2% HCl (Spex CertiPrep), As5 1000 ppm in 2% HNO_3 (PerkinElmer), Dimethylarsinic acid 98% (Sigma), Monosodium methylarsonate 99.0% (Chem Service). All calibration standards were prepared in the aqueous component of the mobile phase.

Instrumental Conditions and Parameters

All analyses were performed with a PerkinElmer Altus™ HPLC system coupled to a PerkinElmer NexION® 350D ICP-MS. Details of the HPLC and ICP-MS method conditions are shown in Tables 1 and 2 and were based on our previous work[1]. Since any chloride present in the apple juice samples did not cause arsenic interferences, Standard mode was used for analysis, although Reaction mode could also be used. All data collection and analysis was done with Waters® Empower® 3 Software. The following reagents were used for mobile phase preparation: 1-octanesulfonic acid, sodium salt (98%, Sigma-Aldrich), malonic acid (99%, Acros Organics), and methanol (Optima grade, Fisher Scientific).

Results and Discussion

From our previous work, all apple juice samples were found to contain both forms of inorganic arsenic (As3 and As5) and one form of organic arsenic (dimethylarsinic acid, DMA). A second form of organic arsenic (monomethylarsonic acid, MMA) was only found in a few samples. Given that both forms of inorganic arsenic were present in all juices, the pH of all samples in this study was measured. The pH ranged from 3.4 – 3.6, which suggests that As3 and As5 are stable at these pHs. Measurement of apple juice samples before and after nine days of storage in a refrigerator confirmed species were stable: the percent differences between results were within 7% for all species present at significant levels to be quantified accurately. With the stability of As species established at the acidic pH of apple juice, a 1 µg/L mixed standard was prepared in mobile phase, which has a similar pH as apple juice, and analyzed daily over eight days. It was observed that the relative abundance of the species did not change, confirming the stability of all As species in slightly acidic conditions. The implication is that the calibration standards can be prepared in the mobile phase without worrying about the relative abundance of the species changing.

To examine any matrix effects of the undiluted apple juice on the chromatography, the chromatogram of an apple juice sample was compared to those of a spiked apple juice sample and a standard prepared in mobile phase, as shown in Figure 1. These chromatograms indicate that the apple juice matrix has almost no effect on the chromatography, with only a very slight retention time shift seen for As3. This demonstrates the robustness of the separation method and confirms that apple juice samples can be analyzed undiluted, thus simplifying sample preparation.

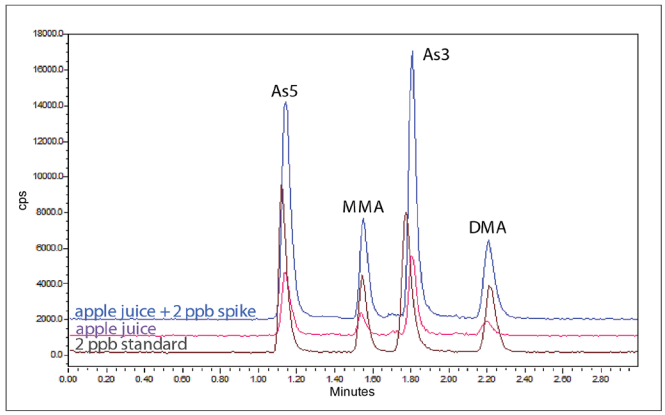


Figure 1. Chromatograms of a 2 µg/L mixed standard, undiluted apple juice, and undiluted apple juice spiked with 2 µg/L of all arsenic species.

Table 1. Altus HPLC Conditions

Parameter	Condition
Column	C18, 4.6 x 250 mm, 5 µm
Mobile Phase	Octanesulfonic Acid (2 mM) + Malonic Acid (2 mM) + Methanol (1%)
pH	4.0 (adjusted with 10% NH_4OH)
Flow Rate	1.5 mL/min
Separation Scheme	Isocratic
Column Temperature	50 °C
Injection Volume	20 µL
LC Vials	Plastic, 1.5 mL

Table 2. NexION 350D ICP-MS Conditions

Parameter	Condition
Nebulizer	Glass Concentric
Spray Chamber	Glass Cyclonic
RF Power	1600 W
Nebulizer Flow	Optimized for < 2% oxides
Mode	Standard
Isotope	^{75}As
Dwell Time	500 ms
Sampling Rate	2 points/second

With the chromatography established, calibration standards were run, and the seven apple juice samples analyzed. All species yielded calibration curves with $r^2 > 0.999$. Figure 2 shows overlaid chromatograms of the blank (i.e. mobile phase) and five lowest calibration standards. Based on our previous studies, we expect arsenic to be present at low levels; therefore, multiple low-level standards were run for accuracy at low concentrations. To accurately measure concentrations at the action level (10 µg/L), additional higher-level calibration standards were also run (5, 10, 15 µg/L) but are not shown in Figure 2, since the low-level standards would not be visible.

After the calibration curves were established, the seven apple juice samples were measured, with the results appearing in Table 3. These results indicate that all samples have arsenic concentrations significantly below the action level of 10 µg/L. It is interesting to note that the inorganic arsenic is always present at levels greater than the organic arsenic, which is consistent with our previous study which analyzed different commercial apple juice samples[1].

Since there are no certified reference materials for arsenic species in apple juice, spike recovery studies were used to assess the accuracy of this method. Each sample was spiked at two different levels: 2 µg/L (to represent typical low levels) and 10 µg/L (the action level). Table 4 shows the spike recovery results, which were all within ± 10%, demonstrating the accuracy of the methodology.

Table 3. Results for Apple Juice Samples (all units in µg/L)

Sample	As5	As3	MMA	DMA	Sum
1	0.94	0.52	< DL	0.49	1.95
2	0.76	0.64	< DL	0.38	1.78
3	1.50	1.10	0.09	0.33	3.02
4	0.73	0.19	0.05	0.20	1.17
5	0.82	0.98	0.56	0.43	2.79
6	0.32	0.75	0.09	0.48	1.64
7	0.75	1.1	0.18	0.42	2.45

With the accuracy established, both short- and long-term stability of the methodology were examined. Short-term stability was evaluated by looking at consecutive injections of the same sample. Figure 3 shows an overlay of seven consecutive injections of one of the apple juice samples (Sample 5) over 30 minutes, along with the concentrations and relative standard deviations (RSDs) for all As species. The low RSDs indicate good short-term stability, with the lowest-concentration species (DMA) having the highest RSD, as expected.

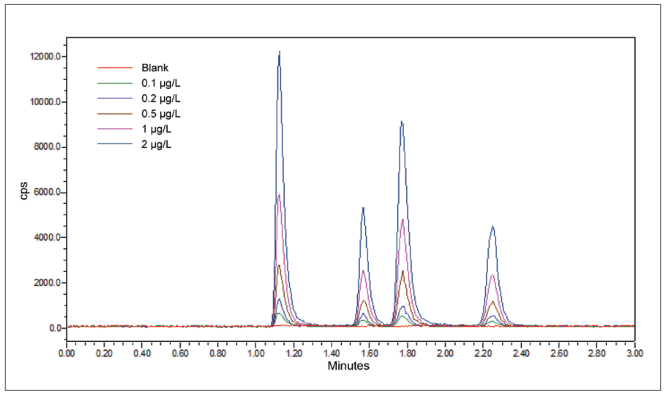
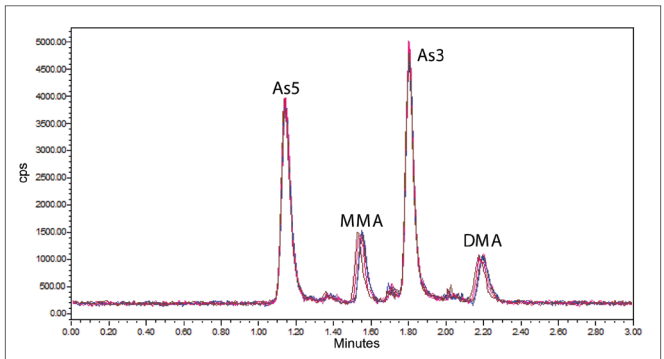


Figure 2. Chromatograms of low-level calibration standards.



	As5	As3	MMA	DMA
Conc (ppb)	0.85	0.98	0.57	0.44
Std. Dev.	0.02	0.02	0.01	0.03
RSD	2.6%	2.5%	1.7%	5.8%

Figure 3. Overlay of seven consecutive injections of Apple Juice Sample 5, along with the associated concentrations and RSDs.

Table 4. Spike Recoveries for Apple Juice Samples (all results expressed as %)

	2 µg/L Spike Recoveries				10 µg/L Spike Recoveries			
Sample	As5	As3	MMA	DMA	As5	As3	MMA	DMA
1	92	100	93	91	99	104	96	94
2	96	102	99	93	95	102	94	90
3	97	106	100	95	98	103	97	94
4	98	109	99	95	101	109	101	100
5	99	106	96	95	99	105	98	95
6	99	104	101	95	102	107	99	95
7	97	104	98	96	101	107	101	98

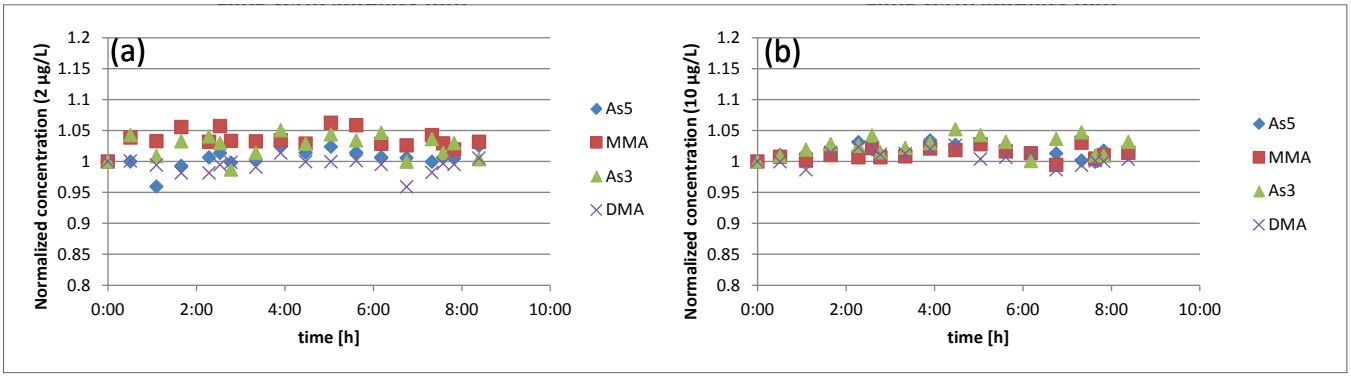


Figure 4. Stability of (a) 2 µg/L and (b) 10 µg/L As check standards run every 30 minutes during an 8-hour run of apple juice samples.

Long-term stability was evaluated by measuring 2 and 10 µg/L check standards every thirty minutes during an eight-hour analysis of all the apple juice samples. The check standard levels were selected to represent both low (i.e. typical) and elevated (i.e. action levels) concentrations of arsenic. Figure 4 shows both stability plots, where each reading has been normalized to the first measurement. Both plots indicate exceptional stability, with all measurements being within ± 6% of the initial reading.

Detection limits can be determined in a variety ways, each giving different results. In general, chromatographic detection limits are a function of many factors, including the injection volume, baseline noise, and peak shape. The commonly-accepted way of determining chromatographic detection limits is to find the concentrations (for a given injection volume) which produce peaks that are three times the amplitude of the baseline noise. Figure 5 shows a chromatogram from a 20 µL injection of standard containing arsenic species ranging from 0.02 to 0.07 µg/L. These peaks are clearly visible above the baseline and can be integrated, indicating that they are near the detection limit.

Since detection limits are not an issue for the measurement of As in apple juice (given that the action level is 10 µg/L), smaller injection volumes can also be used. Smaller injection volumes have two main benefits: extending column lifetime and producing taller, narrower peaks. Since sharper peaks result from lower injection volumes for the same concentration, we found that detection limits do not suffer proportionally to the volume reduction. Likewise, if lower detection limits are desired, larger injection volumes can be used. However, effects of the juice matrix on the chromatography may be more pronounced. Larger injection volumes will also shorten column lifetimes and produce broader peaks.

The work presented here was accomplished without using an internal standard. The good reproducibility of individual injections (Figure 3) and excellent long-term stability (Figure 4) eliminate the need for an internal standard. However, if the use of an internal standard is desired, arsenobetaine (AsB) can be used. As shown in Figure 6, AsB elutes after the DMA, so its presence will not interfere with the peaks of interest.

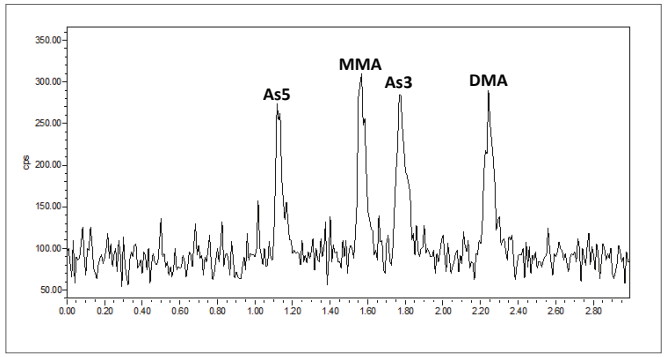


Figure 5. Chromatogram of arsenic species near the detection limit with a 20 µL injection: As5=20 ng/L; MMA=70 ng/L; As3=40 ng/L; DMA=70 ng/L.

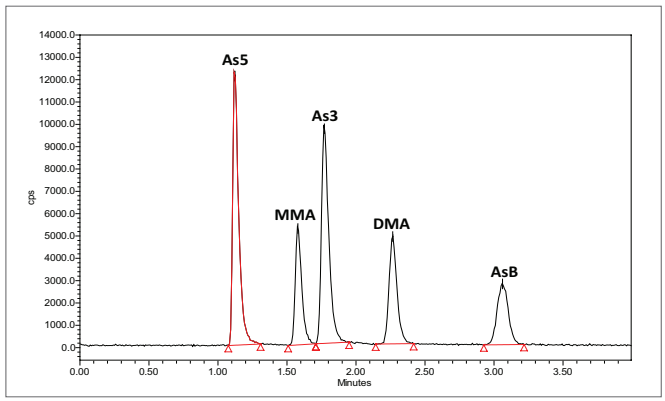


Figure 6. Chromatogram of a mixed arsenic standard containing 2 µg/L of each species, including arsenobetaine (AsB).

Conclusions

This work has demonstrated the rapid and accurate measurement of arsenic species in apple juice. By using reversed-phase chromatography with a cation-pairing reagent, the elution order of the species is reversed compared to the more traditional anion exchange chromatography. The separation is faster than traditional methods with associated benefits of shorter run time and taller, narrower peaks, enabling lower levels to be measured. Sample preparation is simplified as only filtration is required. Commercial apple juice samples were analyzed by this methodology and all found to contain significantly lower arsenic concentrations than the action level. The methodology allows separation of As species in a three-minute run time and was shown to be accurate at both low and high concentrations through spike recovery studies. In addition, the methodology produces excellent short- and long-term stability.

Consumables Used

Component	Part Number
Column: C18, 4.6 x 250 mm, 5 µm	N8145326
Autosampler Vials, clear, 1.5 mL (package of 100, with caps)	N9301736
Disposable Syringes, 10 mL, Luer-Lock (package of 100)	02542893
PTFE Syringe Filters, 0.45 µm, 25 mm (package of 100)	02542927
PEEK Tubing, 0.007" ID x 1/16" OD (5 feet)	N9302678
PEEK Finger Tight Fittings	09920513

Component	Part Number
PEEK Solvent Filter, 10 µm	N8122249
PEEK In-line Filter, 10 µm	N8122250
Nebulizer Connector for HPLC	WE024372
Connector for Peristaltic Pump Tubing to PEEK Tubing	N8122258
Finger Tight Connector for 1/16" OD PEEK Tubing	09920513

References

1. Neubauer, K., Perrone, P., Reuter, W., "Determination of Arsenic Speciation in Apple Juice by HPLC/ICP-MS", PerkinElmer Application Note, 2012.

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