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Elemental Analysis Manual

for Food and Related Products

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4.10 High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometric Determination of Arsenic Species in Fruit Juice

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4.10.1 SCOPE AND APPLICATION

EAM 4.10 is used to learn how arsenic that may be present in juice is distributed among different chemical forms, or species; and, most importantly, how much is in the inorganic arsenic forms. The method could be called for after an elevated level of total arsenic is found, such as via an EAM 4.7 analysis. Or, speciation information could be desired in routine monitoring.

High performance liquid chromatography (HPLC) is used in combination with inductively coupled plasma-mass spectrometry (ICP-MS) to analyze fruit juice and determine mass fractions for arsenic species. The method targets two inorganic arsenic species — arsenite [As(III)] and arsenate [As(V)] — and two organic arsenic species — dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA). Typically, and depending on laboratory customer needs, the two inorganic species are summed and reported simply as total inorganic arsenic (iAs). Should other (unknown) arsenic species be detected, it is also possible to estimate mass fractions for these.

Juice is analyzed in ready-to-drink (RTD) condition. When received in concentrated form, the juice is reconstituted.

A variety of juices were included in the validation studies. Three juices (apple, grape, and pear) were analyzed using a simple “dilute-and-shoot” procedure and three juices (prune, pomegranate, and cherry) were analyzed using an additional acid extraction step.

The method was also validated for wine but using a variation of the method that incorporates a different standardization procedure that compensates for a signal enhancement caused by ethanol. This variation of the method is provided in Appendix A.

EAM 4.10 should be used by analysts experienced in the use of HPLC and ICP-MS, including the identification of chromatographic and matrix interferences and procedures for their correction and should be used only by personnel thoroughly trained in the handling and analysis of samples for determination of trace elements in food products.

4.10 Table 1 lists analytical limits typically found but these will vary depending on instrumentation and actual operating conditions used. As shown, one value (the largest found) is assigned, by definition, to all four species.

4.10 Table 1. Typical Analytical Limits (µg As / kg juice)

Analyte	Abbreviation	ASDL[a,b]	ASQL[a,b]	LOD[b,c]	LOQ[b,c]
Arsenite	As(III)	0.05	0.4	0.25	2.0
Arsenate	As(V)	0.05	0.4	0.25	2.0
Monomethylarsonic acid	MMA	0.05	0.4	0.25	2.0
Dimethylarsinic acid	DMA	0.05	0.4	0.25	2.0

[a] Based replicate injections of fortified method blanks, results taken from EAM Method 4.11's multilaboratory validation reports where average ASDLs were 0.047 ng/g As(III), 0.056 ng/g As(V), 0.041 ng/g DMA, and 0.041 ng/g MMA).

[b] Calculated as in EAM §3.2.1

[c] Based on 5-fold dilution of ready-to-drink juice.

4.10.2 SUMMARY OF METHOD

Ready-to-drink (RTD) clear (i.e., no solids) juice is prepared by diluting an analytical portion approximately 5-fold with water. Juice concentrates are diluted to a reconstituted RTD state prior to this 5-fold dilution. Arsenic species are analyzed by HPLC-ICP-MS. The HPLC uses a PRP-X100 anion exchange column for separation. Arsenic species are identified by peak retention time match with arsenic species standards. The ICP-MS is used as an arsenic-specific detector to monitor for arsenic-containing chromatographic peaks. It is operated in helium collision cell mode or oxygen reaction mode, either of which minimize interference from co-eluting chloride species, which are commonly encountered in food analysis. Mass fractions are calculated based on peak area from analytical solutions compared to response of standard solutions.

A (separate) total arsenic analysis, such as via EAM 4.7, is to be performed in conjunction with an EAM 4.10 species analysis so that when total arsenic is ≥10 µg/kg (in RTD condition), the sum of the individual arsenic species mass fractions can be compared with the total arsenic mass fraction. This calculation indicates whether the arsenic is adequately accounted for.

4.10.3 SAFETY CONSIDERATIONS

Use appropriate personal protective equipment including safety glasses, gloves and lab coat and have proper ventilation and other physical safeguards in place. Such precautions are especially important when working with toxic arsenic standards (powders and/or concentrated solutions), acids, and ammonium hydroxide.

Analysts should consult and must be familiar with their lab's chemical hygiene and safety plan as well as Material Safety Data Sheets for all reagents and standards listed. Refer also to instrument manuals for safety precautions regarding use. Laboratory waste must be handled appropriately.

4.10.4 EQUIPMENT AND SUPPLIES

Disclaimer:

The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration. Several instrumentation-specific and software-specific instructions are provided but equivalent performance may be achievable using apparatus and materials other than those cited here. Should other instrumentation and/or software be used, their performance characteristics must meet or exceed those noted here. Operational details such as how to proceed through software will be different and this is permitted. But, any modification of the analysis procedures given herein would require validation according to FDA guidelines and the quality control elements detailed below in §4.10.10 must pass.[1]

Notes on inorganic As and contamination in labware:

QC verifies the chemical measurement process is in control but experience shows that labware can introduce inorganic As contamination. Labware such as pump tubing is inherently cleaned and monitored during use and therefore require no extra attention. Other labware, however, such as disposable vials must be checked and possibly cleaned.

Glass vials must be washed. Select three at random, fill with 2% nitric acid, and let sit for at least 4 hours (e.g., overnight or over a weekend). Rinse at least 4x with DIW then refill with DIW and check for As (i.e., analyze the water via LC-ICPMS). If As is not detected in any of the vials, then use this procedure to clean all glass vials in that lot. If As is detected in any of the vials, the entire lot is unusable.

Plastic vials are usually As-free but need to be checked and may require washing. Select three at random, add 2% nitric acid, cap, shake or vortex for 15 seconds, rinse at least 4x with DIW then refill with DIW and check for As (i.e., analyze the water via LC-ICPMS). If As is not detected in any of the vials, then this batch/lot is ok. If As is detected, then take a few different (unused) vials, fill with 2% nitric acid, and let sit for a period of time (overnight is usually adequate). Rinse 4x with DIW, fill with DIW, and analyze. If As is not detected, then use this procedure for all vials from this lot. If As is still detected, discard the entire lot.

Once a supply of plastic labware has been shown to be arsenic-free and unless contamination is found during analysis, new shipments of this labware do not, necessarily, need to be checked — as long as they come from the same source (e.g., same supplier and same catalog number).

(1) **Inductively coupled plasma-mass spectrometer (ICP-MS) —** Agilent model 7500 equipped with MassHunter software for instrumental control was used for development of this method (note disclaimer above). The ICP-MS must be equipped with a collision/reaction cell using either helium as collision gas or oxygen as reaction gas to reduce interference from $^{40}Ar^{35}Cl$. The ICP-MS should at minimum be configured to remote start by the HPLC instrument for integrated operation.

(2) **High performance liquid chromatograph (HPLC) —** Agilent 1200 series HPLC control was used for development of this method (note disclaimer above). The HPLC must be equipped with a pump, autosampler and degasser. Column compartment module and an autosampler thermostat module are recommended, but not mandatory.

Ideally, for ease of operation, the HPLC and ICP-MS will be be from the same manufacturer and controlled by the same software. At a minimum, the HPLC needs to be able to trigger the ICP-MS to start recording data after each injection.

(3) **HPLC analytical column —** Hamilton PRP-X100 anion exchange column, 250 ×4.1 mm, stainless steel, 10µm particle size (Hamilton cat. no. 79433) with PRP-X100 guard column (Hamilton cat. no. 79446 for 5-pack of cartridges).

(4) **6-port switching valve —** either integrated in the HPLC column compartment or externally attached; used to inject a post column marker peak solution via the ICP's peristaltic pump with a combination of polyetheretherketone (PEEK) tubing and appropriate pump tubing.

(5) **Autosampler vials —** plastic or acid-cleaned glass HPLC vials (see labware contamination note above).

(6) **High density polyethylene (HDPE) amber bottles —** for preparation and storage of stock standards (~100 mL).

(7) **Centrifuge tubes —** polypropylene conical tubes with caps, 15 mL.

(8) **Vortex mixer —** for mixing diluted fruit juices.

(9) **Plastic Syringes —** used in filtering juice samples, disposable, general use and non-sterile, 5 or 10 mL, Luer-Loc tip.

(10) **Syringe filters —** used in filtering juice samples; disposable, 0.45 µm Nylon or PTFE membrane with polypropylene housing and Luer-Loc inlet.

(11) **Analytical balance —** precision of 0.0001 g.

(12) **Pipettes/pipettors —** delivery range 10 µL up to 10 mL.

(13) **pH meter —** with appropriate calibration buffers (pH 7 and 10).

(14) **Refractometer% (needed only if analyzing concentrates that do not have directions for reconstitution) —** handheld, capable of measuring Brix range 0-80.

(15) **Hot block digestion system (needed for acid extraction procedure) —** 48-place, 50 mL, temperature range from ambient to at least 100°C (SCP Science model DigiPREP MS)

4.10.5 REAGENTS

(1) **Reagent water —** Water that meets specifications for ASTM Type I water[2], such as 18 MΩ•cm de-ionized water (DIW) from a Millipore Milli-Q system.

(2) **Arsenobetaine (AsB) —** CAS 64436-13-1, F.W.178.06, purity ≥95%. [**Arsenocholine bromide (AsC) may also be used** — CAS 71802-31-8, F.W. 249.99, purity ≥95%.]

(3) **Arsenite [As(III)] stock solution —** 1,000 mg/L As(+3) in 2% HCl with the certified arsenic value traceable to a NIST Standard Reference Material.

(4) **Dimethylarsinic acid (DMA) —** CAS 75-60-5, F.W. 138.01, purity ≥98%, commercially available either as solid or as 10 µg/mL solution.

(5) **Disodium methyl arsonate hexahydrate [exists as monomethylarsonic acid (MMA) in the on-column chemical environment] —** Purity ≥98.5%, formula wt. 291.9, commercially available either as solid or as 10 µg/mL solution.

(6) **Arsenate [As(V)] stock solution —** 1,000 mg/L As(V) in H_2O with the certified arsenic value traceable to a NIST Standard.

(7) **Certified Reference Material (CRM) —** National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1643f Trace Elements in Water. Certified total arsenic mass fraction 56.85 µg/kg. This is the current version of the CRM used during method development and a good example of the type of CRM desired.

(8) **Ammonium phosphate dibasic [$(NH_4)_2HPO_4$] —** CAS 7783-28-0, F.W. 132.06, purity ≥ 99%. Due to arsenic contamination in various lots from several manufacturers, the $(NH_4)_2HPO_4$ used in this procedure must be verified to have a low arsenic content (see Section 4.10.8, 4a-4e).

(9) **Ammonium hydroxide (NH_4OH), 20% —** CAS 1336-21-6, F.W. 35.05, ultrapure.

(10) **Nitric acid (HNO_3), 68-70% (used only when acid extraction procedure is needed) —** CAS 7697-37-2, F.W. 63.01, ultra-pure grade, Fisher Scientific (cat. No A467-500)

4.10.6 SOLUTION PREPARATION

Notes on mass and volume:

All calculations are based on mass. When volumes are used herein (e.g., "pipet 2 mL"), they only indicate nominal amounts of material. The use of pipettes is assumed but this is only a convenient mode of transferring liquids. Pipettes therefore do not need to be calibrated because masses will always be used.

Prepare solutions and perform calculations in units of mass of elemental arsenic per mass of solution or sample. Do not use mass/volume and do not use the mass of arsenic compound.

If a standard solution is obtained in mass/volume units or is made from a solid compound, use density and/or molecular weights and convert to the needed mass/mass basis.

When As levels are indicated below in the method procedures (e.g., "1,000 mg/kg"), these are approximate. Exact values will vary. Exact values, however, must be recorded and used in the laboratory in all manipulations. Adjust sample dilution amounts, as needed, to accommodate the actual levels (e.g., if As is at an unexpected level in a commercial solution).

Stock Standards

Stability — Stock standard solutions may be used for up to one year if kept in tightly sealed HDPE or polypropylene containers and in the dark at 4°C. Bring to room temperature and mix well prior to use.

Levels — Arsenic stock standard solutions are typically at ~1,000 mg/kg. If commercial levels differ, adjust the dilution amounts accordingly. Certified solutions have been available commercially for all of the needed arsenic analytes but availability can vary, especially for

MMA and DMA. Therefore, some stock solutions may need to be prepared from solids. Directions are given here for preparing MMA, DMA, and AsB from solids.

Preparation from solids — For each material, tare a 15 mL polypropylene centrifuge tube, add the appropriate amount of material into the tube (see below), then add DIW to 10 g total.

AsB	0.025 g (or 0.033 g AsC)
DMA	0.0184 g
MMA	0.039 g

The stoichiometry of DMA and MMA solids is always in question. Therefore, final values for DMA and MMA standard solutions are determined experimentally, typically using ICP-MS via the following approach:

After preparation of the DMA and MMA stock solutions (at ~1,000 µg/g) and intermediate standards (at ~1 µg/g), a further 1/100 dilution produces solutions at ~10 ng/g which are appropriate for ICP-MS analysis (e.g., via EAM 4.7). This analysis is used as a reference to set the levels for the DMA and MMA standard solutions, so it must include the full set of QC steps plus analysis of a relevant certified reference material such as NIST SRM 1643f. After measuring the actual level of the ~10 ng/g solutions, use the dilution records to back-calculate and assign the levels for the intermediate and stock standards.

Purity — Stock solutions must always be checked for purity via HPLC-ICP-MS analysis. Dilute the 1 ug/g single-analyte intermediate standard solutions to ~100 ng/g and analyze. Impurity peaks must be manageably small.

Notes on Purity:

Ideally, significant impurities will not be detected in stock solutions and arsenic species inter-conversion will not occur. When significant impurities are found, however, they must be dealt with, by either obtaining new solutions or adjusting the calculations (see quality control section below 4.10.10).

Impurities are considered insignificant if ≤2% and are ignored in subsequent mathematical calculations. If >2% impurities are present, new stock solutions should be procured (or re-prepared from solid) because this is usually the most straightforward approach. When obtaining new stock solutions is not a viable option (e.g., sufficient purity not available, budget or time constraints, etc.), adjusting the calculations is necessary for those with >2% impurity.

Whenever impurity issues arise, always review the storage instructions provided herein to be sure inter-conversion is minimized as much as reasonably possible.

Intermediate Standards

(1) **Single-analyte intermediate (purity check) standards (needed only when new stock solutions are obtained; 1 µg/g; 3-month stable) —** For each analyte [AsB, As(III), DMA, MMA, and As(V)], tare a 125 mL HDPE or polypropylene bottle, add ~0.1 g (~100 uL accurately weighed) of the 1,000 µg/g stock solution, and dilute to 100 g total with DIW. Discard after purity is checked.

(2) **Multi-analyte intermediate (spiking) standard [1 µg/g, each analyte, As(III), DMA, MMA, and As(V); 3-month stable] —** Tare a 125 mL HDPE or polypropylene bottle, add ~0.1 g (~100 uL accurately weighed) of each of the 1,000 µg/g stock solutions, and dilute to 100 g total with DIW.

(3) **Diluted multi-analyte intermediate standard (200 ng/g, each analyte; 1-week stable) —** Add 1.0 g of the multi-analyte spiking standard to a tared 15 mL HDPE or polypropylene tube then dilute to 5 g total with DIW. Prepare fresh at the same time as the calibration standard solutions are prepared.

Notes:

Advance planning of analytical work is strongly recommended to minimize standard solution storage time. Store intermediate and working solutions and in tightly sealed HDPE or polypropylene containers at 4°C, in the dark, and no longer than the indicated times. Also watch for changes such as As(III)/As(V) interconversion.

If commercial solutions of DMA and/or MMA are not at ~1,000 µg/g, adjust dilution amounts, as needed. (E.g., if commercial DMA is 10 µg/g, then 10 g are added to the tared spiking solution bottle instead of only 0.1 g).

AsB (or AsC) used for resolution check only, not included with calibration standard solutions.

Working Solutions:

Working solutions are those used during the day(s) of analysis. Four multi analyte standards (used for instrument calibration) and a continuing calibration verification (CCV) standard must be prepared fresh on the first day of analysis then re-used for up to one week if kept in the dark at 4°C and standard chromatograms do not show evidence of inter-conversion of arsenic species.

(1) **Mobile phase solution [10 mM $(NH_4)_2HPO_4$ at pH 8.25] —** Add 1.32 g $(NH_4)_2HPO_4$ to 1 L HPLC reservoir bottle, add 990 g DIW, adjust pH to 8.25 (±0.05) with 20% ammonium hydroxide, and fill to 1,000 g with DIW.

(2) **Individual-species purity check solutions —** New standard stock solutions must be checked for purity. Perform appropriate dilutions to obtain ~100 ng/g levels.

(3) **Retention time check solutions —** Retention times are set at the start of an analysis. This can be accomplished using the 10 ng/g calibration standard (see below).

(4) **Multi-analyte Instrument calibration standards:**

a. **Calibration standard (10 ng/g) —** Add 0.500 g of the 200 ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with DIW.

b. **Calibration standard (4 ng/g) —** Add 0.200 g of the 200 ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with DIW.

c. **Calibration standard (1 ng/g) —** Add 1.0 g of the 10ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with DIW.

d. **Calibration standard (at or slightly above ASQL; i.e., ~0.4 to 0.5 ng/g) —** Add

0.500 g of the 10 ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with DIW. (see 4.10.10 for ASQL).

(5) **Continuing calibration-verification (CCV) standard (~2 ng/g) —** Add 0.1 g of the 200 ng/g multi-analyte intermediate standard solution into a tared HDPE or polypropylene tube and dilute to 10 g with DIW.

(6) **Resolution check solution, 5 ng/g each of AsB (or AsC) and As(III) —** Add 0.05 g, each, of the AsB (or AsC) and As(III) 1 µg/g intermediate standard solutions into a tared HDPE or polypropylene tube and dilute to 10 g with DIW. A new resolution check solution must be prepared when significant oxidation of As(III) to As(V) is noted.

(7) **Post-column marker peak solution [As(V); 2 ng/g] —** Add 1 g of the 1 µg/g As(V) intermediate standard solution into a tared HDPE or polypropylene bottle and dilute to 500 g total with mobile phase solution.

(8) **Certified reference material —** Add 0.5 g of NIST 1643f *Trace Elements in Water* into a tared 15 mL tube and dilute to 7.5 g total with DIW (a 15-fold dilution).

(9) **Extraction solution 0.28 M HNO_3 (used only when acid extraction procedure is needed) —** Add 25.3 g concentrated HNO_3 to ~500 mL DIW and dilute to 1,000 g.

4.10.7 SAMPLE PREPARATION

Two sample preparation procedures are given below — a simple "dilute-and-shoot" procedure and a nitric acid extraction procedure. The acid extraction is needed when arsenic is present in an unknown form that does not otherwise elute from the HPLC column. Per the mass balance control element in 4.10.10, the acid extraction is needed for low mass balance juices (LMBJ). While there is no strict rule as to when the extraction will or will not be needed, experience has been that it usually is needed for prune, pomegranate, and cherry juices but not for apple, grape, and pear juices.

The addition of nitric acid (to analytical samples) results in an extra dilution with DIW to mitigate effects on retention time. The amount of extra dilution is minimized to keep LOQs below ~10 ng/g.

Juice

Juice is analyzed in ready-to-drink condition (RTD; also called single strength or 100% juice). If received in concentrate form, reconstitute to RTD condition according to the manufacturer's directions. If no directions, measure the degree Brix (°Bx) and dilute to the minimum RTD strength using 4.10 Table 2. (see example below). When analyzing juice concentrates, only one dilution (to RTD condition) is necessary. The appropriate number of replicate preparations should be prepared from that (RTD condition) juice; this applies to both "dilute-and-shoot" and nitric acid extraction procedures.

Bring juice, as needed, to room temperature and invert juice container several times to mix and ensure homogeneity.

(1) **Dilute and shoot procedure —**

a. Pipet 2 mL (~ 2 g) RTD juice into a tared 15-mL polypropylene centrifuge tube (record analytical portion mass), dilute to ~10 g with DIW (record analytical solution mass), cap, and mix thoroughly.

b. Draw at least 4 mL of the analytical solution into a syringe, attach a 0.45 µm Nylon or PTFE syringe filter,

- discard the first ~1 mL to waste
- transfer ~1 mL to an autosampler vial (for analysis)
- dispense remainder (reserve) into a second 15mL polypropylene centrifuge tube

c. Store the reserve for up to 48 hours at 4°C in the event this sample solution needs to be re-analyzed.

(2) **Acid extraction procedure —**

a. Weigh a 50 mL tube with lid and record the mass.

b. Pipet 5 mL (~ 5g) RTD juice into the pre-weighed 50 mL tube (record the mass).

c. Add 6 mL 0.28M HNO_3, cap tube tightly, and mix thoroughly (vortex ~15 seconds).

d. Place tubes in hot block (preheated to 95°C) for 90 minutes.

e. Remove, cool, and add 9 mL DIW (record mass).

f. Centrifuge (3000 rpm) for 10 minutes.

g. Draw at least 4 mL of the analytical solution into a syringe, attach a 0.45 µm Nylon or PTFE syringe filter, and dispense into a second 15mL polypropylene centrifuge tube (discard the first ~1 mL to waste).

h. Transfer ~1 mL of the filtered extract to a tared 15 mL centrifuge tube (record mass), add ~4 g DIW (record mass) and mix.

i. Transfer ~1 mL of diluted juice to an autosampler vial for analysis.

j. Store the remainder for up to one week at 4°C in the event this sample solution needs to be re-analyzed. (Dilution factor is ~20 following this procedure.)

Example - Using Brix to Reconstitute Juice Concentrate:

An apple juice concentrate (without reconstitution directions) is found to be 70 ***°Bx*** *and 4.10 Table 2 shows the RTD apple juice Bx is 11.5. The dilution amount is therefore 6.1 (i.e., 70/11.5=6.1) and 1 g juice concentrate can be combined with 5.1 g DIW in a 15 mL centrifuge tube and mixed thoroughly to get it in RTD condition.*

4.10 Table 2. Minimum °Bx Values for Ready-to-Drink Juices[3]

Juice	°Bx		Juice	°Bx
Apple	11.5		Mango	13.0
Apricot	11.7		Nectarine	11.8
Blackberry	10.0		Orange[a]	11.8
Blueberry	10.0		Papaya	11.5
Carrot	8.0		Passion Fruit	14.0
Cherry, dark, sweet	20.0		Peach	10.5
Cherry, red, sour	14.0		Pear	12.0
Cranberry	7.5		Pineapple	12.8
Currant (Black)	11.0		Pomegranate	16.0
Currant (Red)	10.5		Prune	18.5
Grape	16.0		Raspberry (Black)	11.1
Grapefruit[a]	10.0		Raspberry (Red)	9.2
Guava	7.7		Strawberry	8.0
Kiwi	15.4		Tomato	5.0

[a]Brix values determined by refractometer for citrus juices may be corrected for citric acid.

Replicate analytical portions (RAP)

Use the sample preparation procedures described above for multiple (replicate) juice samples (dilute-and-shoot or acid extraction, as appropriate).

Fortified analytical portions (FAP)

Prepare an analytical portion fortified with As(III), DMA, MMA and As(V) at a level of 25 µg/kg each by combining 2 mL (~2 g) RTD juice and 0.05 mL (~ 0.05 g) of the 1,000 ng/g multi-analyte spiking solution in a 15 mL polypropylene centrifuge tube. It is recommended that the same sample be used for both FAP recovery and replicate precision. Proceed with analysis as would for unfortified sample (dilute-and-shoot or acid extraction, as appropriate).

Method blank (MBK)

Take 2 g DIW through the sample preparation procedures described above for juice samples (dilute-and-shoot or acid extraction, as appropriate).

Certified Reference Material (CRM)

Depending on CRM matrix, prepare as if a sample but dilute (with DIW) appropriately according to the known As level so the analytical solution's As level will be within the calibration. Although NIST SRM 1643f is not certified for arsenic species, As(V) and possibly As(III) should be the only peaks detected. For this CRM, a dilution factor of ~15× is appropriate to dilute the acid content.

4.10.8 DETERMINATION PROCEDURE

4.10 Table 3 shows operating conditions for ICP-MS and HPLC. Set any parameters not listed according to laboratory SOP for ICP-MS tuning.

4.10 Table 3. Typical HPLC-ICP-MS Operating Conditions

ICP and HPLC conditions	
ICP-MS Conditions	
Spray chamber drain	≥1 mL/min
Ions (mass-to-charge ratio)	75, 77 (He) 91 (O_2)
Dwell time	0.8 s (*m/z* 75) and 0.2 s (*m/z* 77) or 1s (*m/z* 91)
Reaction/collision cell mode	1.5-3 mL/min He
HPLC Conditions	
Mobile phase composition	10 mM $(NH_4)_2HPO_4$
Mobile phase pH	8.25 (±0.05)
Mobile phase flow rate (mL/min)	1.0
Injection volume (µL)	100
Column temperature	Ambient
Column compartment time table for introduction of marker peak	0.1 min, Column Position 1
	1.0 min, switch to Column Position 2
	2.0 min, switch back to Column Position 1
Acquisition time	1200 s (20 min)

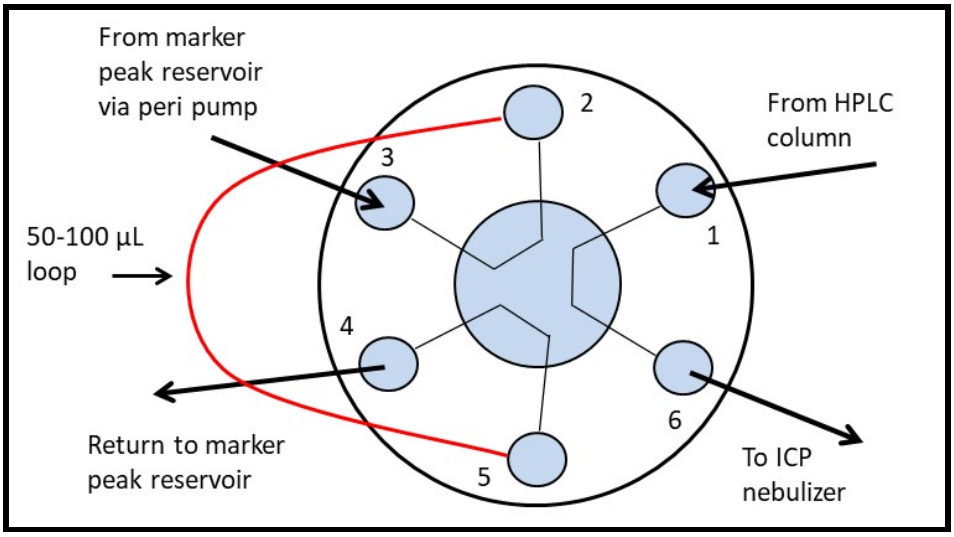
Instrument Setup

(1) Follow instrument manufacturer recommendations for startup and initialization. After ~30 min warm-up, ensure that the ICP-MS is operating normally and tune the instrument for use with helium as a collision gas or oxygen as a reaction gas. In general, the helium flow rate should be optimized to minimize polyatomic interferences, while preserving m/z 75 signal. Oxygen flow rates/conditions will vary. (For Agilent ICPs, such as used in method development, the He gas flow rate for chromatographic analysis needs to be 2-3 mL/min less than what is used for typical total arsenic analysis using He mode. I.e., ~1.5-3.0 mL/min and typically ~2 mL/min).

(2) Use peristaltic pump to introduce the 2 ng/g As(V) marker peak (MPk) solution directly into the nebulizer. Ensure signal for *m/z* 75 (or 91) response is within normal range. Rinse the ICP-MS system well when finished tuning.

(3) For post-column introduction of the As(V) marker peak solution, connect a small (50 to 100 µL) loop across 2 ports of the 6-way 2 position column switching valve, with LC flow and peristaltic pump marker pump solution reservoir flow tubes connected similar

to 4.10 Figure 1. In the HPLC method timetable, column switching valve should be triggered at 1 min and triggered to switch back at 2 min. Start the peri-pump and verify that no bubbles are present. Timing and flow rate are shown in Table 3.



4.10 Figure 1. Switching valve diagram

(4) Connect ICP-MS and HPLC via LAN and/or remote start cables, connect LC outflow to nebulizer, and start HPLC flow (1 mL/min mobile phase).

a. Ensure proper flow and adequate drainage of ICP spray chamber (>1mL/min).

b. Check for leaks and correct, if needed.

c. Allow time for column and plasma to equilibrate (<15 min).

d. Ensure that backpressure is acceptable. Note that increasing backpressure can be indicative of column problems.

Check Mobile Phase and Instrument Operation

(1) If this is the first time using a source of $(NH_4)_2HPO_4$ for the mobile phase, it needs to be tested for arsenic contamination. Perform the steps below and if acceptable proceed to step (2). If the $(NH_4)_2HPO_4$ source has already been found to be acceptable, proceed to directly to step (2).

a. Set the ICP-MS conditions as in 4.10 Table 3 (if using Agilent instrumentation), but rather than setting up an acquisition method, test the following in the tune window.

b. After eluting DIW through the HPLC to the ICP-MS (through the HPLC column) for at least 30 minutes, monitor *m/z* 75 (integration time of 0.8 seconds) in the tune window for at least 30 seconds and record the average response (in CPS).

c. Switch the eluent to mobile phase [using the new source of $(NH_4)_2HPO_4$]. After eluting mobile phase for ≥30 minutes, monitor *m/z* 75 (integration time 0.8 seconds) in the tune window for ≥30 seconds and record the average response (in CPS).

d. Compare the average responses of DIW and mobile phase for *m/z* 75. The ratio of mobile phase response (cps) to DIW response (cps) must be less than 6 to 1. If it is not, try another source of $(NH_4)_2HPO_4$. If it is <6, proceed to step 5. This threshold is equivalent to ~50 ng/g in the ammonium phosphate solid. An acceptable ammonium phosphate source (same lot number) can be used until depleted without retesting.

(2) Set ICP-MS acquisition method for time-resolved collection of *m/z* 77 and 75 with integration (dwell) times of 0.2 and 0.8 s, respectively (or 1 s for *m/z* 91), and 1 replicate (read) per point. (see 4.10 Table 3)

(3) Inject DIW to verify that the system is arsenic-free and monitor instrument conditions to ensure the chromatogram remains steady and within normal functioning range.

(4) Inject resolution check solution to ensure adequate resolution.

(5) Create/edit the sequence file on the ICP-MS data system. If controllers for the HPLC and ICP-MS are separate, ensure that the injection lists match.

Analysis

(1) Proceed according to the batch sequence — A typical analytical batch is shown in 4.10 Table 4. Standardize then inject/analyze the various QC solutions (MBKs, resolution check solution, CCV, RAPs, FAPs, CRMs) and sample solutions.

(2) **QC analyses**

a. **Standardize** using a minimum of four calibration levels for each arsenic species.

b. Analyze **method blanks** at start and with every 10 or fewer analytical solutions.

c. Analyze the **CCV standard** after every 10^{th}analytical solution and at the end (after the last analytical solution analyzed) to monitor retention time and calibration.

d. Analyze two replicate analytical portions **(RAPs)** at a frequency of 10% (i.e., at least one RAP for every ten samples) and at least one for each type of juice. Analysis of a greater number of RAPs (e.g., triplicate) is permitted but not required.

e. Analyze at least one **FAP** for each type of juice to verify peak identification and quantitative recovery.

f. Include at least one **CRM** or in-house reference material in each batch. Since juice is comprised largely of water, reference materials such as NIST 1643f trace elements in water represent a reasonable matrix match.

4.10 Table 4. Typical Analytical Batch Sequence

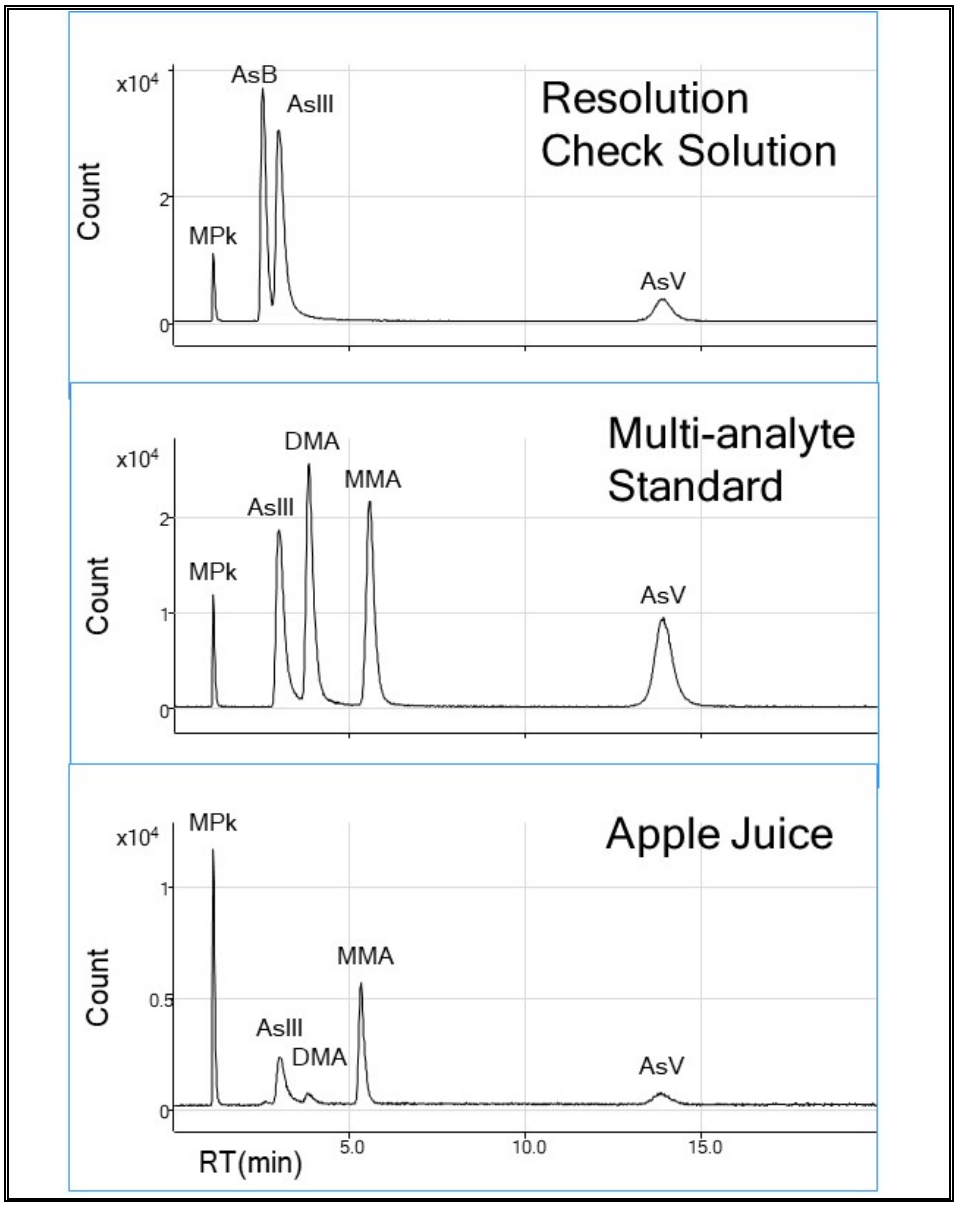
Solution	Purpose	QC Criteria
DIW blank	Verify clean auto-sampler vials	≤ ASDL
Resolution check solution	Check separation between unretained species (represented by AsB) and As(III)	near-baseline separation
Multi-analyte calibrations standards	Standardize instrument	r^2>0.99
MBK 1	Verify absence of contamination	≤ ASDL
SRM NIST 1643f	Demonstrate accuracy	80-120% recovery
Ten (10) analytical solutions (includes replicates and FAPs)	Determine As species conc.	within calibration range, RPD ≤ 15%
CCV	Verify standardization	85-115% of expected
MBK 2	Verify absence of contamination	≤ ASDL
Ten (10) analytical solutions (includes replicates and FAPs)	Determine As species conc.	within calibration range, RPD ≤ 15%
CCV	Verify standardization	85-115% of expected

[a] This and any subsequent standard solution injections should agree with previous standard solution injection within ±10%.

(3) Establish RTs and check peak shapes and responses of the marker peak and the arsenic analytes in the *m/z* 75 chromatograms. Typical retention times (in minutes) are 2.9 for As(III), 3.9 for DMA, 5.5 for MMA, and 12.7 for As(V). To some extent, the retention times and peak shapes are dependent on the age and performance of the LC column and the As(V) peak is especially sensitive to the column's condition. Significant differences (~7%, based on method validation data) between RT of standards and samples (including spiked samples) within the same batch are not anticipated but must be investigated and corrected if noted.

a. 4.10 Figure 2 shows example chromatograms obtained for the resolution check solution, a 5 ng/g standard, and an apple juice sample.

b. If using He collision mode, check the *m/z* 77 sample chromatograms for indications that argon chloride interferences might occur in the *m/z* 75 chromatograms (arising from $^{40}Ar^{35}Cl^{+}$). Peaks detected in the *m/z* 77 chromatograms (arising from $^{40}Ar^{37}Cl^{+}$) indicate that there will also be peaks with matching retention time in the *m/z* 75 chromatograms. Note, however, that selenium ($^{77}Se^{+}$) will cause a *m/z* 77 peak without a *m/z* 75 peak.



4.10 Figure 2. HPLC-ICP-MS chromatograms. Resolution Check Solution 5 ng/g arsenobetaine (AsB) and As(III); Multi-analyte standard 5 ng/g each of As(III), DMA, MMA and As(V); MPk = marker peak.

(4) Integrate *m/z* 75 chromatograms.

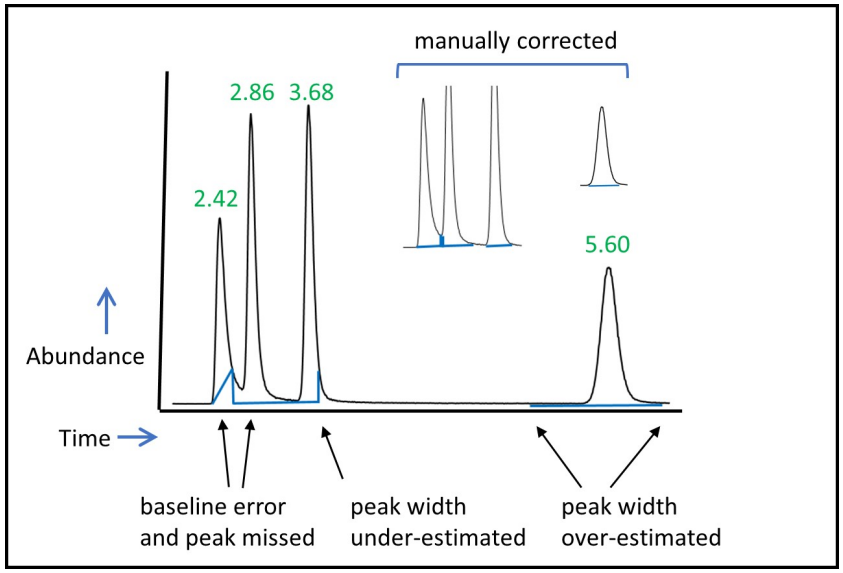
a. Recommended software integration settings (applicable for *m/z* 75 and 91) are given in 4.10 Table 5. These settings reflect programming in the Agilent MassHunter software but other software packages will have similar parameters. Inspect all chromatograms visually to verify the peaks are properly identified and the integration fits appear logical. If automated fitting is abnormal, the effect may be slight or extreme and obvious as is shown in EAM 4.10 Figure 3. If abnormal fitting is noted, integrate manually and use a consistent approach throughout the

analytical batch.

4.10 Table 5. Integration parameters[a] for m/z 75 or 91

Data Point Sampling: 1	Start threshold: 0.3
Smoothing: (enabled)	Stop threshold: 0.5
Detection filtering: 5 point	Peak location: Top
Baseline Allocation	
Baseline reset (number of points): > 10	
If leading or trailing edge: < 50	
Baseline preference: Drop else tangent skim	
Peak Area [counts]: >2000 (fill in this bullet only)	

[a] Specific to Agilent MassHunter software



4.10 Figure 3. Examples of peak integration errors and manual corrections.

b. Unknown peaks — Unknown peaks are defined as peaks that do not match the retention times of As(III), As(V), DMA, or MMA. Such peaks may occur but are unlikely and not expected.

- If unknown peaks are detected (S/N> 3), add them to the analyte list (in DA Method Editor) with the names "Unk RT" (where retention times are inserted for RT). Ignore small questionable peaks with S/N<3.
- Unknown peaks can be integrated using the above parameters, but ensure that they are not mistakenly identified as known peaks and vice versa.
- Unknown peak areas will be used to calculate approximate arsenic mass fractions for the unknowns in the sample by using he calibration for the closest RT species (see below).

4.10.9 CALCULATIONS

Standardization calibration and Mass fractions

Use the integrated peak areas and the weighted calibration curves ($1/x^2$) to calculate (via the instrument software) mass fractions of individual arsenic species in the analytical solutions. Do not choose an algorithm type where the y-intercept must pass through zero (for example, using MassHunter, use the IGNORE option for Intercept).

Calculate the mass fraction of individual arsenic species in the samples as follows:

$$MF_{sample} = MF_{AS} \cdot DF \cdot \underbrace{\left(\frac{1\ \mu g}{1{,}000\ ng}\right)}_{\text{for As}} \cdot \underbrace{\left(\frac{1{,}000\ g}{1\ kg}\right)}_{\text{for juice}}$$

(unit conversions)

Where: MF_{sample} = mass fraction of arsenic species in the sample (µg/kg)
MF_{AS} = mass fraction of arsenic species in the analytical solution (ng/g)
DF = dilution factor (mass analytical solution / mass RTD juice)

$$DF = \frac{M_{juice\ sample} + M_{DIW}}{M_{juice\ sample}}$$

Calculate the mass fraction of inorganic arsenic in the juice.

$$MF_{iAs} = MF_{As(III)} + MF_{As(V)}$$

Where: MF_{iAs} = mass fraction of inorganic arsenic in juice (µg/kg)
$MF_{As(III)}$ = mass fraction of arsenite in juice (µg/kg)
$MF_{As(V)}$ = mass fraction of arsenate in juice (µg/kg)

Notes:
Use only mass fractions that are ≥ LOD [for As(III) and As(V)] to calculate MF_{iAs}.

Mass fractions will be for juice in RTD condition. If need to report relative to a concentrate form, use the reconstitution/dilution amounts to convert.

Unknown Mass fractions

Mass fractions for unknown As species in the analytical solution can be estimated using the calibration curve for the known species having the closest retention time. This would ideally be accomplished automatically via the data analysis software. However, it can be calculated manually using the following equation:

$$MF_{Unk} = A_{Unk} \cdot m + b$$

Where:
MF_{Unk} = mass fraction for unknown arsenic species in the analytical solution
A_{Unk} = area of unknown peak
A_{marker} = area of post-column marker solution peak
m = slope of calibration curve of nearest eluting arsenic species
b = Y-intercept of calibration curve of nearest eluting arsenic species

Obtain mass fraction(s) for unknown As species in the juice sample using the same calculation to account for dilution as is used for the known species (see above).

4.10.10 QUALITY CONTROL ELEMENTS

Prior to the Sample Analysis

(1) Find retention times for the As species.

(2) Verify purity of As in the single component intermediate standards.

When new standard stock solutions are obtained, prepare and analyze 100 ng/g single-species purity check solutions.

Impurity peaks should make up ≤2% of total peak area for any solution; impurities below this level are considered insignificant and should not be used to adjust the analyte concentration (i.e. assume the standard concentration is 100% pure). If impurity peaks are >2% of total peak area for any solution, remake the purity check solution and reanalyze. If impurity peaks are still >2%, a new stock solution is typically obtained (or re-prepare from solid). See purity notes above in SOLUTION PREPARATION section.

If an impurity remains, then it must be taken into account by adjusting the concentration. For instance, a DMA stock standard containing 1,000 µg/g total As with 5% As(V) impurity should be re-labeled as 950 µg/g DMA + 50 µg/g As(V). The additional As(V) must be taken into account when assigning levels in the mixed-species standard solutions.

(3) If DMA and MMA are prepared from solids, determine (experimentally) the mass fractions of arsenic in the stock standards. (See §4.10.6)

(4) Calculate ASDL and ASQL for each species. Base these on the standard deviations of replicate (n=10) analyses of a low-level multi-species standard (equivalent to a fortified method blank) with mass fractions just above the estimated ASDLs (*e.g.*, ≈ 0.1-0.3 ng/g). The ASDL and ASQL calculations are shown below (according to EAM§3.2).

(5) Calculate LOD and LOQ (using ASDL and ASQL, respectively).

$$ASDL = t_{0.95} \cdot \sqrt{1 + \frac{1}{n}} \cdot s$$

$$ASQL = 30 \cdot s$$

$$LOD = ASDL \cdot DF$$

$$LOQ = ASQL \cdot DF$$

Where: s = standard deviation of replicates (ng/g)
DF = dilution factor (typically 5 for RTD juice and ~30 for concentrate; unitless)

Note:

Because these are estimates, the largest ASQL and ASDL obtained from each of the four arsenic species can be assigned universally to all species.

During Analysis of Samples

Failure of any of the QC elements described below to meet performance criteria shall require an explanation of what was done to correct the problem and may require reanalysis of samples analyzed prior to the loss of method control measures.

(1) Calibration Curve

The calibration curves must be linear over the entire analytical solution mass fraction range with r^2>0.99. If failure to meet these criteria, repeat calibration and prepare new working standard solutions, if necessary.

(2) Continuing Calibration Verification (CCV) Standard

The CCV standard is monitored repeatedly during analysis. If QC criteria not met, re-analysis allowed only once. Additional failure requires re-standardization and re-analysis of samples that were analyzed after the last acceptable CCV standard analysis.

Control limits are 100 ± 15% for DMA, MMA, and iAs [As(III)+As(V)]. There are no control limits for As(III) and As(V) individually. Retention times (when compared to the 10 ng/g calibration standard) are As(III) RT ± 0.2 minutes, DMA RT ± 0.2 minutes, MMA RT ± 0.3 minutes, and As(V) RT± 0.5 minutes.

(3) Method Blanks

Monitor method blanks during analysis. If the control limit is not met, identify the source(s) of contamination and eliminate it/them prior to continuing with the analyses. As indicated above, ammonium phosphate dibasic is used to prepare mobile phase and is a common source of contamination that can interfere.

Control limit — Arsenic below detection (<ASDL) for all species.

(4) Fortified Analytical Portion (FAP) - peak characterization

Monitor peak characteristics during analysis. For each species, compare the unfortified and fortified chromatograms to verify that (for the main analytical peaks) the peak retention times are identical, there is an appropriate increase in peak area, the peak shapes are similar, and there is no significant band broadening or appearance of shoulders. Also verify the absence of unexpected peaks.

FAP recovery is verified after analysis.

After Sample Analysis

(1) Replicate Analytical Portions (RAP) - precision

Check RAP precision by calculating the relative percent difference for duplicates (RPD) or relative standard deviation for triplicates (RSD). If the control limit is not met, re-analyze the sample replicates that are involved and determine whether a high RD/RSD is most likely due to nonhomogeneity or analysis imprecision. If the latter, find and eliminate the source of imprecision then re-analyze the batch.

Precision control limit for iAs, DMA and MMA (for levels ≥LOQ) is RPD (or RSD) ≤15%

$$PD(\%) = \frac{C_1 - C_2}{MF_{avg}} \times 100\%$$

(or) $$RSD(\%) = \frac{s}{MF_{avg}} \times 100\%$$

Where: C_1 = mass fraction of first measurement (µg/kg)
C_2 = mass fraction of second measurement (µg/kg)
MF_{avg} = average mass fraction of replicates (µg/kg)
s = standard deviation of replicates (µg/kg)

Caution on juice mixing:

Take care that the juice is well mixed when analytical portions are taken. This will assure that non-homogeneity will be negligible and the observed RPD will be an effective indicator of analytical imprecision.

(2) Fortified Analytical Portion (FAP) - recovery

If a recovery is not acceptable, ensure that the spiking level is appropriate then re-prepare and analyze the new FAP solution. Re-analysis of the entire sample batch may be required.

FAP recovery control limit for iAs, DMA and MMA is 100 ± 20%. Note that As(III) and As(V) are evaluated only as the sum (i.e., iAs). Neither of these two species are evaluated independently.

For DMA, and MMA: $$\%\ Recovery = \left[\frac{(MF_{FAP} - MF_{sample})}{\left(\frac{MF_{fort\ sol} \cdot M_{fort\ sol}}{M_{sample}}\right)}\right] \times 100\%$$

Where:
- MF_{FAP} = mass fraction for As in FAP (µg/kg)
- MF_{sample} = mass fraction for As in unfortified sample (µg/kg)
- $MF_{fort\ sol}$ = mass fraction for As in fortification solution (µg/kg)
- $M_{fort\ sol}$ = mass of fortification solution added to create the FAP (kg)
- M_{sample} = mass of sample (kg)

$$iAs\ \%\ Recovery = \left[\frac{\left(MF_{FAP\ As(III)} + MF_{FAP\ As(V)}\right) - \left(MF_{sam\ As(III)} + MF_{sam\ As(V)}\right)}{\left(\frac{MF_{fort\ sol\ As(III)} \cdot M_{fort\ sol}}{M_{sample}}\right) + \left(\frac{MF_{fort\ sol\ As(V)} \cdot M_{fort\ sol}}{M_{sample}}\right)}\right] \cdot 100\%$$

Where:
- $MF_{FAP\ As(III)}$ = mass fraction for As as As(III) in FAP (µg/kg)
- $MF_{FAP\ As(V)}$ = mass fraction for As as As(V) in FAP (µg/kg)
- $MF_{sam\ As(III)}$ = mass fraction for As as As(III) in unspiked sample (µg/kg)
- $MF_{sam\ As(V)}$ = mass fraction for As as As(V) in unspiked sample (µg/kg)
- $MF_{fort\ sol\ As(III)}$ = mass fraction for As as as As(III) in spiking solution (µg/kg)
- $MF_{fort\ sol\ As(V)}$ = mass fraction for As as As(V) in spiking solution (µg/kg)
- $M_{fort\ sol}$ = mass of multi-species spiking solution added to sample portion (g)
- M_{sample} = mass of juice sample (g)

(3) Mass Balance (MB)

If total arsenic is ≥10 µg/kg (in RTD condition), verify that the speciation analysis has accounted for the majority of the arsenic in each sample. Calculate the MB in percent as the sum of all the arsenic found in the species analysis relative to the total As found using a total As analysis, e.g., using EAM 4.7. Control limit for MB is 100 ± 25%. If mass balance via a "dilute-and-shoot" procedure is below 75%, analyze the juice via the acid extraction procedure. If MB remains low, then document this finding on the analysis report.

$$Mass\ Balance\ (\%) = \frac{MF_{iAs} + MF_{DMA} + MF_{MMA} + MF_{Unknown}}{MF_{Total\ As}} \cdot 100\%$$

Where:
- MF_{iAs} = mass fraction for As as iAs (sum of III and V) (µg/kg)
- MF_{DMA} = mass fraction for As as DMA (µg/kg)
- MF_{MMA} = mass fraction for As as MMA (µg/kg)
- $MF_{Unknown}$ = sum of mass fraction(s) for As in unknown species (µg/kg)
- $MF_{Total\ As}$ = mass fraction for As measured using ICP-MS (µg/kg)

(4) Reference Material

Check CRM and/or in-house reference material recovery. The control limit will vary according to certified values. Use an appropriate metrological procedure to evaluate. For NIST SRM 1643f, the control limit for iAs [sum As(III) + As(V)] relative to the certified total As value of 56.85 µg/kg is 100 ± 20%. As(V) and possibly As(III) should be the only peaks detected.

CRM Notes:

The availability and matrices produced vary over time. NIST SRM 1643 is mentioned above several times only because it was relied on heavily during method development. At the time of this revision, 1643f is the current version of this SRM. Other CRMs may be used as long as they are not out-of-date and certificate instructions are followed.

Practicality issues may also affect CRM usage. For example, NIST SRM 3035 *Arsenic Species in Apple Juice* is certified for all four arsenic species. But, it is not normally considered useful for routine analysis because it has unique storage/use specifications (store at -80°C and use within 4 hours of thawing). And, it can be cost-prohibitive since it is supplied in only a small unit size (7.5 mL). It could, however, be quite useful for applications such as method validation and/or training.

4.10.11 REPORT

Follow the guidelines below.

(1) **QC —** Report results for a batch only when quality control criteria have been satisfactorily met.

(2) **Species —** Report results according to customer needs. Any of the following are available from EAM 4.10:

- DMA
- MMA
- As(III)
- As(V)
- total inorganic arsenic [As(III) + As(V)]
- $As_{(unknown\ species)}$

Total arsenic (the independently-obtained value used in the mass balance calculation) is obtained outside the scope of EAM 4.10 and therefore does not need to be reported.

(3) **Units —** Give mass fractions in units of mass of elemental arsenic per mass of juice in RTD condition (i.e., reconstituted, if necessary). Do not use mass/volume and do not use the mass of arsenic compound.

(4) **Juice Concentrates —** Results would not typically be reported for juice in an as-received concentrate condition. However, if required, report them in addition to the RTD juice results and very clearly identify both sets of results so the findings will be very clear. Mass fractions for arsenic in concentrate condition would be calculated

using the dilution amounts.

(5) **LOD and LOQ —** Report laboratory LOD and LOQ (i.e., do not report the values in 4.10 Table 1 as these are presented only as examples).

(6) **Qualifiers —** For a result ≥LOQ, report mass fraction and units without any accompanying qualifier. If it is ≥LOD and <LOQ, report mass fraction and units followed by the trace qualifier (i.e., "TR"). If it is <LOD (and probably not even picked up by the auto-integrator), report zero (i.e., "0") followed by the units of measurement.

Qualifier Example for iAs [LOQ=3.5 and LOD=0.45 (µg/kg)]:
Found (for three juices) 5.0, 1.0, and 0.2 (µg/kg)

Found	*Condition*	*Report*
5 µg/kg	*≥LOQ*	*5 µg/kg*
1 µg/kg	*≥LOD and <LOQ*	*1 µg/kg (TR)*
0.2 µg/kg	*<LOD*	*0 µg/kg*

4.10.12 METHOD VALIDATION

Use of the PRP-X100 column with ammonium phosphate mobile phases for arsenic speciation has been previously reported with good results[4-7].

Single Laboratory Validation

Dilute-and-shoot procedure — EAM 4.10 (using the dilute-and-shoot procedure) was validated by replicate analyses of 18 commercial juices (five juices n=10/ea and 13 juices n=3/ea)[8]. In total, the juices included five grape (2 purple, 1 red, and 2 white), three apple, seven pear, and three juice blends. Three of these were marketed for infants (one/each white grape, apple, and pear) and six of the seven pear juices were obtained in concentrated form (all others received in RTD condition). One blend was primarily cherry, one primarily cranberry, and the third was a variety.

certified reference materials were analyzed (to show accuracy).

(1) Replicate analysis showed excellent repeatability. Precision was ≤10% RSD for species present at mass fractions ≥LOQ.

(2) Fortification analysis showed good recovery which ranged 80-120% for all four species in all juices.

(3) CRM analysis showed excellent accuracy. A z-score evaluation was performed for NIST SRMs 1640a (Trace Elements in Natural Water) and 2669 [Arsenic Species in Frozen Human Urine (Level II)].

SRM 1640a — In addition to z-scores all being <2, the iAs mass fractions were exceptionally close to certified values - differing by less than 0.1%.

SRM 2669 — Z-scores were all <2 for DMA, MMA, and As(V) (all present at levels above LOQ). As(III) was not in agreement but total inorganic arsenic [sum

of As(III) and As(V)] was [using combined uncertainty for As(III) and As(V) calculated as root-sum-square].

Acid digest procedure — EAM 4.10 (using the dilute-and-shoot procedure) was validated for prune, pomegranate, and cherry juices using a set of low mass balance juice samples found to have total arsenic above 10 ng/g.[9] EAM 4.10 Table 6 shows single-laboratory validation results for nine juice samples analyzed using both the dilute-and-shoot and acid digestion procedures. As evidenced, mass balances were low using the dilute-and-shoot procedure but excellent using the acid digestion procedure.

QC Supporting Data from Surveys

EAM 4.10 was used successfully in two FDA surveys conducted in 2011 to gather information on arsenic species in fruit juices[10]. Two FDA laboratories were involved and a total of 396 juice samples were analyzed (254 apple and 142 pear; including both RTD and concentrates). The findings and QC data from those surveys provided useful validation information.

(1) Check standard recovery ranges verified overall system stability.

93-115% for iAs,
90-112% for DMA
93-114% for MMA

(2) Replicate analytical portion analyses showed excellent repeatability with RSDs for iAs, DMA, and MMA (when ≥LOQ and n≥3) ranging 1.1-7.5% in juices.

(3) Fortified analytical portion analysis showed good recoveries.

83-120% for iAs (101% average, n=24),
86-106% for DMA (97% average, n=17)
83-111% for MMA (100% average, n=17)

(4) Mass balance results verified that the dilute-and-shoot procedure, which was used exclusively for these surveys, is acceptable for the vast majority of apple and pear juices. The overall mass balance average was 85% and the range was 64-111%. Note that the mass balance QC check is still necessary because the extraction may be necessary for an unusual juice preparation.

(5) CRM analysis results (NIST SRM 1643e, n=34) showed excellent accuracy, repeatability, and reproducibility. The iAs mass fraction ranged 92-107% and averaged 99.8% relative to the certified value.

Multi Laboratory Validation

Dilute-and-shoot procedure — EAM 4.10 (using the dilute-and-shoot procedure) was validated for apple, grape, and pear juices[11,12]. Thirteen juice samples were analyzed by 8 laboratories, including 6 apple (3 obtained as concentrates), 4 grape, and 3 pear. Each laboratory analyzed method blanks, fortified method blanks, triplicate portions of each juice sample, and duplicate fortified juice samples (one for each matrix type) at three fortification levels. Two water Standard Reference Materials (SRMs) were also analyzed.

LODs and LOQs were ~0.3 and ~2 ng/g, respectively. Repeatability and reproducibility (when >LOQ) were in general ≤15% RSD. Fortification recovery averages ranged 98-104% (for iAs,

DMA, and MMA) and SRM iAs results ranged 96-98% of the certified values (NIST SRMs 1640a and 1643e).

Acid digest procedure — EAM 4.10 was also validated specifically for prune, pomegranate, and cherry juices which are common juices that typically exhibit low mass balance without the added acid extraction. Tables 7 and 8 show excellent results from a multi-laboratory validation study involving 3 samples (1/each prune, pomegranate, and cherry) analyzed by two laboratories. Speciation results are in Table 7 and fortification recovery results are in table 8. Fortifications were at 8, 16, and 32 ng/g [for each species — As(III), DMA, MMA, and As(V)].

EAM 4.10 Table 6. Single-Laboratory Validation — Procedure Comparison for Total Arsenic in LMBJ

	EAM 4.7[a]	dilute-and-shoot[b]		acid digest[b]	
	(ng/g)	(ng/g)	(MB %)[c]	(ng/g)	(MB %)[c]
Prune	7.1	4.5	64%	6.6	93%
Prune	31.6	16.7	53%	28	87%
Prune	19.3	10.0	52%	15.8	82%
Pomegranate	13.1	5.7	44%	11.4	88%
Pomegranate	7.7	3.5	46%	6.9	90%
Pomegranate	22.7	11.7	52%	19.1	84%
Cherry	7.5	6.1	80%	7.8	104%
Cherry	25.6	17.9	70%	23.3	91%
Cherry	10.6	7.7	73%	10.3	98%

[a] Total arsenic via EAM 4.7
[b] EAM 4.10 procedures [sum As(III) + As(V); see section 4.10.7]
[c] Relative to the EAM 4.7 result

EAM 4.10 Table 7. Multi-Laboratory Validation — Arsenic Speciation for Three LMBJ Samples Using Acid Digestion Procedure

LMBJ	lab #	iAs (ng/g; %)[a]	DMA (ng/g; %)[a]	MMA	SUM	MB[b]
prune	1	27.1 ± 0.1 (0.4)	<LOD	<LOD	27.1	86%
	2	31.6 ± 1.4 (1.4)	<LOD	<LOD	31.6	100%
pomegranate	1	18.6 ± 1.7 (9.1)	2.30 ± 0.11 (5)	<LOD	20.9	92%
	2	18.8 ± 0.8 (4.3)	2.40 ± 0.12 (5)	<LOD	21.2	94%
cherry	1	10.8 ± 0.6 (5.6)	<LOD	<LOD	10.8	102%
	2	10.8 ± 0.3 (2.8)	<LOD	<LOD	10.8	102%

[a] Mean ± standard deviation (n=3, expressed in ng/g) followed by the relative standard deviation (in parentheses, expressed in %)

[b] Species sum relative to total-arsenic level (determined separately).

EAM 4.10 Table 8. Multi-Laboratory Validation Exercise - FAP Summary

Sample Type	FAP % Recoveries[a]		
	iAs[b] $\bar{x}$ (Min - Max)	DMA $\bar{x}$ (Min - Max)	MMA $\bar{x}$ (Min - Max)
Prune (n=12)	99% (85-112%)	109% (95-116%)	106% (89-139%)
Pomegranate (n=12)	96% (80-103%)	103% (99-108%)	97% (88-107%)
Cherry (n=12)	98% (86-118%)	108% (99-119%)	99% (90-116%)
MTB (n=10)	102% (89-112%)	104% (92-119%)	102% (92-112%)

[a]Juice fortification levels each species at 8, 16, 32 µg/kg [As(III) and As(V) both fortified].
[b] iAs = As(III) + As(V).

Uncertainty

A result above LOQ has an estimated combined uncertainty of 10%. Use a coverage factor of 2 to give an expanded uncertainty at about 95%. A result above LOD, but below LOQ is considered qualitative and is not reported with an uncertainty.

A detailed discussion of method uncertainty is presented in EAM §3.3. This method conforms to the information contained in that discussion. Derivation of an estimated uncertainty specific to an analysis is also discussed EAM §3.3.2.

4.10.13 REFERENCES

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U.S. Department of Health & Human Services

FDA U.S. Food and Drug Administration

Elemental Analysis Manual

for Food and Related Products

4.10 Appendix A
High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometric Determination of Arsenic Species in Wine

Version 1.1 (May 2020)
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GLOSSARY

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4.10A.1 SCOPE, APPLICATION, AND BACKGROUND

This appendix presents modifications to EAM 4.10 that enable arsenic species to be determined in wine, which contains 7-24% (v/v) ethanol[1]. EAM 4.10 is followed almost in entirety except for the few modifications given below.

Wine is of particular interest because it is made from grapes and therefore, like grape juice, may have elevated inorganic arsenic[2]. However, EAM 4.10 can not be used for wine because its ethanol interferes with MMA quantitation. Ethanol coelutes with MMA (see Figure 1) and its carbon increases arsenic's mass spectrometry sensitivity[3] (i.e., invalidates the MMA standard calibration; see figure 2). This was evident in EAM 4.10 analysis of wine which resulted in MMA FAP recoveries that averaged 129% and ranged up to 155% (see Table 1). The ethanol effect is also evident in an ethanol-spiked blank chromatogram (see Figure 3; peak at ~5 min).

Although the usual EAM 4.10 calibration for MMA cannot be used, an appropriate calibration is established if ethanol is added to the calibration standards (and associated QC samples). The use of 3% ethanol mimics wine's matrix because it corresponds with 15% ethanol (per the 5-fold dilution in EAM 4.10). The 15% is logical because it is midway for the 10-20% range that applies for most wines and is a reasonable approximation.

Multi-laboratory validation study of this modified method included analysis of wines representing the five main classifications of wine found in the marketplace - red, white, rose, sparkling, and high-alcohol fortified.

4.10A.2 SUMMARY OF METHOD (See EAM 4.10 but subject to changes below)

4.10A.3 SAFETY CONSIDERATIONS (See EAM 4.10)

4.10A.4 EQUIPMENT AND SUPPLIES (See EAM 4.10)

4.10A.5 REAGENTS (See EAM 4.10 plus the following:)

Ethanol — CAS Number 64-17-5 High purity, such as "UV HPLC gradient 99.9%" 200 proof ethanol from Sigma-Aldrich (cat. no. V002075).

4.10A.6 SOLUTION PREPARATION (See EAM 4.10 except for the following:)

Working Solutions

(10) **(Addition to EAM 4.10) 3% Ethanol —** Prepare a 3% (v/v) ethanol solution by measuring 15 mL (11.84) absolute ethanol into a 500 mL polypropylene bottle. Add 485 mL (485 g) DIW; shake to mix.

(11) **(Changes from EAM 4.10) Multi-analyte Instrument calibration standards:**

e. **Calibration standard (10 ng/g) —** Add 0.500 g of the 200 ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with **3% ethanol**.

f. **Calibration standard (4 ng/g) —** Add 0.200 g of the 200 ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with **3% ethanol**.

g. **Calibration standard (1 ng/g) —** Add1.0 g of the 10 ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with **3% ethanol**.

h. **Calibration standard (at or slightly above ASQL; i.e., ~0.4 to 0.5 ng/g) —** Add 0.500 g of the 10 ng/g level solution into a tared HDPE or polypropylene tube and dilute to 10 g with **3% ethanol**. (see 4.10.10 for ASQL).

(12) **(Change from EAM 4.10) Calibration-check standard (~2 ng/g) —** Add 0.1 g of the 200 ng/g multi-analyte intermediate standard solution into a tared HDPE or polypropylene tube and dilute to 10 g with **3% ethanol**.

(13) **(Change from EAM 4.10) Certified reference material —** Add 0.5 g of NIST 1643f *Trace Elements in Water* into a tared 15 mL tube and dilute to 7.5 g total with **3% ethanol** (a 15-fold dilution).

4.10A.7 SAMPLE PREPARATION

Sample, RAP, FAP - Unchanged from EAM 4.10 (i.e., dilute with **DIW**).

Method blank (MBK)

Take 2 g DIW through the sample preparation procedures described in EAM 4.10 but dilute to 10 g with **3% ethanol**. Note that the 3% ethanol will give a peak at around 5 minutes (Figure 3) due to carbon enhancement of the background. This is due to low level arsenic contamination in the mobile phase. Do not integrate or blank-subtract this.

Certified Reference Material (CRM)

Depending on CRM matrix, prepare as if a sample but dilute (**with 3% ethanol**) appropriately according to the known As level so the analytical solution's As level will be within the calibration. Although NIST SRM 1643f is not certified for arsenic species, As(V) and possibly As(III) should be the only peaks detected. For this CRM, a dilution factor of ~15× is appropriate

to dilute the acid content.

4.10A.8 DETERMINATION PROCEDURE (See EAM 4.10)

4.10A.9 CALCULATIONS (See EAM 4.10)

4.10A.10 QUALITY CONTROL ELEMENTS (Unchanged from EAM 4.10)

4.10A.11 REPORT (See EAM 4.10)

Note concerning MMA FAP bias:
When wine has ethanol near the limits of the range herein mentioned (7-24%), the MMA FAPs will have an inherent bias to a lower or higher FAP recovery, respectively. Based on data presented in Tanabe, et al.[4], the predicted MAA recovery in 7% wine would be low (~89%) and high (113%) in 24% wine. The room for error is therefore less in these cases but the quality control measure (80-120%) remains.

4.10A.12 METHOD VALIDATION

This method extension has been validated using a set of samples intended to represent a majority of commonly consumed wine styles: red, white, rose, sparkling, and high-alcohol fortified. The issues and the steps taken to resolve the ethanol enhancement issue are discussed in detail in the Tanabe et al. manuscript[4]. Due to the low levels of MMA in typical wine samples, the issue was only noted in FAP solutions. EAM 4.10 Appendix A Tables 1 and 2 show the difference between spike recovery for DMA, MMA, and iAs using EAM 4.10 as written (Table 1), and then using the modified method described in this appendix (Table 2), respectively. The modified method has gone through a multi-laboratory validation exercise as well[4], and the results of this are summarized in Table 3. Additional summary tables are presented below to demonstrate the performance of this altered method to include analysis of wine samples.

4.10A.13 REFERENCES

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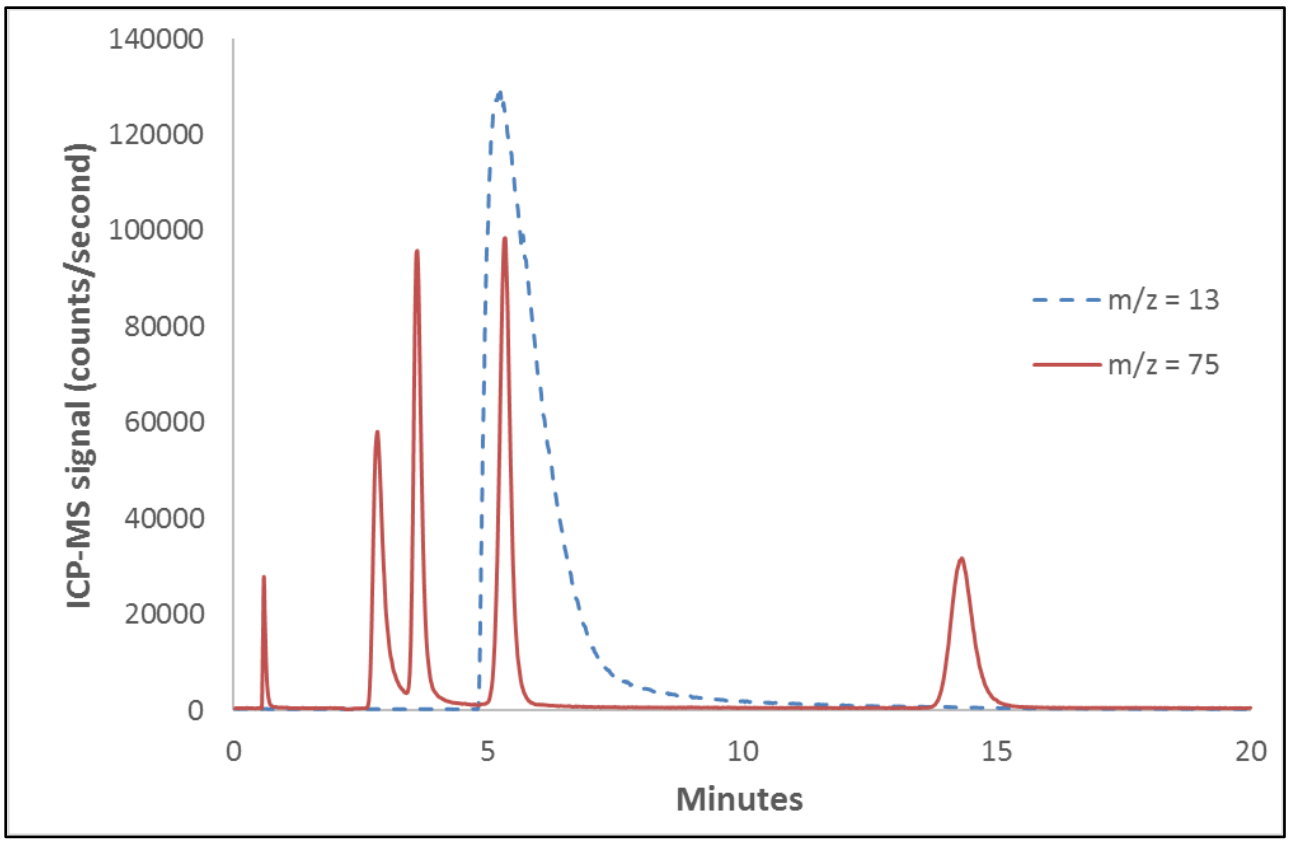
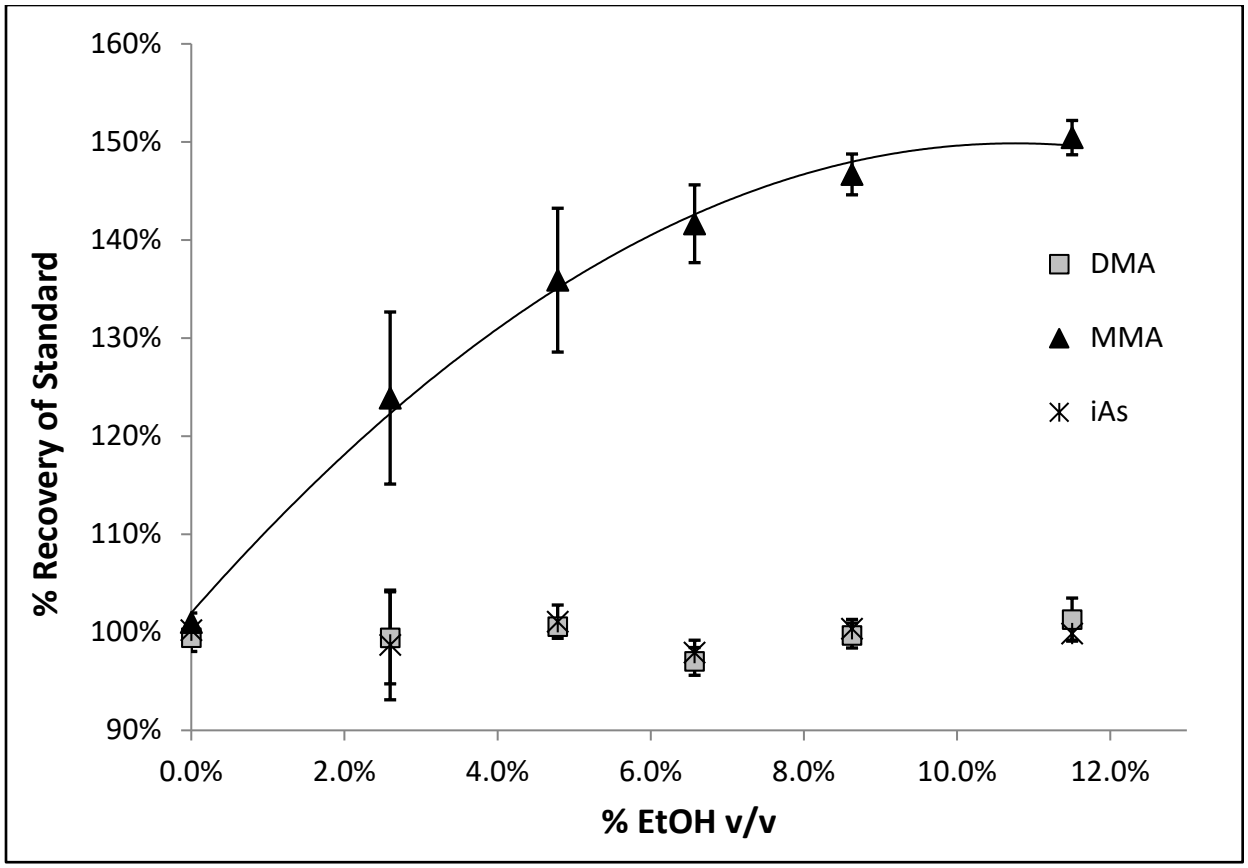
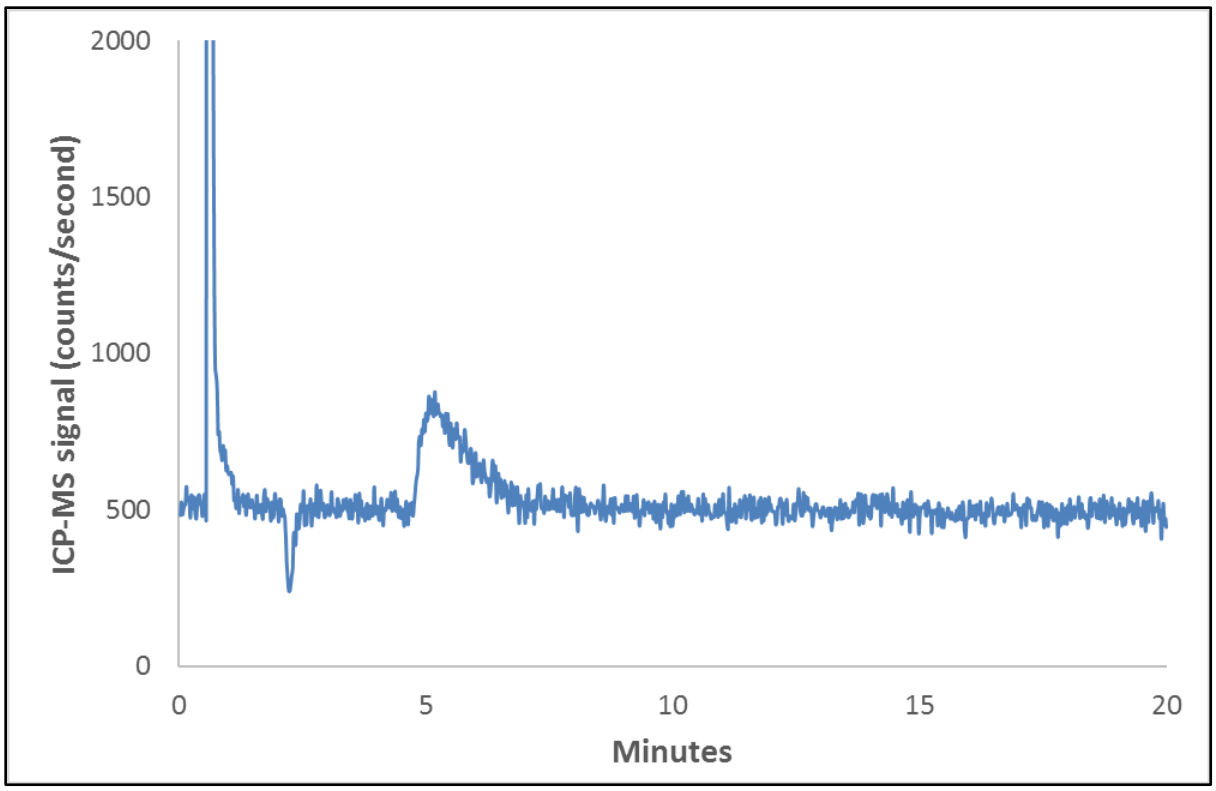


4.10A Figure 1. Chromatograms (^{13}C and ^{75}As) from 20 ng/g calibration standard in 3% ethanol



4.10A Figure 2. Spike recoveries for iAs, DMA and MMA at various ethanol concentrations



4.10A Figure 3. Chromatogram (m/z 13) for method blank with 3% ethanol

4.10A Table 1. Wine analysis using EAM 4.10

Sample	DMA	MMA	iAs
A (µg/g)[a]	0.39	0.25	15.43
B (µg/g)[a]	0.35	0	10.83
C (µg/g)[a]	0.45	0.24	9.33
D (µg/g)[a]	1.45	0	1.15
E (µg/g)[a]	0	0	1.62
FAP recovery[b] (%)	103 (96-113)	129 (116-155)	104 (92-117)

[a]Average mass fraction, n=4 replicates for each wine
[b]Average recovery with range in parentheses, n=12 (3 levels x 2 replicates x 2 labs = 12)

4.10A Table 2. Comparison of Multilaboratory Validation Results for Five Fortified Samples from Three Laboratories

Laboratory		DMA	MMA	iAs
Lab 1	average[a]	99%	99%	93%
	range	84-121%	84-111%	82-106%
Lab 2	average[a]	106%	110%	105%
	range	88-113%	94-121%	83-113%
Lab 3	average[a]	99%	92%	102%
	range	93-107%	72-119%	97-107%
Overall	average	101%	100%	101%
	range	84-121%	72-121%	82-113%

[a]Average fortification recovery for duplicates at 5, 10, and 30 µg/kg for DMA, MMA and iAs in five samples for a total of n = 30 per laboratory

4.10A Table 3. Comparison of Multilaboratory Validation Results for Five Samples from Three Laboratories

Lab	Wine Sample[a]	DMA[b]	MMA[b]	iAs[b]	Sum of Species	Total As[c]	% Mass Balance
LOD		0.17	0.15	0.17			
LOQ		1.3	1.2	1.4			
Lab 1		0.46 ± 0.2 [d]	<LOD	14.0 ± 0.3	14.4 ± 0.3		94%
Lab 2	1 (9.5)	0.66 ± 0.01 [d]	<LOD	14.2 ± 0.1	14.9 ± 0.1	15.3 ± 1.2	97%
Lab 3		0.81 ± 0.1 [d]	<LOD	14.4 ± 1.0	15.2 ± 1.1		99%
Lab 1		0.47 ± 0.1 [d]	<LOD	9.4 ± 0.1	9.9 ± 0.2		89%
Lab 2	2 (13)	0.51 ± 0.1 [d]	<LOD	10.6 ± 0.3	11.1 ± 0.3	11.1 ± 0.8	100%
Lab 3		0.74 ± 0.04 [d]	<LOD	10.7 ± 0.2	11.4 ± 0.2		103%
Lab 1		0.80 ± 0.1 [d]	<LOD	7.3 ± 0.2	8.1 ± 0.2		87%
Lab 2	3 (12)	0.58 ± 0.1 [d]	<LOD	9.0 ± 0.3	9.6 ± 0.3	9.3 ± 1.1	103%
Lab 3		0.75 ± 0.1 [d]	<LOD	9.2 ± 0.4	9.9 ± 0.4		107%
Lab 1		1.8 ± 0.1	<LOD	1.4 ± 0.1 [c]	3.2 ± 0.1		89%
Lab 2	4 (20)	1.6 ± 0.1	<LOD	1.6 ± 0.1	3.2 ± 0.2	3.6 ± 0.3	89%
Lab 3		1.7 ± 0.1	<LOD	2.1 ± 0.3	3.8 ± 0.3		105%
Lab 1		<LOD	<LOD	1.2 ± 0.1 [c]	1.2 ± 0.1		54%
Lab 2	5 (14.5)	<LOD	<LOD	1.4 ± 0.1 [c]	1.5 ± 0.1	2.2 ± 0.1	68%
Lab 3		0.45 ± 0.01 [d]	<LOD	1.5 ± 0.3	2.0 ± 0.3		91%

[a] Wines randomly identified as #1-5, ethanol content given in parentheses as % v/v
[b] Values are in (µg/g), lab results are averages ± 1σ, n=3
[c] Values are in (µg/g), averages ± 1σ, n≥5 for the Total As values reported from laboratories 1 and 2
[d] Value between LOD and LOQ