Analysts Report Amendment

Study No.: 275.30

Amendment No.: 1

Effective Date: 3 Feb 2011

Study Title: Provide data on various arsenic species present in broilers treated with roxarsone: Comparison with untreated birds

Changes:

The following minor errors were noted by the Quality Assurance Unit in review of the Analysts Report after it had been signed:

1. On page 10, line 203, the Jackson and Bertsch reference number should be (8), not (7).

2. On page 20, line 359, should read "Tables 3a though 3d" not 3c.

3. On page 23, line 424, should read "(Table 3b)" not 3a.

Mary C. Carson — 2/3/2011

Analyst Mary C. Carson (CVM) — Date

Sean Conklin — 2/3/11

Analyst Sean D. Conklin (CFSAN) — Date

[signature] — 03-03-11

Philip J. Kijak, Director, Division of Residue Chemistry (CVM) — Date

Stephen G. Capar — 2/3/2011

Stephen G. Capar, Chief, Chemical Contaminants Branch (CFSAN) — Date

Office of Research Final Report:

Analysts' Report for Liver, Feed and Premix Analyses

Provide data on various arsenic species present in broilers treated with roxarsone: Comparison with untreated birds.

OR Study 275.30

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I. Abstract

We were tasked with developing and applying methods to characterize the arsenic (As) profiles in tissue and excreta from control chickens and those treated with roxarsone. A literature search showed that ion chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS) is the most sensitive and versatile instrumentation for this purpose. Validated methods to accomplish As speciation in roxarsone treated poultry were not available. The animal phase of Study 275.30 was completed before we could develop such methods. We determined total As in the breast and liver samples from the study birds using a standard method. Total As concentrations were much higher (ca 40x) in liver than in breast, so method development focused on liver. A preliminary speciation method developed using standards and fortified control tissue proved inadequate when used with the study samples due to the presence of unknown As compounds (presumed roxarsone metabolites) which interfered with the chromatography. The IC-ICP-MS speciation method was refined with the primary purpose of identifying and quantifying trace levels of arsenite (AsIII) or arsenate (AsV) in liver in the presence of large amounts of roxarsone and other unknown organic As species. This method was used to characterize the study liver samples. Livers from untreated birds did not have any As species above the method's lower limit of quantification. Livers from treated birds all had roxarsone present, as well as several other As-containing compounds. A modified version of the tissue speciation method was also applied to feed and type A medicated articles to further characterize them.

II. Narrative

Introduction

The fact that arsenic comes in a variety of forms (with greatly differing toxicological effects) has been known for over 100 years. Arsenic speciation was limited to determination of arsenate and arsenite, until the development of hydride-generation (HG) techniques coupled with atomic absorption detection in the 1970s, which enabled measurement of AsIII, AsV, dimethylarsinic acid (DMA) and monomethylarsenic acid (MMA). Hydride generation is limited, though, because many arsenicals are not amenable to formation of hydrides. Liquid chromatography (LC), and in particular ion chromatography (IC), was more suitable as a method of separating a larger number of species, but success was limited for lack of a suitable detector. This changed with the commercialization of inductively coupled plasma-mass spectrometers (ICP-MS) in the 1980s. ICP-MS represented a new detector with superb detection limits which was easy to interface with LC, a powerful tool for separating a large number of compounds in the same solution. The element-specific ICP-MS is especially well suited for detection of arsenic species, as the As heteroatom (*m/z* 75) allows discrimination from all the other organic material in biological samples. The combination of the two—LC-ICP-MS—enabled a proliferation of arsenic speciation research from the 1990s to the present. A number of other techniques are still used, in part because of the expense associated with ICP-MS. The main weakness of ICP-MS is that it offers no molecular information, meaning that species identification is primarily based on retention time matching with known standards. *(History based on Francesconi & Kuehnelt's review on the state of arsenic speciation)*(1)

Previous arsenic speciation in chicken tissue

Falnoga (2) used LC-HG-AAS to analyzed extracts of freeze-dried liver tissue from hens given feed enriched with arsenic trioxide (30 µg/g), and found AsIII (29 ng/g) and DMA (189 ng/g). Species were separated using a Hamilton PRP-X100 column with 15 mM potassium phosphate pH 6 mobile phase.

In 2004, Polatajko and Szpunar (3) reported on the speciation of arsenic in chicken meat, in the context of characterizing the species identification and stability in a freeze-dried chicken meat candidate reference material (source not specified). The authors used methanol-water extraction and LC-ICP-MS detection to find 106 ng/g DMA, 37 ng/g arsenobetaine (AsB, which could come from the fish meal which was a feed ingredient), and ~15 ng/g unknown As (60-65% extraction efficiency reported, 157 ng/g total As in tissue). Species were separated using an AS7 column with 0.5 and 50 mM nitric acid mobile phase with 0.5% methanol added.

In her Ph.D. dissertation (4), Dr. Tyre Grant developed an LC-ICP-MS method for extraction and analysis of arsenic species in chicken tissue. Several different chromatographic conditions were compared in her work, including PRP-X100 column with 10mM ammonium phosphate + 10mM ammonium nitrate pH 6.3, PRP-X100 column with 20mM ammonium carbonate, and AS7 column with 10mM nitric acid. While the PRP-X100 column was good at separating AsIII, DMA, MMA and AsV, she found that roxarsone did not elute from the PRP. Roxarsone was successfully detected using the AS7 column for separation. In tetramethylammonium hydroxide (TMAH) extracts of freeze-dried store-bought chicken liver she found AsIII (0.043 mg/kg), AsV (0.116 mg/kg), MMA (0.106 mg/kg) and roxarsone (0.644 mg/kg), claiming confirmation of roxarsone in edible tissues “for the first time.” Several unidentified peaks presumed to be

126 roxarsone metabolites were also reported. Total arsenic values ranged from 0.02 to 0.057 mg/kg
127 in muscle, and 0.606 to 1.999 mg/kg in liver. The average mass balance (sum of total species
128 found chromatographically divided by total As) was 51%.

129 Another group's report focused on extraction methods for liberating arsenic from chicken meat
130 (5). Using LC-UV-HG-AFS analysis of methanol-water (+ heat + ultrasonics) extracts of freeze-
131 dried store-bought chicken breast, the authors found AsB (48 µg/kg) and Nitarsone (227 µg/kg)
132 (reported extraction efficiency 80-100%, 270 ng/g total As). Species were separated using a
133 PRP-X100 column with 25mM potassium phosphate pH 5.8 mobile phase.

134 Pizzaro, et al., focused primarily on extraction of arsenic species from various matrices (6).
135 They determined arsenic species in chicken tissue extracts, again as part of a characterization of
136 candidate reference materials. Using methanol/water, they achieved 70-75% extraction
137 efficiency of 0.168 mg/g total arsenic. Of the arsenic extracted, ~15% was AsB, 50% DMA and
138 34% was an unidentified arsenical. Anionic species were separated using a PRP-X100 column
139 with 10mM phosphate pH 6 mobile phase.

140 Using protease digestion and an ultrasonic probe extraction, Sanz et al (7) found AsB (4.7
141 µg/kg), AsIII (2.3 µg/kg) and DMA (133 µg/kg) in freeze-dried chicken muscle tissue. These
142 species represented an 83% mass balance compared to the 169 µg/kg total. Of the matrices
143 examined (rice, fish, chicken muscle, and soil), only chicken required enzymatic digestion to
144 optimize extraction. Speciation was performed using the PRP-X100 anion exchange column
145 with 10 mM ammonium phosphate mobile phase pH 8.5 and 2% methanol and ICP-MS
146 detection. The chicken muscle analyzed in that study was from 70 day old cockerels given an
147 AsIII-enriched diet.

For the analytical portion of Study 275.30, we evaluated existing literature methods, combined or modified them as necessary to meet study needs, validated the resulting method and applied it to the analysis of study tissues. Total As levels were much lower in muscle than liver, and muscle was more problematic to extract, so initial efforts focused on speciation of As in liver. This report is limited to the analysis of liver tissues from the control and treated chickens.

Personnel

Mary Carson has over 20 years experience developing and validating methods for veterinary drug residue analysis. She is also trained in biochemistry, and is familiar with mass spectrometry and multiple modes of liquid chromatography. However, at the start of this study she had no experience with As speciation. She contacted a colleague at CFSAN, Stephen Capar an expert in trace metal analysis, for advice. He referred her to a member of his branch. **Sean Conklin** has several years experience in IC-ICP-MS, with emphasis on As speciation in fruit juice and seafoods. Throughout this project, he was primarily responsible for instrumentation maintenance, calibration and tuning, and took the lead on chromatographic optimization. Mary Carson took the lead in sample preparation and data processing, and as she learned ICP-MS, routine instrument operation. **Karyn Howard** assisted with sample preparation.

Procedures

Total As Determination

Total As concentration was determined in sliced portions of liver and muscle as described in Attachment 1, "ICP-MS analysis for total arsenic." This method is based on an FDA Elemental Analysis Manual standard analytical procedure for total metals determination. The nitric acid

digestion procedure results in a very stable solution which can be stored at room temperature for extended periods of time with no noticeable effect. Instrumental calibrants varied from one set to the next, depending on the expected As concentrations of the samples. Performance of the method was assured by analysis of a NIST standard reference material (SRM 1577c - Bovine Liver, 19.6 ± 1.4 ng As/g certified value, found values in acceptable data sets 15.9 to 22.8 ng/g), as well as by analysis of negative and positive (fortified) controls, with each set. The amount of nitric acid leftover after digestion can affect signal response, so nitric acid concentration in the calibrants needs to match that of the sample digests. Some sets of digests required reanalysis with calibrants prepared in a different concentration of nitric acid.

As Speciation in Liver

Speciation was conducted as described in Attachment 2, "Speciation of arsenic compounds related to roxarsone use in chickens." The method was developed for this project. Once developed and optimized, method performance was validated concomitantly with sample analysis by inclusion of control and fortified samples with each set. We had standards available for AsIII, AsV, DMA, MMA, Rox, and possible metabolites 3-amino-4-hydroxyphenylarsonic acid (3-Amino) and N-acetyl-4-hydroxyphenylarsonic acid (N-Acetyl). Mixed standards and mixed fortification solutions had equal amounts of As for all seven of these species.

Several extraction schemes and chromatographic separations were evaluated during the course of method development. Extraction with water and methanol, similar to that previously reported (3-5) was promising with fortified muscle samples, but could not distinguish a treated muscle sample (total As = 93 ppb) from a control muscle sample (total As = 3 ppb). Subsequent flow injection analysis of the treated muscle extract suggested that extraction efficiency was very low.

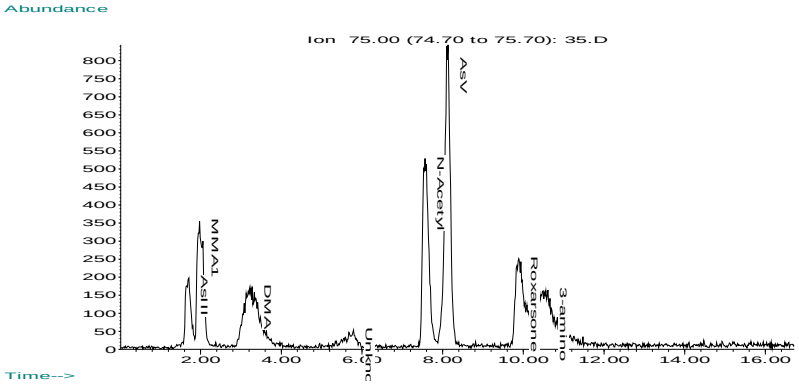
Extraction with an aqueous alkaline solution, TMAH, (as in Dr. Tyre Grant's dissertation) appeared more efficient. TMAH is a strong base and causes AsIII to be oxidized to AsV. It can also strongly affect ion chromatography (IC) results.

IC separation is based on ionic interactions between the analyte, the solid phase, and the mobile phase. These interactions are strongly affected by pH and ionic strength of the mobile phase; ideally samples are in a solvent that is identical to the mobile phase. Many As compounds have a pKa value below 7, making them negatively charged under neutral or basic pH conditions and therefore amenable to separation via anion exchange. A number of ion chromatography methods have been applied to arsenic speciation in a wide variety of samples, but as indicated above, a survey of papers describing arsenic speciation in chicken tissue extracts reveals only 2 different columns have been used for this purpose—the Hamilton PRP-X100 and the Dionex AS7. Other methods and columns have been applied to speciation of As in poultry litter and manure—Jackson and Bertsch (7) used AS14 and AS16 as well as AS7. We confirmed early on the observation by Grant (4) that roxarsone is not easily eluted from the PRP-X100 and therefore that column is not the best choice for this project. However, since the PRP-X100 is often used for determination of AsV (among other species), this column was useful for confirming the presence of AsV in liver extracts.

We evaluated various dilution, neutralization, and cleanup steps following extraction of 0.5 g samples with 3 mL 0.625% aqueous TMAH. This concentration of TMAH solubilized most of the liver tissue, with little solid material left. The resultant extract was quite crude. Trichloroacetic acid both neutralized TMAH and precipitated protein, enabling separation on an AS7 column with a nitric acid mobile phase, similar to Grant's method. This method looked

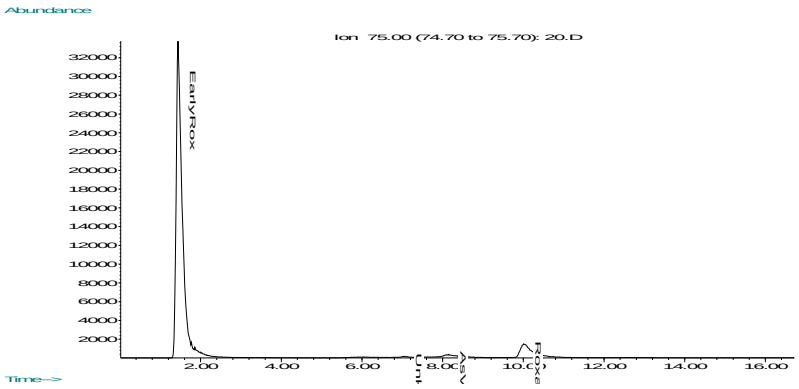
promising with fortified samples, though inter-conversion between AsIII and AsV was highly variable, and MMA tended to split between two peaks, MMA1 and Unknown, as shown in the chromatogram of a 10 ppb (mix of 7 compounds) fortified liver extract below:

Figure 1 Chromatography on AS7 with acid mobile phase, 10 ppb stds



Unfortunately, the first analysis with an incurred roxarsone liver sample resulted in a very large peak at the front, which was suspected to be unretained roxarsone (or a metabolite closely related to roxarsone) and not AsIII:

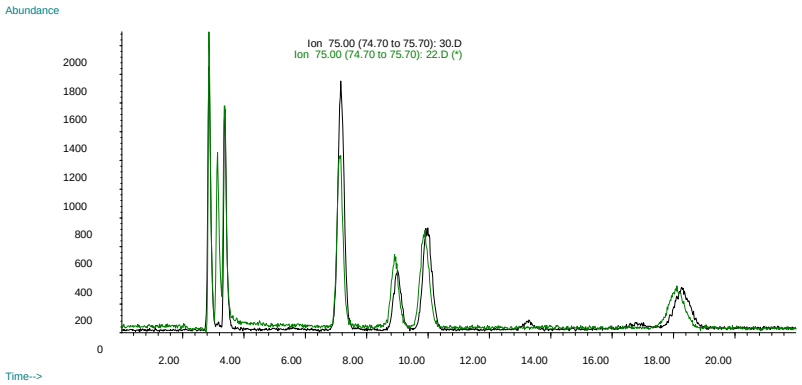
Figure 2 AS7 chromatogram of a treated liver extract.



Once the AS7 separation was found to be unsuitable, we evaluated a Dionex AS16 column, based on the work of Jackson and Bertsch (8) which showed good separation of several As species in under 10 min. On the AS16, good separation was achieved for most of the species in our standard mix, but two species (N-Acetyl and 3-Amino) co-eluted and the peak shape of AsV was poor.

The Dionex AS18 column was the next choice, and it showed a good ability to separate all 7 species in the standard mix within 20 min. Fortuitously, the separation was achieved using 45 mM tetramethylammonium hydroxide with 1% methanol mobile phase, so it was completely compatible with the TMAH tissue extracts without need for neutralization. In the figure below, the green trace is a 1 ng/g mixed standard in water (equivalent to 20 ppb in tissue), and the black trace is a 20 ppb fortified liver extract.

Figure 3 Standards and fortified liver extract on an AS18 column with 45 mM TMAH mobile phase. Retention times: DMA (2.85), AsIII (3.05), MMA (3.3), AsV (7.1), 3-Amino (8.9), N-Acetyl (9.9), Rox (18).



Injections of incurred tissues showed As-containing compounds that were strongly retained on the column and eluted in subsequent runs. A comparison of the two treated liver extracts below shows the difference between the first treated sample in a set and a subsequent (several runs later) treated sample with interfering late eluters.

Figure 4 AS18 chromatogram of a treated liver extract at the beginning of a set.

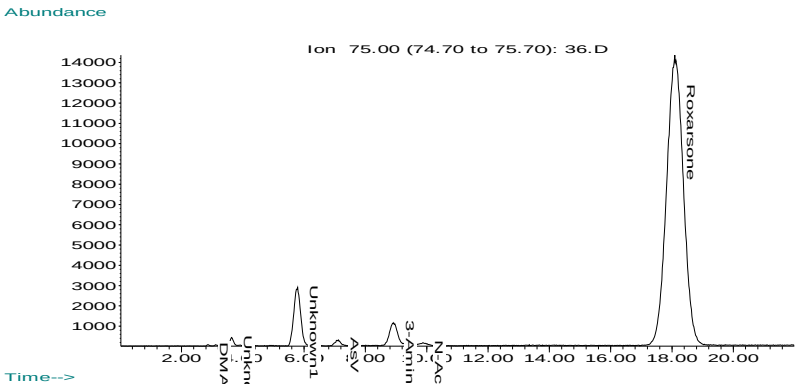
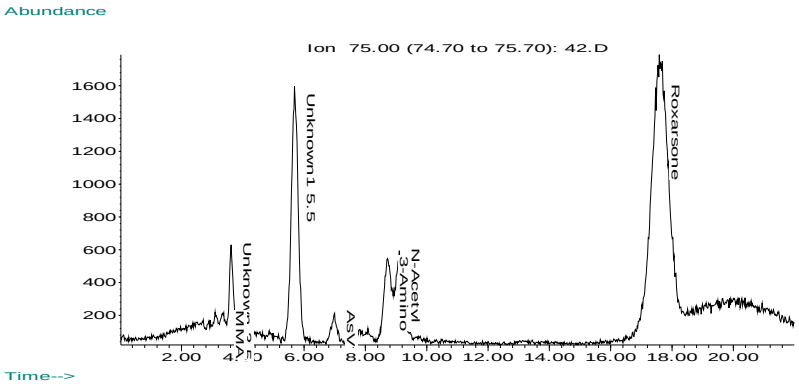


Figure 5 AS18 chromatogram of a treated liver extract later in the set, showing late-eluting peaks from a previous injection.



In order to get all peaks, including late eluters, out in the same run, the mobile phase was ramped from 45 mM TMAH to 70 mM for 25 min after roxarsone eluted. It was also necessary to

introduce an ultrafiltration cleanup of the extracts, as the unfiltered extracts quickly caused column back pressure to increase, which eventually resulted in column leakage and system shutdown.

The chromatograms below illustrate chromatographic results obtained with the final method. The known standards elute in the order DMA, AsIII, MMA (a tight grouping in the first 4 min), AsV, 3-Amino, N-Acetyl, and Roxarsone. The first chromatogram is a standard equivalent to 20 ppb, the second is a commercial control liver sample, the third is a 20 ppb fortified liver sample, and the bottom one is from a liver extract from treated bird #18. Several of the peaks in this chromatogram do not correspond to known As compounds, including the large peaks at 6 min and after 40 min. We did inject a number of species that were not included in the multi-species standard, including arsenobetaine (RT = 2.8 min), trimethylarsine oxide (RT = 2.8min), 4-arsanilic acid (RT = 3.6min), tetramethylarsonium ion (RT = 3.9 min), arsenocholine (RT = 3.92 min), and nitarsone (4-nitrobenzenearsonic acid, RT = 7.6 min). None of these compounds is a retention time match with any of the unknowns mentioned above, although arsenobetaine and tetramethylarsine oxide both partially co-elute with DMA. Most of these species are of little concern, because species eluting in less than 4 min from treated bird tissue extracts were present only at very low levels, mostly less than the lower limit of quantification (LLOQ).

Figure 6 Gradient AS18 chromatograph of a standard equivalent to 20 ppb in tissue.

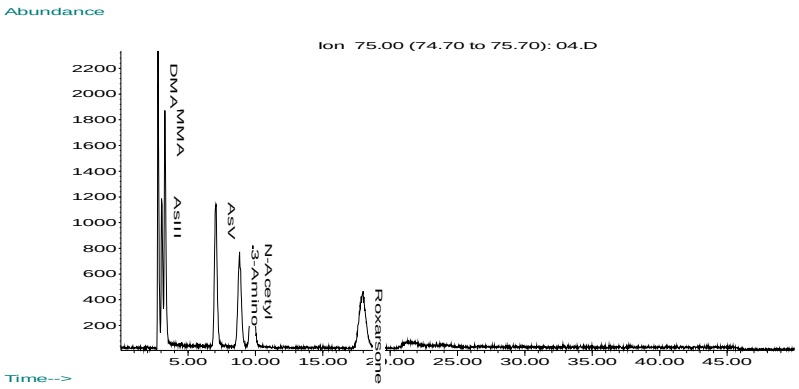


Figure 7 Chromatogram of a liver extract from (commercial) control livers. The AsV peak shown is below the LLOQ.

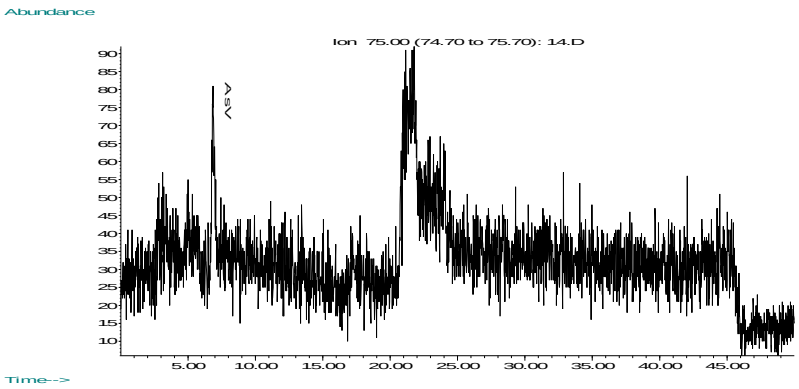


Figure 8 Chromatogram of an extract from 20 ppb fortified liver.

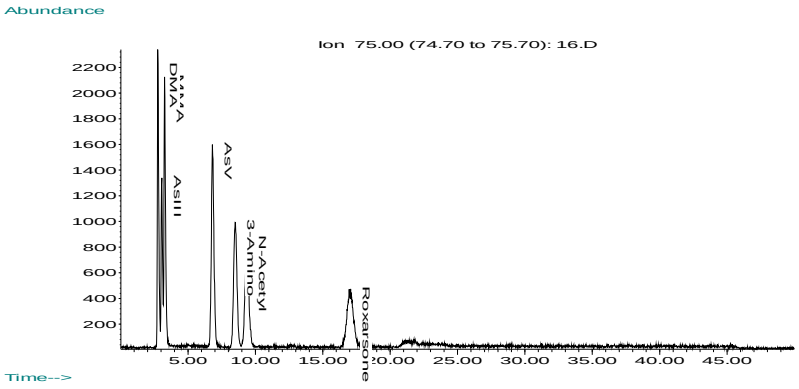
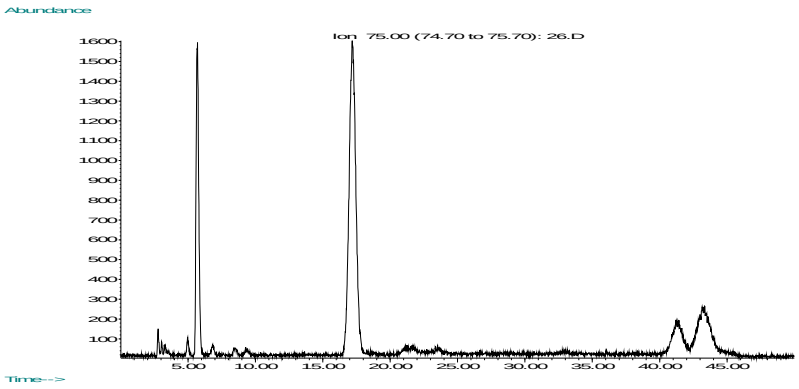


Figure 9 Chromatogram of an extract from a treated liver.



Analysis Dates

Total As analyses were conducted on dates listed in Table 1.

Attempts at speciation analysis began in Nov 2009. However, the speciation method was not finalized until Mar 2010. The reported results come from analyses conducted from 22 Mar 2010 through 23 Aug 2010.

Statistical Methods and Calculations

All results are reported as mass elemental As per mass wet tissue.

Total As calculations are described in Attachment 1. Values for each sample are generally from replicate analyses with mean and standard deviation reported, though most muscle results are from a single analysis.

Species concentrations of individual extracts were determined by comparison to an external standard curve for the known analytes, and by reference to known analytes for unknown concentration (using the slopes of the adjacent known analytes as a response factor for the

unknowns). The dilution factor [(9.5+ sample wt)/sample wt] was included in the sequence information, so the numbers reported by the chromatographic data system were in ng As/g tissue. Each extraction set included a Method Blank (water substituted for the tissue, so essentially just the TMAH and water), Method Blank + fortification standards, and quality control (QC) samples consisting of control liver (purchased at a local store and found to have very low levels of As) and fortified control liver samples. If an analyte value in the Method Blank significantly exceeded the LLOQ (or >20% above the lowest calibrant included), the set was repeated as the critical reagent criterion had not been met (see section J.5.a of the liver speciation method SOP). Otherwise, all positive Method Blank values were subtracted from the tissue values for that set. Only AsV ever had a positive Method Blank value, and only in some of the sets. Since As is a naturally occurring element and common environmental contaminant, method blank subtraction is routinely used (9). Reported results are the average of two speciation analyses for each tissue.

Summary of Results

Total As Determination

Table 2 summarizes the findings for total As determination using microwave assisted nitric acid digestion and ICP-MS analysis. The feed had to be ground in an IKA mill prior to sampling as the original analyses were too variable, which was likely due to heterogeneity of the feed. The entire laboratory portion of both the control and medicated feeds were ground to a fine powder before reanalysis. This laboratory sample had been subsampled from the Control and Test Materials in September 2009. The control feed had 156 ppb of As and the medicated feed had 11.3 ppm, which is equivalent to 39.6 ppm of roxarsone.

Day 3 muscle samples were not analyzed. All of the livers and all but 3 of the muscles from the untreated birds had total As concentrations below 5 ppb at each time point. Many of the muscle concentrations were below the lowest point of the calibration curves. Total As values for the livers from the treated birds were much higher and varied considerably from bird to bird. They ranged from a high of over 5 ppm for a 0 day withdrawal bird to a low of 275 ppb for one of the 5 day withdrawal birds. Values for total As in muscle from treated birds were much lower than liver values. There was approximately 40 times as much total As in liver as in muscle for both 0 and 5 day withdrawal treated birds. All muscle values at both 0 and 5 days were below the tolerance value of 0.5 ppm. Some birds at each time point exceeded the liver tolerance of 2 ppm.

Arsenic Speciation in Liver

Detection and Quantification Limits of the Speciation Method

CVM policy strongly discourages extrapolation beyond upper or lower limits of a calibration curve(10), therefore the lowest level below which AsV was not reliably quantifiable (the lower limit of quantification, LLOQ) was by default defined as the lowest level calibration standard used in the analysis. The lowest calibration mixed standard used was either 0.1 (earlier analyses) or 0.03 ng As/g solvent for each analyte (most analyses used in this report). These standard solutions were equivalent to 2 or 0.6 ppb in tissue.

The Limits of Detection (LOD) and LLOQ were statistically estimated by a variety of methods. All methods were based on estimating noise, multiplying it by factors for LOD and LLOQ, and converting the values obtained to equivalent tissue ppb. The initial estimate was derived while still using 0.1 ng/mL (2 ppb tissue equivalent) as the lowest calibrant. A 0.2 ng/mL mixed standard was prepared and analyzed ten (10) times. The standard deviation of the found

concentrations was multiplied by $2\times1.833\times\sqrt{1.1}$ for LOD and by 10 for LLOQ. The results are shown in Table 3a.

Shortly after this experiment, we discovered that soaking the autosampler vials in 2% Trace Metal Grade Nitric Acid significantly reduced the method blank values for AsV, enabling lower detection and quantification limits. We added a lower calibrant (0.03 ng/mL, equivalent to 0.6 ppb in tissue) to the method. With assistance from Dr. Idowu of the CVM Office of New Animal Drug Evaluation, LOD and LLOQ for AsV were estimated for both the older calibration curves (range 0.1 to 100 ng/mL) and the new curves (0.03 to 100 ng/mL) by determining the standard errors of the intercepts (SE Intercept) of the calibration curves used for analysis. A total of 21 curves for AsV were evaluated—10 with the 0.03 ng/mL calibrant and 11 early curves only going as low as 0.1 ng/mL. The evaluation also compared no weighting, 1/x, and $1/x^2$ weighting and verified that $1/x^2$ weighting consistently resulted in the best fit. The SE Intercepts for each $1/x^2$ weighted curve were multiplied by 3.28 (LOD) or 10 (LLOQ) and converted to tissue ppb. The average results are shown in Table 3a. With the 0.03 calibrant included, the estimated LLOQ for AsV was less than 0.5 ppb.

The data shown in Table 3b provide a point of reference to the accuracy of analyses near these low levels, with 5 replicate analyses of 1 ppb AsV only fortified liver giving an average reading of 1.02 ppb with standard deviation of 0.13 ppb (13% RSD). For AsV, the LOD and LLOQ based on 3× or 10× this standard deviation (0.4 or 1.3 ppb, respectively) are comparable to the LLOQ values of 0.6 or 2 ppb based on lowest point of the calibration curve. For the other analytes, 2 ppb was used as the LLOQ even if the lowest calibrant was 0.03 ng As/g solvent. Roxarsone, 3-Amino and N-Acetyl were not always detectable in the 0.03 ng/g standard, and

353 DMA and MMA showed a pronounced rise in analytical variance when fortification
354 concentrations decreased from 2 ppb to 1 ppb.

355 *Speciation Method Evaluation*

356 Due to time constraints, the speciation method performance evaluation was conducted using the
357 fortified QC samples prepared and analyzed with each set, along with some additional analyses
358 performed after completion of the study liver samples. The results are summarized in Tables 3a
359 through 3c. AsIII and AsV readily interconvert via oxidation and reduction, and the extraction
360 procedure oxidizes most AsIII to AsV (single analyte fortified sample analyses results, data not
361 shown). This is evident in Table 3b, where accuracy for AsIII is quite low, while accuracy of
362 AsV approaches 200% in all the mixed standard fortified samples. If one sums AsIII and AsV
363 nominal values in the mixed fortified QC samples, the accuracy for AsV determination ranges
364 from 78% to 102% across all concentrations tested, with relative standard deviations (RSDs) of
365 13% to 23%. These results meet CVM criteria (60-110% accuracy and RSD < 20%) for a
366 determinative procedure for residue concentrations less than 100 ppb.

367 The method is clearly incapable of distinguishing AsIII from AsV. The high RSDs (~30%) also
368 mean the method is not suitable for quantifying Rox or its two metabolites 3-Amino and N-
369 Acetyl. The accuracy of the Rox determination was also too low (~70%) for a residue whose
370 concentration is ≥ 100 ppb (CVM criteria 80-110% accuracy and <10% RSD).

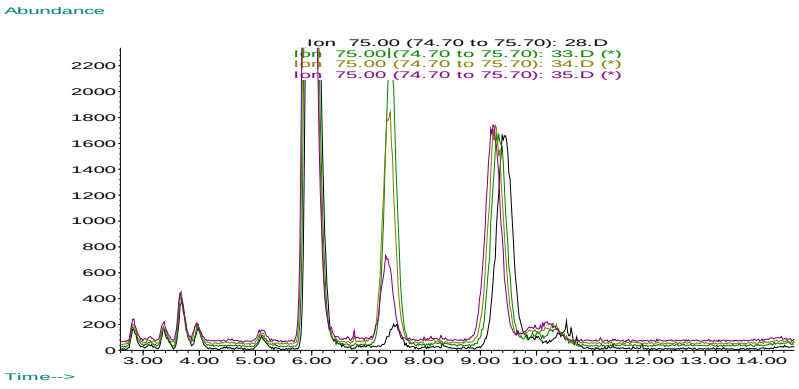
371 The performance characteristics for DMA and MMA were generally acceptable for
372 quantification between 2 and 20 ppb based on CVM criteria for a determinative procedure for
373 residue concentrations less than 100 ppb. The analytical variance for MMA is slightly high.
374 Because the determination of iAs was the most crucial measurement, and the method was

optimized for this measurement, the less than ideal performance characteristics for DMA, MMA, 3-Amino, N-acetyl, and Rox were deemed acceptable. The speciation method should only be used to estimate concentrations of 3-Amino, N-Acetyl, and Rox. MMA and DMA should only be determined above 2 ppb.

A liver from a treated bird was selected for replicate analysis to evaluate precision in a "real" sample. Five replicate portions of liver tissue from the same bird were extracted and analyzed. The results are in Table 3c and are consistent with precision results in the fortified samples.

AsV is clearly one of the smallest peaks in a liver extract chromatogram from a treated bird. To demonstrate that this peak is truly AsV, we conducted two additional experiments. First, we took portions of an extract from a putative AsV-containing liver, added amounts of AsV standard equivalent to roughly 4x, 2x, and 1x the estimated AsV concentration, and analyzed them. The resulting AsV peak was still a single, symmetrical peak, with area increased as expected, as shown in the chromatograms below:

Figure 10 AsV standard addition to a treated liver extract.



AsV is the peak at 7.4 min. The peak at 9.3 min is 3-Amino and shows there is a slight shift forward of retention times over the course of this experiment. The amount of AsV in the original extract was quantified as 4.4 ppb by external calibration and as 5.0 ppb by standard addition.

The second experiment was to chromatograph the extracts from a treated liver on an alternate system. In this case, we set up a second IC-ICP-MS instrument with a PRP-X100 column and isocratic mobile phase of pH 9 ammonium carbonate. AsIII elutes before DMA in this system, and AsV elutes at 30 min rather than 7.4 min as on the AS18 system. Some of the extracts from the replicate incurred analyses were divided and analyzed concomitantly on both systems to avoid any concerns of extract stability. The number of injections of Rox-containing extracts had to be limited on the PRP to avoid interference. The results are shown in Table 3d. The amount of AsV found, 4.4 ppb, agrees very well with the 4.2 ppb found on the AS18 analyses in Table 3c. Together these two experiments provide strong evidence that the peak we identified as AsV truly is AsV.

Speciation Analysis of Roxarsone-Treated and Control Livers

The speciation results for the study livers are tabulated in Tables 4a and 4b. Values that were below the LLOQ are indicated by greyed-out cells. The only analyte above the LLOQ in any untreated bird at any time point was Rox in Day 3 bird #67, which was due to residual contamination of the system with Rox following speciation analysis of the 3-Nitro 20% Premix (see below). The AsV concentrations are higher in the roxarsone-treated birds than in the control birds.

In addition to Rox, 3-Amino was readily detected in all treated samples and a probable peak for N-Acetyl was seen in most. However, a striking feature of the speciation analyses was the

presence of several unknown As-containing compounds. Until they can be identified, they are simply labeled by their approximate retention time. Some of these unknowns comprised >10% of the total detectable As in the speciation chromatogram.

Extraction Efficiency and Mass Balance of Arsenic

We compared the sum of all As species found chromatographically with the total As determined in each sample. The sums in general do not add up to the total. The average mass balance (sum/total) was 39%, but the values ranged from 8 to 98% We investigated this discrepancy by determining total As at each step of the extraction for two incurred liver samples (Day 0 #11 and Day 5 #27). Of the total As concentrations of 1760 and 2940 µg/kg, 93% and 108% were extracted by the TMAH, respectively, indicating nearly quantitative extraction of As compounds from the tissue. The ultrafiltrates contained 67% and 69% of the total tissue As. This loss through ultrafiltration was consistent with the loss seen for roxarsone recovery in fortified experiments (Table 3a). However, the species for these two samples, including estimated concentrations of the unknown As-containing metabolites, added to only 53% and 32%, suggesting additional loss of As analytes during chromatography. To verify this possibility, we removed resin from a used column and guard cartridge, determined the total As concentration by microwave-assisted nitric acid digestion, and compared those values with one obtained from new guard cartridge resin. Used resin had approximately 2 mg/kg As at the inlet end of the column, and 0.5 mg/kg at the outlet end of the same column, while new guard cartridge resin had 0.02 mg/kg As. Clearly, some As compound(s) bound very tightly to the AS18 column under the conditions we used.

The low and variable mass balances suggest that we do not have a full accounting of all the As species that may exist in livers from roxarsone-treated birds, and that the numbers we are reporting should be treated as minimum numbers. Actual concentrations may be higher.

Speciation in Control Feed, Medicated Feed, and Type A Medicated Articles

When inorganic arsenic in livers from chickens treated with roxarsone medicated feed was found, we wanted to be sure it was not because the medicated feed itself contained inorganic arsenic. Control feed, the medicated feed, and the premix used to make the medicated feed were tested for the two inorganic arsenic species and several organic arsenic species.

An *ad hoc* method based on the instrumental procedure used for the chicken tissue samples was used. Analysis consisted of mixing a small portion of each feed and premix with water or TMAH to dissolve the drug and any water soluble contaminants, and then analyze the extract for arsenic species by IC-ICP-MS using the same chromatographic conditions employed for the liver speciation. The water extraction has the potential to distinguish AsIII from AsV, while the TMAH extraction converts most AsIII to AsV. The *ad hoc* method is not the same as the official feed analysis procedure. The official feed method will not distinguish inorganic arsenic from roxarsone.

The results are shown in Table 5a. In the first analysis of the original 20% roxarsone premix, a random sample was taken from the top of the 50lb premix bag and analyzed. The premix contained 1 part per million AsIII, 12 parts per million AsV, and 38,000 parts per million roxarsone (equivalent to 14% roxarsone) using the water extraction. Based on the total arsenic in the premix, 0.03% was inorganic arsenic and 99.97% was roxarsone or other organic arsenic. The control feed had 0.024 parts per million AsIII and 0.032 parts per million AsV. The premix

455 is diluted 4000-fold with control feed to make medicated feed. The amount of inorganic As
456 contributed by the Rox premix to the medicated feed is in the vicinity of 3-4 ppb, or ≤ 10%
457 above control feed levels. We therefore find it unlikely that the AsV found in the treated bird
458 livers is a result of direct inorganic As contamination of the medicated feed.

459 It was decided to further investigate the inorganic arsenic concentrations in organic arsenical
460 premixes. A penicillin premix (a presumed negative control), a premix containing 50%
461 nitarsone, and a second lot of a 20% roxarsone premix were purchased. Greater care was taken
462 to ensure that representative samples of the premixes were obtained using a "core sampling
463 probe" that is approximately one (1) inch in diameter and about three (3) feet long. The probe
464 was pushed into the bags of medicated premix until it reached the bottom of the bag and was
465 rotated several times to enable the test article to fill the probe. The probe's outer tube was
466 rotated to close off the probe and the collected materials were poured into a sample collection
467 bag. This process was repeated about 12-15 times by sampling in a clockwise rotation within the
468 bag, thereby collecting material from all parts of the bags of each of the test articles. During the
469 sampling process, it was noted that despite the fact that these four (4) preparations were
470 "supposed" to have about the similar proportions of inert ingredients, there was a marked
471 difference in the texture of the various products.

472 The results are shown in Table 5b. The amount of inorganic arsenic in the penicillin premix was
473 very low, in the parts per billion range. The results for the original roxarsone premix were
474 consistent with the initial analysis. However, the second lot of roxarsone premix had a much
475 higher percentage of inorganic arsenic--0.4%. The percent of inorganic arsenic in the nitarsone
476 premix was also high, but within the manufacturing specifications for this compound.

Conclusions

The CVM study wherein chickens were raised on feed medicated with roxarsone according to label directions along with chickens raised on unmedicated feed was specifically designed to determine which arsenic species are present in chicken tissue as a direct result of roxarsone treatment. The liver tissues collected from these birds were found to contain higher levels of total arsenic than had been reported in store-bought tissues in previous work (4), and more arsenic species were found in our samples than in previous work. This was at least partially due because we included more species in our multi-species standard than in previous work, *i.e.,* we found more peaks because we were looking for them. While some inorganic arsenic had been reported in chicken tissues (4) (7), the source of the arsenic contamination cannot be ascertained since the tissues were purchased from stores and the birds' treatment prior to the point of purchase was unknown to the researchers. By comparing treated and untreated birds raised under carefully controlled conditions, it should be possible to determine whether roxarsone treatment represents a food safety risk based on increased inorganic arsenic concentration in the treated birds.

Signatures

Mary C. Carson 1/24/2011

Analyst Mary C. Carson (CVM) Date

Sean Conklin 1/24/2011

Analyst Sean D. Conklin (CFSAN) Date

Philip J. Kijak 01/24/11

Philip J. Kijak, Director, Division of Residue Chemistry (CVM) Date

Stephen G. Capar 1/24/2011

Stephen G. Capar, Chief, Chemical Contaminants Branch (CFSAN) Date

505 **III. Tables**

506 Table 1. Dates for digestion and total As determination in study samples

Digestion Date	Analysis Date	Sample Set	Comments
14 Oct 2009	15 Oct 2009	Feed and standards	Sequence SDC1015b
12 Nov 2009	12 Nov 2009	Day 0 Breast	Preweighed breast, instrumental problems
12 Nov 2009	13 Nov 2009	Day 0 Breast	Preweighed breast, reanalysis
12 Nov 2009	13 Nov 2009	Day 0 Liver Ground Feed	Preweighed liver
30 Nov 2009	2 Dec 2009	Day 5 Liver	Preweighed liver
2 Dec 2009	3 Dec 2009	Day 5 Breast	Preweighed breast
12 Nov 2009	4 Dec 2009	Day 0 Breast	Reanalysis with optimized tune file
12 Nov 2009	4 Dec 2009	Day 0 Liver Ground Feed	Reanalysis with optimized tune file
2 Dec 2009	4 Dec 2009	Day 5 Breast	Reanalysis with optimized tune file
9 Apr 2010	9 Apr 2010	Selected Day 0 and Day 5 liver repeats	Freshly weighed samples
12 Apr 2010	12 Apr 2010	Selected Day 5 liver re-repeats	Freshly weighed samples
1 Jun 2010 and 2 Jun 2010	2 Jun 2010	Day 3 Liver	Freshly weighed, duplicate or triplicate
4 Oct through 12 Oct 2010	4 Oct through 1 Nov 2010	Day 0 and Day 5 Liver	Freshly weighed, triplicates, reanalysis with calibrants in different % nitric

507 Table 2. Total Arsenic Concentrations in Feed, Liver, and Muscle Samples

Bird #	Treatment	Withdrawal time (days)	Total As (ng/g wet wt)	n	Total As (ng/g wet wt)	n
Feed	Control	N/A	156	3		
Feed	Roxarsone	N/A	11300	3		
			LIVER		**MUSCLE**	
66	Control	0	1.3±0.2	3	5.0	1
69	Control	0	1.8±0.2	3	3.9	1
75	Control	0	3.1±0.5	4	3.6	1
77	Control	0	4.6±1.3	3	3.5	1
82	Control	0	3.2±.0.6	3	2.7±0.1*	3
84	Control	0	4.4±0.5	4	4.7	1
97	Control	0	3.5±0.2	4	10.7	1
99	Control	0	4.9±0.8	4	5.4	1
11	Roxarsone	0	1760±60	4	72.9±17.1	3
12	Roxarsone	0	2920±260	4	55.4	1
21	Roxarsone	0	5430±220	5	68.9	1
26	Roxarsone	0	4200±580	4	72.7	1
28	Roxarsone	0	4140±240	4	126.	1
36	Roxarsone	0	1950±330	4	64.4	1
40	Roxarsone	0	899±106	4	54.2	1
46	Roxarsone	0	4300±120	4	78.7	1
48	Roxarsone	0	2920±410	4	80.7	1

Bird #	Treatment	Withdrawal time (days)	Total As (ng/g wet wt)	n	Total As (ng/g wet wt)	n
52	Roxarsone	0	2460±360	4	81.6	1
53	Roxarsone	0	3560±650	4	91.8	1
54	Roxarsone	0	4410±150	4	71.2	1
56	Roxarsone	0	652±35	5	54.1	1
58	Roxarsone	0	2180±420	5	61.1	1
59	Roxarsone	0	1360±170	4	57.7	1
60	Roxarsone	0	2240±230	4	38.6	1
67	Control	3	3.7±0.5	6		
68	Control	3	4.7±1.1	3		
74	Control	3	4.3±0.3	3		
78	Control	3	2.3±0.2	3		
79	Control	3	3.5±0.1	3		
83	Control	3	3.4±0.2	3		
91	Control	3	4.5±0.2	3		
100	Control	3	2.9±0.1	3		
17	Roxarsone	2	3170±110‡	3		
34	Roxarsone	2	2250±210	3		
1	Roxarsone	3	4590±410	3		
2	Roxarsone	3	1600±760	3		

Bird #	Treatment	Withdrawal time (days)	Total As (ng/g wet wt)	n	Total As (ng/g wet wt)	n
5	Roxarsone	3	988±42	2		
9	Roxarsone	3	2510±290	2		
13	Roxarsone	3	3090±850	3		
18	Roxarsone	3	843±24	3		
23	Roxarsone	3	3270±150	2		
30	Roxarsone	3	2520±940	3		
31	Roxarsone	3	941±135	3		
33	Roxarsone	3	1720±350	3		
38	Roxarsone	3	1070±170	2		
47	Roxarsone	3	1110±580	3		
50	Roxarsone	3	469±38	3		
61	Roxarsone	3	2010±1120	3		
62	Roxarsone	3	2640±15	2		
65	Roxarsone	3	1930±290	2		
70	Control	5	4.3±0.6	4	3.2	1
71	Control	5	4.6±1.2	4	3.4	1
72	Control	5	2.8±0.3	3	2.5	1
73	Control	5	2.8±0.4	4	2.5	1
81	Control	5	3.6±0.7	4	3.1	1
86	Control	5	3.2±2.3	3	3.2	1
87	Control	5	3.0±0.3	4	3.3	1

Bird #	Treatment	Withdrawal time (days)	Total As (ng/g wet wt)	n	Total As (ng/g wet wt)	n
89	Control	5	2.5±1.2	4	1.8	1
92	Control	5	3.0±0.8	4	4.9	1
94	Control	5	2.7±1.0	4	2.9	1
98	Control	5	3.3±0.5	4	3.6	1
3	Roxarsone	5	1060±310	4	30.6	1
4	Roxarsone	5	1150±170	4	31.5	1
6	Roxarsone	5	655±190	4	24.7	1
7	Roxarsone	5	1910±540	4	44.7	1
8	Roxarsone	5	1590±230	4	23.1	1
14	Roxarsone	5	691±416	6	19.1	1
15	Roxarsone	5	427±44	5	23.2	1
16	Roxarsone	5	480±45	6	17.4	1
19	Roxarsone	5	301±12	4	13.9	1
24	Roxarsone	5	1640±180	6	39.9	1
27	Roxarsone	5	2940±360	6	46.6	1
29	Roxarsone	5	581±61	8	21.5	1
32	Roxarsone	5	1220±210	7	19.8	1
37	Roxarsone	5	2170±220	5	48.4	1
39	Roxarsone	5	275±22	6	21.4	1
44	Roxarsone	5	633±136	4	14.0	1
49	Roxarsone	5	388±53	4	39.0	1

Bird #	Treatment	Withdrawal time (days)	Total As (ng/g wet wt)	n	Total As (ng/g wet wt)	n
51	Roxarsone	5	907±160	4	25.0	1
55	Roxarsone	5	499±23	6	23.9	1
63	Roxarsone	5	1030±160	4	21.6	1
64	Roxarsone	5	1745±52	4	30.5	1

508 *Two replicates below that set's lowest calibrant—this value was censored in the statistical
509 analysis.

510 ‡One replicate above that set's highest calibrant

511 Table 3a. Estimates of LOD and LLOQ by various methods. Values are in tissue ppb.*

Analyte	DMA	AsIII	MMA	AsV	3-Amino	N-Acetyl	Rox
Method	10 analyses of 0.2 ng/mL standard; lowest calibrant 0.1 ng/mL						
LOD	0.8	1.6	0.6	1.1	1.5	0.4	1.4
LLOQ	2.0	4.0	1.6	3.0	3.9	1.0	3.5
Method	SE Intercept of 11 calibration curves, range 0.1 to 100 ng/mL						
LOD				0.7			
LLOQ				2.1			
Method	SE Intercept of 10 calibration curves, range 0.03 to 100 ng/mL						
LOD				0.15			
LLOQ				0.45			

512 *The dilution factor from equivalent tissue ppb (µg As/kg tissue) to standard ng/mL is 20.

513 Table 3b. Validation Results for Arsenic Speciation Method—Fortified Liver Accuracy and
514 Precision

Fortification Level		**DMA**	**AsIII***	**MMA**	**AsV***	**3-Amino**	**N-Acetyl**	**Rox**
2000 ppb Rox	Average Found ppb	0.3			0.9	2.7		1490
n = 16	Accuracy							74%
	RSD	97%			103%	173%		28%
20 ppb mix	Average Found ppb	21.3	2.1	16.4	38.7	16.6	14.9	14.5
n = 12	Accuracy	106%	10%	82%	194%	83%	75%	72%
	RSD	10%	275%	23%	15%	33%	33%	33%
4 ppb mix	Average Found ppb	4.6	0.0	3.77	8.0	4.1	3.8	3.4
n = 7	Accuracy	116%	0%	94%	201%	103%	95%	86%
	RSD	9%		17%	15%	32%	30%	28%
2 ppb mix	Average Found ppb	2.3	0.04	1.7	3.6	1.7	1.6	1.4
n = 9	Accuracy	117%	2%	86%	180%	86%	82%	70%
	RSD	7%	300%	21%	18%	32%	29%	33%
1 ppb mix	Average Found ppb	1.2	0.00	0.8	1.6	1.1	0.8	0.6
n = 10	Accuracy	119%	0%	85%	157%	113%	78%	62%
	RSD	23%		32%	23%	25%	42%	86%

Fortification Level		**DMA**	**AsIII***	**MMA**	**AsV***	**3-Amino**	**N-Acetyl**	**Rox**
1 ppb AsV	Average Found ppb	0.3			1.0			
n = 5	Accuracy				102%			
	RSD	16%			13%			

*The extraction procedure oxidizes most AsIII to AsV.

516 Table 3c. Validation Results for Arsenic Speciation Method—Precision with an Incurred Roxarsone Liver Sample. Cells that are
517 grey indicate a censorable value below the LLOQ. Concentration units are ppb or µg As/kg wet weight liver.

Set	Sample	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.6	AsV	3-Amino	N-Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
10H23 #10	Treated #11	1.2	1.1	1.8	5.0	1.7	62	4.5	65	9.7	2.1	543	8.0	8.4	121
10H23 #11	Treated #11	1.3	1.0	1.4	5.4	1.2	63	3.2	44	7.9	0.0	377	4.9	7.0	81
10H23 #12	Treated #11	1.3	1.3	1.8	6.0	1.9	60	4.7	71	10.1	2.2	605	7.6	10.9	122
10H23 #13	Treated #11	1.1	1.2	1.4	3.7	1.8	42	4.6	41	6.9	2.0	328	6.1	8.4	96
10H23 #14	Treated #11	1.2	0.9	1.6	4.7	1.8	51	4.2	49	7.4	2.4	375	5.4	9.3	98
	Average	1.2	1.0	1.6	4.9	1.7	56	4.2	54	8.4	1.7	446	6.4	8.8	104
	Std Dev	0.1	0.1	0.2	0.9	0.3	8.8	0.6	13	1.4	1.0	121	1.3	1.4	17.8
	RSD	8%	12%	13%	18%	17%	16%	14%	24%	17%	57%	27%	21%	16%	17%

518 Table 3d. Validation Results for Arsenic Speciation Method—Verification of AsV in Incurred
519 Sample by Alternate Chromatography. Concentration units are ppb or µg As/kg wet weight
520 liver.

521 Column: PRP-X100 anion exchange column

522 Mobile phase: 20 mM Ammonium Carbonate pH 9 (adjusted with ammonium hydroxide)

523 Conditions: 1 mL/min flow rate, 100 µL injections, 40 min run time

524 Instrument: Agilent 7500 ICP-MS, operated in helium collision mode

525 Analysis within 24 hrs of AS18 column analysis.

526

	AsIII	**DMA**	**MMA**	**AsV**
19Aug #12 Bird #11 liver extract	0	0.95	1.90	3.22
19Aug #13 Bird #11 liver extract	0	1.17	2.14	5.25
23Aug #10 Bird #11 liver extract	0	0.93	2.04	3.72
23Aug #10 Bird #11 liver extract	0	0.93	2.04	4.27
23Aug #11 Bird #11 liver extract	0	0.83	2.28	3.93
23Aug #11 Bird #11 liver extract	0	1.04	2.07	4.76
23Aug #12 Bird #11 liver extract	0	0.96	2.11	4.81
23Aug #14 Bird #11 liver extract	0	0.60	2.19	5.17
Average	0	0.92	2.10	4.39
RSD		18%	5%	17%

527 Table 4a. As Species in Liver Samples from Birds Treated with Roxarsone Medicated Feed.

528 Concentrations are in ppb (µg As per kg wet weight liver). Results are the average of duplicate analyses.

529 See the text for a description of LLOQ estimation. Cells that are grey indicate a censorable value below the LLOQ.

530 The extraction solvent (tetramethylammonium hydroxide in water) oxidizes most AsIII to AsV, so AsIII is rarely found.

531 Compounds are listed in chromatographic elution order.

532 Unknown As compound concentrations were estimated by using the slope of the adjoining known compounds as a response factor.

With-drawal Day	Bird	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.5	AsV	3-Amino	N-Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
0	#11	0.4	0.8	1.7	7	2	54	8.2	97.7	12.8	1	588	19	n.d.†	137
0	#12	1.0	0.3	2.0	10	1	71	21.5	245	8.4	3	1640	37	n.d.	216
0	#21	0.0	0.5	0.2	3	1	12	18.1	73.8	7.1	2	333	24	n.d.	90
0	#26	1.2	0.4	2.3	12	2	93	19.3	215	15.4	2	1920	29	n.d.	234
0	#28	0.8	0.4	1.7	10	2	155	8.4	66.0	15.9	2	2810	13	n.d.	107
0	#36	0.8	0.4	1.6	5	2	84	3.8	39.6	9.5	1	1430	7	n.d.	66
0	#40	0.9	0.1	0.8	2	1	70	0.1	3.7	0.5	0	284	0	n.d.	58
0	#46	1.9	0.0	1.3	5	1	123	5.2	48.4	12.5	2	3560	8	n.d.	222
0	#48	1.6	0.0	1.3	4	1	76	4.2	33.3	5.4	0	2040	6	n.d.	164
0	#52	1.8	1.2	1.5	3	2	66	5.4	31.9	8.0	1	865	9	n.d.	204

With-drawal Day	Bird	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.5	AsV	3-Amino	N-Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
0	#53	1.7	0.0	1.6	12	2	175	11.4	118	15.5	3	2560	13	n.d.	240
0	#54	2.1	0.3	1.0	3	1	70	4.0	23.4	4.8	1	1290	2	n.d.	125
0	#56	0.4	0.4	0.5	2	1	15	0.3	8.1	1.4	0	43.1	0	n.d.	19
0	#58	1.7	0.5	1.1	3	2	44	2.2	19.8	3.1	0	367	4	n.d.	57
0	#59	1.3	0.0	1.0	2	1	51	2.3	16.3	3.0	0	322	2	n.d.	41
0	#60	1.3	1.6	1.0	6	3	32	6.8	52.9	9.2	0	306	13	n.d.	88
2	#17	1.2	0.5	0.9	4	1	125	2.5	18.4	10.5	3	1520	5	0	210
2	#34	1.6	0.5	0.9	1	1	51	1.5	8.4	3.9	0	838	0	0	83
3	#1	1.3	0.0	0.1	11	2	105	3.2	103	34.6	1	2150	13	6.5	246
3	#2	0.6	0.0	0.0	2	1	31	0.0	15.9	4.5	0	270	3	0	67
3	#5	1.0	0.0	0.0	1	1	77	0.0	1.3	0.5	0	112	1	0	45
3	#9	1.2	0.0	0.0	2	1	70	0.1	7.2	2.2	0	585	2	0	77
3	#13	1.0	0.0	0.2	5	1	79	2.2	49.3	5.2	0	1490	5	6.5	215
3	#18	1.5	0.5	0.4	1	1	50	0.3	1.6	0.6	0	99.2	0	0	35

With-drawal Day	Bird	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.5	AsV	3-Amino	N-Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
3	#23	1.5	0.3	0.8	5	2	145	3.2	34.8	8.3	2	1670	6	7.7	197
3	#30	0.8	0.0	0.3	5	1	72	1.2	34.8	7.8	2	771	4	7.2	92
3	#31	1.2	0.2	0.1	1	1	34	0.1	1.9	0.5	0	90.9	0	0	20
3	#33	1.0	0.2	0.2	0	1	47	0.3	1.7	0.5	0	299	0.8	0	42
3	#38	1.1	0.3	0.3	1	1	54	0.3	2.3	0.8	0	172	0	0	36
3	#47	1.2	0.5	0.4	1	1	49	0.3	2.0	0.6	0	139	0	0	33
3	#50	1.0	0.4	0.3	1	1	53	0.3	1.4	0.3	0	96.1	0	0	29
3	#61	1.1	0.5	0.8	1	1	53	0.5	1.1	0.2	0	66.9	0	0	35
3	#62	0.7	0.1	1.2	10	0	141	4.5	97.8	11.8	4	1350	10	9.0	217
3	#65	1.2	0.5	0.4	1	1	71	0.5	4.6	1.8	0	310	0	0	60
5	#3	1.1	0.6	0.2	1	1	28	0	1.1	0.9	0	72.0	0	1	25
5	#4	0.9	0.4	0.7	3	1	54	1.7	41.2	8.4	1	630	2	16	105
5	#6	0.0	0.0	0.6	9	1	36	0.5	4.9	12.3	1	32.2	2	17	29
5	#7	0.2	0.3	1.5	21	1	63	5.2	117	31.7	5	443	17	55	187
5	#8	0.7	0.5	0.3	2	1	25	0	7.2	2.4	0	345	0	19	41

With-drawal Day	Bird	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.5	AsV	3-Amino	N-Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
5	#14	0.9	0.1	0.2	0	1	30	0	0.6	0.2	0	28.6	0	0	19
5	#15	1.2	0.2	0.3	1	1	37	0.1	0.6	0.7	0	23.0	1	0	20
5	#16	0.8	0.3	0.6	10	1	57	0	3.6	4.0	0	38.8	0	9	20
5	#19	0.8	0.1	0.5	0	1	20	0.3	0.6	0.6	0	20.8	1	1	20
5	#24	1.2	0.6	0.4	1	2	17	0.8	10.4	2.6	0	316	1	5	75
5	#27	0.0	0.0	0.2	9	1	50	9.1	93.0	15.8	3	592	14	13	134
5	#29	1.0	0.2	0.7	3	1	102	1.3	7.3	4.2	1	175	2	8	74
5	#32	1.2	0.2	0.4	1	1	39	0.3	2.4	1.5	0	287	1	0	47
5	#37 (extra)	1.5	0.3	0.5	1	1	52	0.9	13.3	6.6	0	1010	2	5	103
5	#39 (extra)	0.8	0.1	0.3	0	1	32	0	0.0	0.0	0	10.3	0	4	20
5	#44	1.0	0.5	0.5	0	1	27	0.3	2.2	0.7	0	100	0	0	22
5	#49 (extra)	3.6	1.1	0.7	1	2	49	0	1.1	0.5	0	41.9	1	0	20
5	#51	0.7	0.4	0.5	1	1	31	0.9	8.5	1.8	1	162	1	5	46
5	#55 (extra)	1.4	0.6	0.5	1	1	56	0	1.1	0.6	0	28.8	1	4	27

With-drawal Day	Bird	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.5	AsV	3-Amino	N-Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
5	#63	0.8	0.5	0.5	1	1	27	0.6	9.6	2.0	0	196	1	1	46
5	#64	1.1	0.8	0.6	1	1	35	1.0	14.1	2.4	0	313	1	3	60

533 †Not done. This unknown compound was not added to the list of unknowns in the processing method until later.

534 Table 4b. As Species in Liver Samples from Untreated Birds. Conditions the same as in Table 4a.

With drawal Day	Bird	DM A	AsIII	MM A	Unk 3.5	Unk 4.5	Unk 5.5	AsV *	3- Amino	N- Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
0	#66	0	0	0.0	0	0	0	0	0	0	0	0	0	n.d.	0
0	#69	0.1	0	0.2	0	0	0	0.0	0	0	0	0	0	n.d.	0
0	#75	0.4	0	0.4	0	1	0	1.1	0	0	0	0	0	n.d.	0
0	#77	0.3	0	0.4	0	0	0	0	0	0	0	0	0	n.d.	0
0	#82	0.2	0	0.3	0	0	0	0	0	0	0	0	0	n.d.	0
0	#84	0.5	0	0.6	0	1	0	0.5	0	0	0	0	0	n.d.	0
0	#97	0.3	0	0.5	1	1	0	0	0	0	0	0	0	n.d.	0
0	#99	0.6	0	0.6	0	1	0	0.2	0	0	0	0	0	n.d.	0
3	#67	0.9	0	0	0	0	0	0	0	0	0	2.6	0	0	0
3	#68	0.8	0	0	0	0	0	0	0	0	0	1.0	0	0	0
3	#74	0.8	0	0	0	1	0	0	0	0	0	1.8	0	0	0
3	#78	0.8	0.1	0.6	0	0	0	0.1	0	0	0	0	0	0	0
3	#79	0.6	0	0.5	0	0	0	0.1	0	0	0	0	0	0	0

With drawal Day	Bird	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.5	AsV *	3-Amino	N-Acetyl	Unk 13	Rox	Unk 21	Unk 32	Unk 36
3	#83	0.8	0.0	0.5	0	1	0	0	0	0	0	0	0	0	0
3	#91	0.9	0.3	1.0	0	1	0	0	0	0	0	0	0	0	0
3	#100	0.7	0.1	0.6	0	0	0	0	0	0	0	0	0	0	0
5	#70	0.6	0.1	0.5	0	1	0	0	0	0	0	0	0	0	0
5	#71	0.4	0.3	0.6	0	1	0	0	0	0	0	0	0	0	0
5	#72	0.3	0.1	0.1	0	0	0	0	0	0	0	0	0	0	0
5	#73	0.4	0.0	0.4	0	0	0	0	0	0	0	0	0	0	0
5	#81	0.9	0.1	0.7	0	1	0	0	0	0	0	0	0	0	0
5	#86	0.7	0.0	0.4	0	1	0	0	0	0	0	0	0	0	0
5	#87	0.4	0.0	0.1	0	0	0	0	0	0	0	0	0	0	0
5	#89	0.5	0.0	1.3	0	1	0	0	0	0	0	0	0	0	0
5	#92	1.0	0.5	0.7	0	0	0	0	0	0	0	1.3	0	0	0
5	#94	0.6	0.3	0.5	0	0	0	0	0	0	0	0	0	0	0
5	#98	0.8	0.3	0.8	0	0	0	0	0	0	0	0	0	0	0

535

536 Table 5a. Initial Speciation Analysis Of Control Feed, Medicated Feed, And Premix. **Concentration is in ppm (mg As per kg).**

Sample	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.6	AsV	3-Amino	N-Acetyl	Unk 13	Rox*	Unk 21	Unk 32	Unk 36
Control feed (water extract)	<LLOQ	0.024	<LOD	<LOD	<LOD	<LOD	0.0316	<LOD	<LOD	<LOD	0.00364	<LOD	<LOD	<LOD
Control feed (TMAH extract)	<LLOQ	<LOD	<LLOQ	<LOD	<LOD	<LOD	0.0381	<LOD	<LOD	<LOD	0.0154	<LOD	<LOD	<LOD
Medicated feed (water extract)	<LLOQ	0.0228	<LOD	<LOD	<LOD	<LOD	0.0362	<LLOQ	<LOD	<LLOQ	11.3	0.003	<LOD	0.15
Medicated feed (TMAH extract)	<LLOQ	<LLOQ	<LLOQ	<LOD	<LLOQ	<LOD	0.0470	0.0098	<LLOQ	<LLOQ	8.00	0.003	0.046	0.051
Premix (diluted) (water extract)	<LOD	1.02	<LOD	<LOD	0.44	<LLOQ	12.2	2.03	1.64	0.110	38100	22	810	53
Premix (diluted) (TMAH extract)	<LOD	0.731	<LOD	<LLOQ	0.45	<LLOQ	12.4	2.07	1.63	0.182	38200	12	460	58
Premix (direct) (water extract)	<LOD	1.00	<LOD	0.094	0.11	0.18	11.2	1.72	1.45	<LOD	Off scale	11	670	40
Premix (direct) (TMAH extract)	<LOD	0.770	<LOD	0.074	0.13	0.36	12.5	1.82	1.22	<LOD	Off scale	15	420	52

537 *The conversion from Rox concentration as ppm As to percent roxarsone is: $ppm/10^6 \times 263/75 \times 100$

538 Table 5b. Analysis of Type A Medicated Articles for iAs using Water Extraction

Product	**API**	**Number of assays**	**API Conc.**		**AsIII Conc.**		**AsV Conc.**	
			NADA spec.	**Found**	**Found**	**NADA spec.****	**Found**	**NADA spec.****
3-Nitro 20 (Bag 1/test 1)	Rox	3	18 – 22 %	13.4%	1 ppm	≤50 ppm	12 ppm	≤100 ppm
3-Nitro 20 (Bag 1/test 2)	Rox	5	18 – 22 %	21.1%	1 ppm	≤50 ppm	22 ppm	≤100 ppm
3-Nitro 20 (Bag 2)	Rox	5	18 – 22 %	21.3%	31 ppm	≤50 ppm	865 ppm	≤100 ppm
Histostat 50	Nitarsone	5	45 – 50 %	47.5%	686 ppm	≤5000 ppm	207 ppm	≤5000 ppm
Penicillin 100*	Penicillin G	5	NA	NA	-	NA	30 ppb	NA

539 *contains similar inactive carriers/extenders as arsenical products

540 **Roxarsone API impurity limit is 0.025% for AsIII and 0.05% for AsV. Nitarsone has single

541 specification for limit (1% of API) of inorganic arsenic which would represent the sum of As

542 (III) and As (V).

IV. List of Excel Files Contributing to Tables 2, 3, 4, and 5

Table 2	0- 3- and 5-day liver totals- adjusted for NR audit.xls (muscle and feed)
	All liver totals results.xls (liver)
	Contributing:
	Day 3 liver digest worksheet.xls
	09l02 5-day birdsTOTALS As worksheet.xls
	09l09 0-Day birds TOTAL As worksheet.xls
	10D09 and 10D12 treated liver MWdigest TOTAL As checks.xls
	10J04 digestion form 434-142.xls
	10J04 digestion form.xls
	10J05 digestion form 434-143.xls
	10J05 digestion form.xls
	10J06 digestion form 434-146 HNO3.xls
	10J06 digestion form.xls
	10J06pm digestion form 434-147.xls
	10J06pm digestion form.xls
	10J07 digestion form 434-148.xls
	10J12am digestion form 434-150.xls
	10J12pm digestion form 434-151.xls
	10J13 digestion form 434-152.xls
	434-143 -146 -151 -152 Totals with GOOD SRM.xls
Table 3a	10C11 replicates QUANTTAB.xls
	arsenic_calibration_data_Table_Remi-MCC comments.xls

		Contributing: This file summarizes all valid calibration data acquired from 22 Mar to 20 Sep 2010, so pulls data from all QUANTTAB.xls files listed below for Tables 3b, 3c, 4, and 5, as well as these additional sets:
		10C30QUANTTAB.xls (tissue results not used because reanalyzed later with lower calibrant)
		10D20QUANTTAB.xls (tissue results not used because reanalyzed later with lower calibrant)
		10D22QUANTTAB.xls (tissue results not used because reanalyzed later with lower calibrant)
		10H19QUANTTAB.xls (Method Blank high for this set—but calibration ok)
		10I20QUANTTAB.xls
	Table 3b	MethodEval + ASDL and LOD + orthogonal-MBcorrected.xls, worksheet Fortification recoveries
		Contributing: All files contributing to Table 4, plus
		10E20QUANTTAB.xls
		10F15QUANTTAB.xls
		10H23QUANTTAB.xls
	Table 3c	MethodEval + ASDL and LOD + orthogonal-MBcorrected.xls, worksheet Incurred replicates
		Contributing:
		10H23QUANTTAB.xls
	Table 3d	MethodEval + ASDL and LOD + orthogonal-MBcorrected.xls, worksheet "Orthogonal" results
		Contributing:
		10H19 QUANTTAB PRP-X100.xls
		10H24 QUANTTAB treated extracts on PRP-X100.xls
	Table 4	Chicken liver speciation summary-rev 20110114.xls
		Contributing:
		10C22QUANTTAB.xls

595 10C23QUANTTAB.xls

596 10C25QUANTTAB.xls

597 10D01QUANTTAB.xls

598 10E06QUANTTAB.xls

599 10E17QUANTTAB.xls

600 10E19QUANTTAB.xls

601 10F24QUANTTAB.xls

602 10F29QUANTTAB.xls

603 10F30QUANTTAB.xls

604 Data for extraction efficiency:

605 10I16 liver extract filt + unfilt TOTALS worksheet vs avg totals.xls

606 Table 5a Feed speciation LOQ.xls

607 Contributing:

608 10E03QUANTTAB.xls

609 Table 5b 10I23QUANTTAB.xls

610 Generally contributing to all extraction data: extractionform.xls

611 REFERENCES

612

613 (1) Francesconi, K.A. & Kuehnelt, D. (2004) *Analyst.* **129**, 373-395

614 (2) Falnoga, I., Stibilj, E., Tusek-Znidaric, M., Slejkovec, Z., Mazej, D., Jacimovic, R., &
615 Scancar, J. (2000) *Biol.Trace Elem.Res.* **78**, 241-254

616 (3) Polatajko, A. & Szpunar, J. (2004) *J AOAC Int* **87**, 233-237

617 (4) Grant, Tyre. Dissertation (5-26-2004) *Assessing The Environmental And Biological*
618 *Implications Of Various Elements Through Elemental Speciation Using Inductively*
619 *Coupled Plasma Mass Spectrometry: Chapter 4: Characterization of arsenic species in*
620 *poultry tissue: Identification of 3-nitro-4-hydoxyphenylarsonic acid.* University of
621 Cincinnati

622 (5) Sanchez-Rodas, D., Gomez-Ariza, J.L., & Oliveira, V. (2006) *Analytical and Bioanalytical*
623 *Chemistry* **385**, 1172-1177

624 (6) Pizarro, I., Gomez, M., Camara, C., & Palacios, M.A. (2003) *Analytica Chimica Acta* **495**,
625 85-98

626 (7) Sanz, E., Munoz-Olivas, R., & Camara, C. (2005) *Journal of Chromatography A* **1097**, 1-8

(8) Jackson, B.P. & Bertsch, P.M. (2001) *Environmental Science & Technology* **35**, 4868-4873

(9) **SOP Number T012(001): Determination of Trace Elements in Aqueous Solutions by Simultaneous Inductively Coupled Plasma Atomic Emission Spectrometry**, D Heitkemper, B Barnes, J Urban, and B Zimmer, last updated Feb. 2000, http://inside.fda.gov:9003/ORA/CentralRegion/ForensicChemistryCenter/ucm040360.htm accessed on

(10) Guidance for Industry: **Bioanalytical Method Validation**, FDA CDER and FDA CVM, last updated May 2001, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf accessed on 12/6/2010

SOP for ICP-MS analysis for total arsenic. v. 1.2 8/25/10

Microwave digestion

Accurately weigh ~0.5-1g of sample into clean, dry tared microwave digestion vessel (record actual mass of sample). Include at least two method blanks (0.5g DIW) for each batch, and at least one SRM in each batch. Fortified blank and SRM samples should also be included (fortify by adding a small amount of 10ppm As standard to the vessel along with the sample).

Add 10g (7mL) concentrated (70%) nitric acid (Tracemetal grade or better) to each vessel.

Seal each vessel using the torque device and place it in the numbered carousel slot.

Place carousel containing vessels in microwave. Heat according to the following program: 20 min ramp to 200°C, 20 min hold at 200°C.

Allow vessels to cool to ~50°C before opening. **Use caution when opening vessels, as contents may be under pressure, and hazardous spray may occur when pressure is released.**

Transfer contents of each vessel to a **tared** 50mL polypropylene tube. Rinse each vessel with several portions (~10mL) of deionized (DI) water, and add each rinse to the 50mL tube. Add DI water up to 50g total weight (record final mass of diluted digest solution).

Prepare calibration standards, blanks and check solutions. All standards should be matrix-matched to the digested samples. A final dilution weight of 50g for the 7mL (10g) of 100% nitric acid in the microwave vessel results in 20%(wt/wt) nitric acid. Assuming that about half of the acid is used up in the digestion process, standards should be made up in 10% (wt/wt) HNO_3. The blank should be 10% HNO3. A reasonable set of calibration standards might be 0.05, 0.2, 1, 4, 20, and 50 ng/g As, with a 2ng/g check solution.

ICP-MS analysis

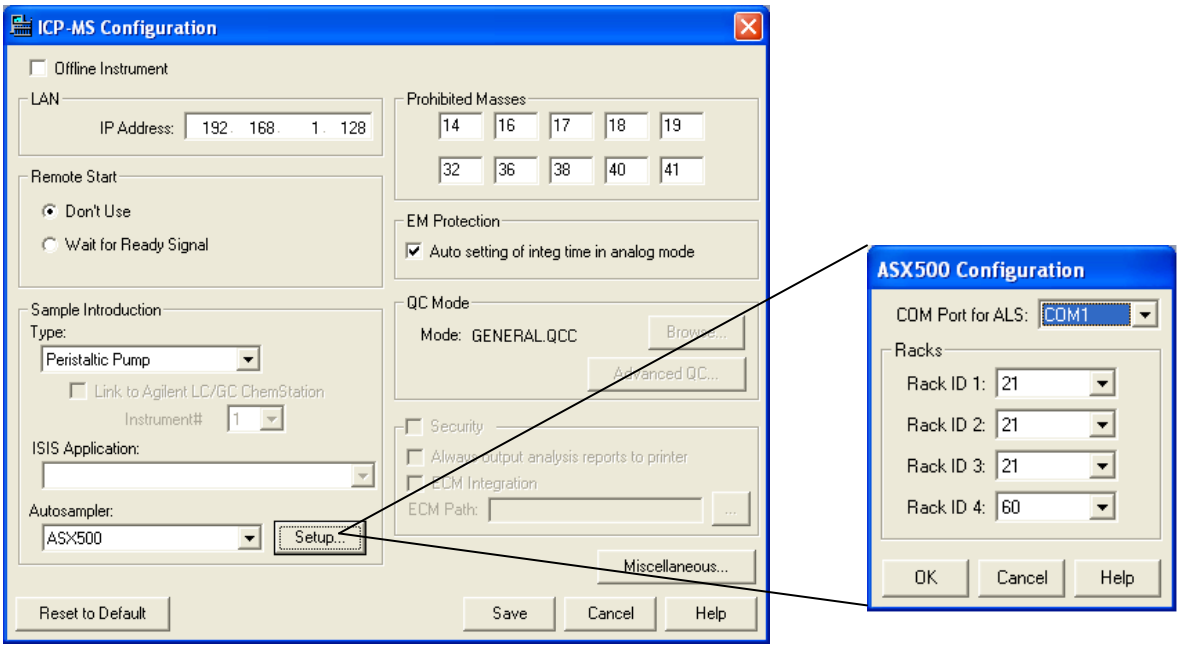
(Note that this SOP is based on the Agilent 7500ce ICP-MS system. Other systems may be used for the analysis, with details of this protocol adapted for that instrument.)

Open main valve on Argon supply.

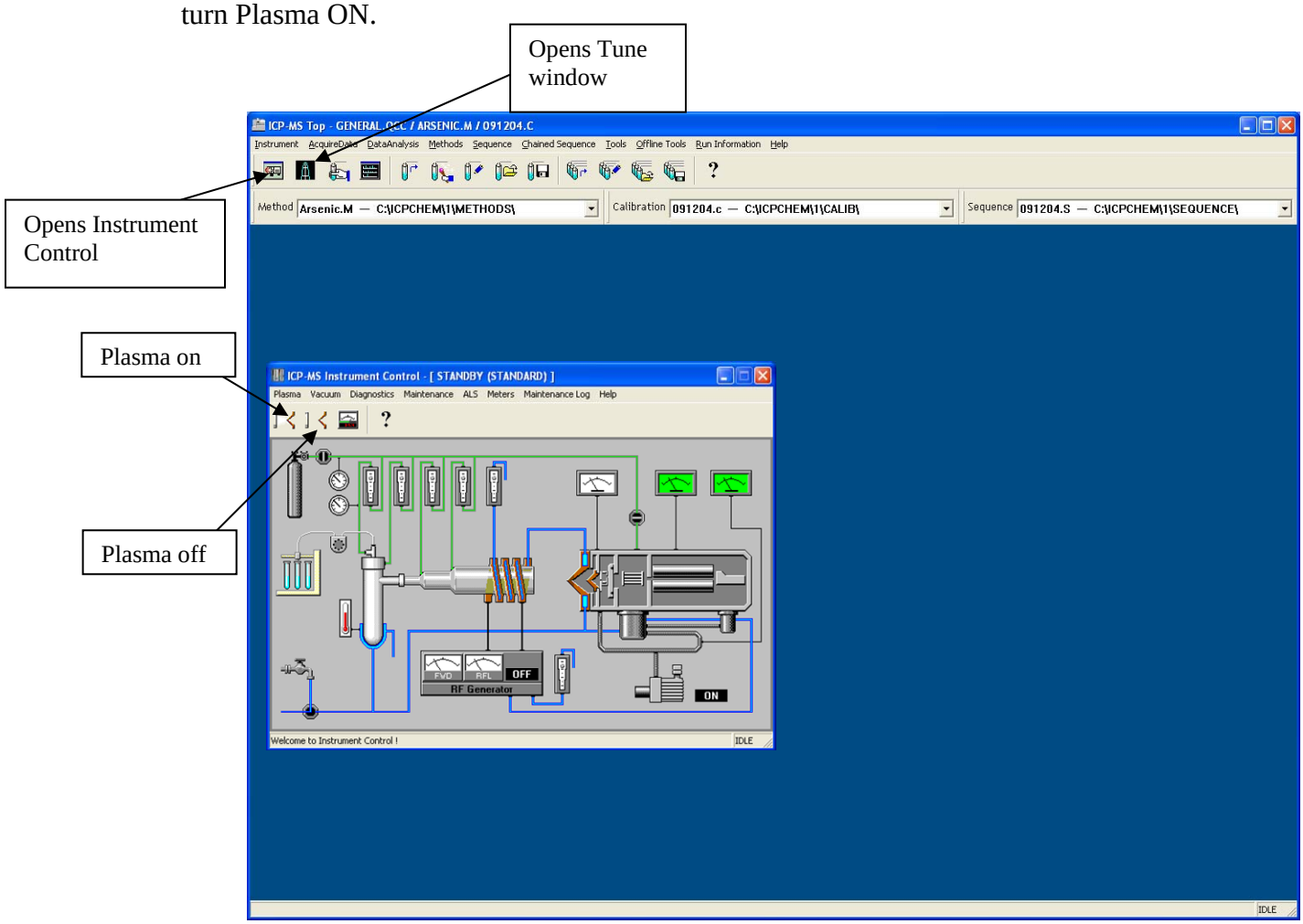
Turn on chiller.

Tighten peristaltic pump tubing for drain, internal standard and sample introduction lines, and tighten clamps on ICP peristaltic pump.

Open “Configuration” software, and setup system for the appropriate autosampler configuration.



Click “save” and exit. Open ICP-MS software, open the Instrument Control panel and turn Plasma ON.

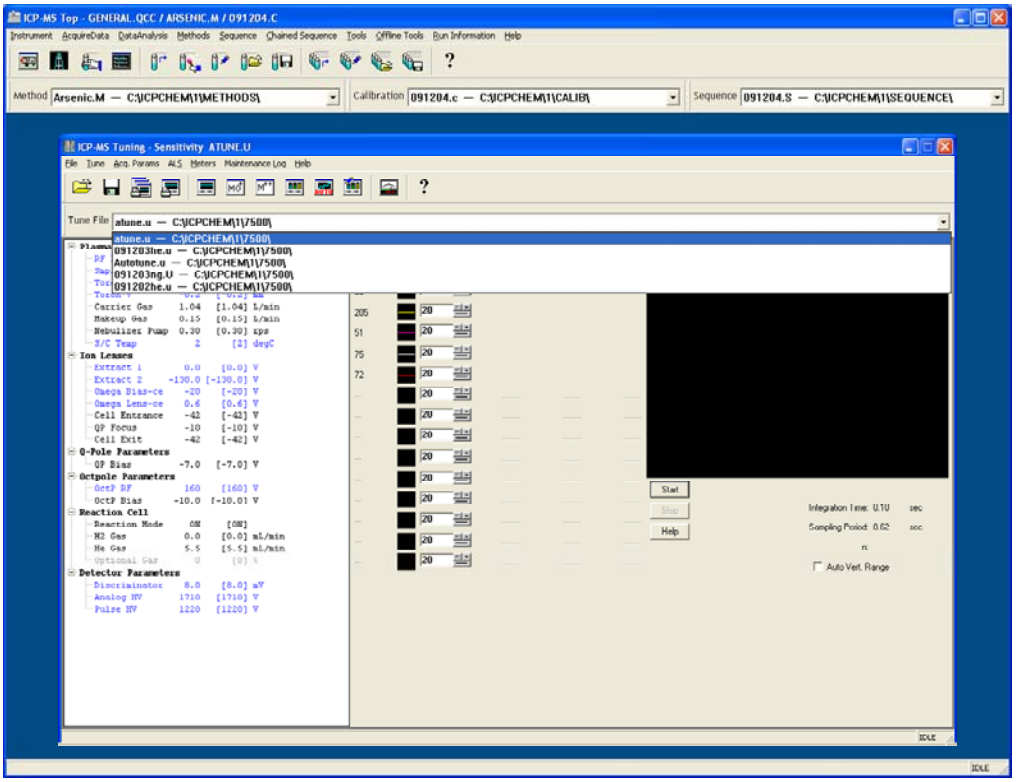


Wait for the plasma to ignite. If it does not come on, make a note of the error message at the bottom of instrument control window and troubleshoot.

Allow 30mins for the instrument to warm up and the plasma to stabilize. This is a good time to edit the sequence table with your sample list and place samples in the racks.

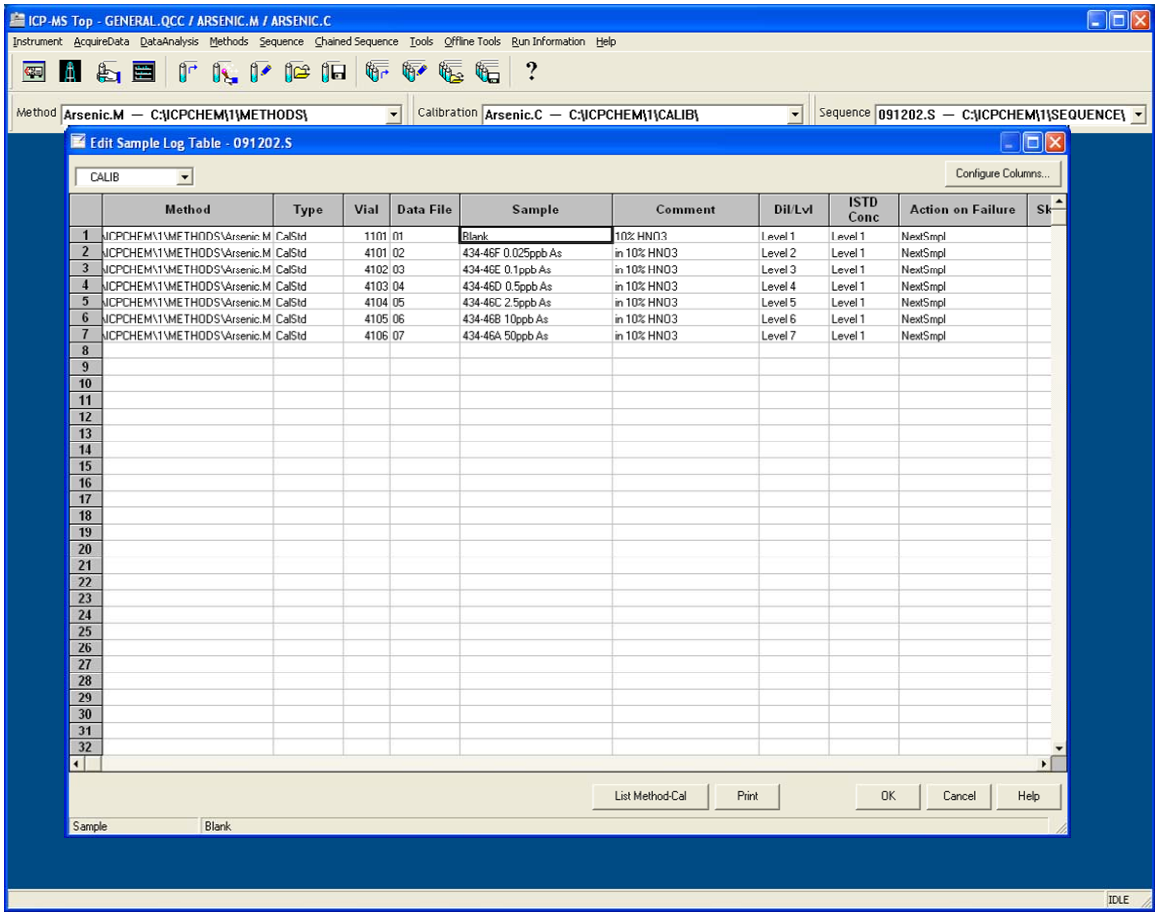
The sample introduction line should be connected to a mixing tee, along with an internal standard line, with the output of the tee going to the nebulizer. The internal standard should be ~100ng/g germanium in 7%(vol/vol) HNO3, with a flow rate about 1/20 of the flow rate from the autosampler. This can be accomplished by using peristaltic pump tubing with three black+white tabs for the sample flow, and tubing with orange/red/orange tabs for the internal standard line.

In the tune window, load the most recent no-gas mode tune file. Check tuning by uptaking a 1ppb tune solution (1ng/g each Li, Y, Tl, Ce and Co in 2% HNO3 + 0.5% HCl). Under the "Tune" dropdown, select "Autotune" and tune the torch horizontal and vertical position (select Hot Plasma only). Ensure that signals meet the requirements specified by the FCC ICP tuning SOP (attached- Hydrogen mode steps may be disregarded, as it is not used), adjusting tune parameters as needed. Generate a tune report, and save the tune file (for example, filename 091204ng.u for the no-gas mode tune file saved on Dec 4, 2009). Load the most recent helium-mode tune file. Check that tuning SOP requirements are met (print a tune window with about 50 data points plotted). Save the tune file. If tuning requirements are not met after adjusting tune parameters, turn off plasma and clean the cones (follow steps in Agilent maintenance video on PC). <u>Make sure to run the analysis with the He mode tune file open.</u>



Close the tune window, and load the method Arsenic.m (this method is set up to acquire data for *m/z* 72, 75 and 77, 0.1 s dwell time and 3 repetitions per mass).

Edit the Sample Log Table. Under “sequence”, click “edit sample log table”, and fill in the table (example below). Make sure the proper method is selected, make sure calibration standards are entered in the right place (calibration stds in the CALIB sequence, samples in the SMPL sequence), and check that the Vial number matches the sample’s placement in the rack. After the calibration standards, and after every ten samples thereafter, run a blank, a check solution, and another blank.



	Method	Type	Vial	Data File	Sample	Comment	Dil/Lvl	ISTD Conc	Action on Failure
1	\ICPCHEM\1\METHODS\Arsenic.M	CalStd	1101	01	Blank	10% HNO3	Level 1	Level 1	NextSmpl
2	\ICPCHEM\1\METHODS\Arsenic.M	CalStd	4101	02	434-46F 0.025ppb As	in 10% HNO3	Level 2	Level 1	NextSmpl
3	\ICPCHEM\1\METHODS\Arsenic.M	CalStd	4102	03	434-46E 0.1ppb As	in 10% HNO3	Level 3	Level 1	NextSmpl
4	\ICPCHEM\1\METHODS\Arsenic.M	CalStd	4103	04	434-46D 0.5ppb As	in 10% HNO3	Level 4	Level 1	NextSmpl
5	\ICPCHEM\1\METHODS\Arsenic.M	CalStd	4104	05	434-46C 2.5ppb As	in 10% HNO3	Level 5	Level 1	NextSmpl
6	\ICPCHEM\1\METHODS\Arsenic.M	CalStd	4105	06	434-46B 10ppb As	in 10% HNO3	Level 6	Level 1	NextSmpl
7	\ICPCHEM\1\METHODS\Arsenic.M	CalStd	4106	07	434-46A 50ppb As	in 10% HNO3	Level 7	Level 1	NextSmpl
8									
9									
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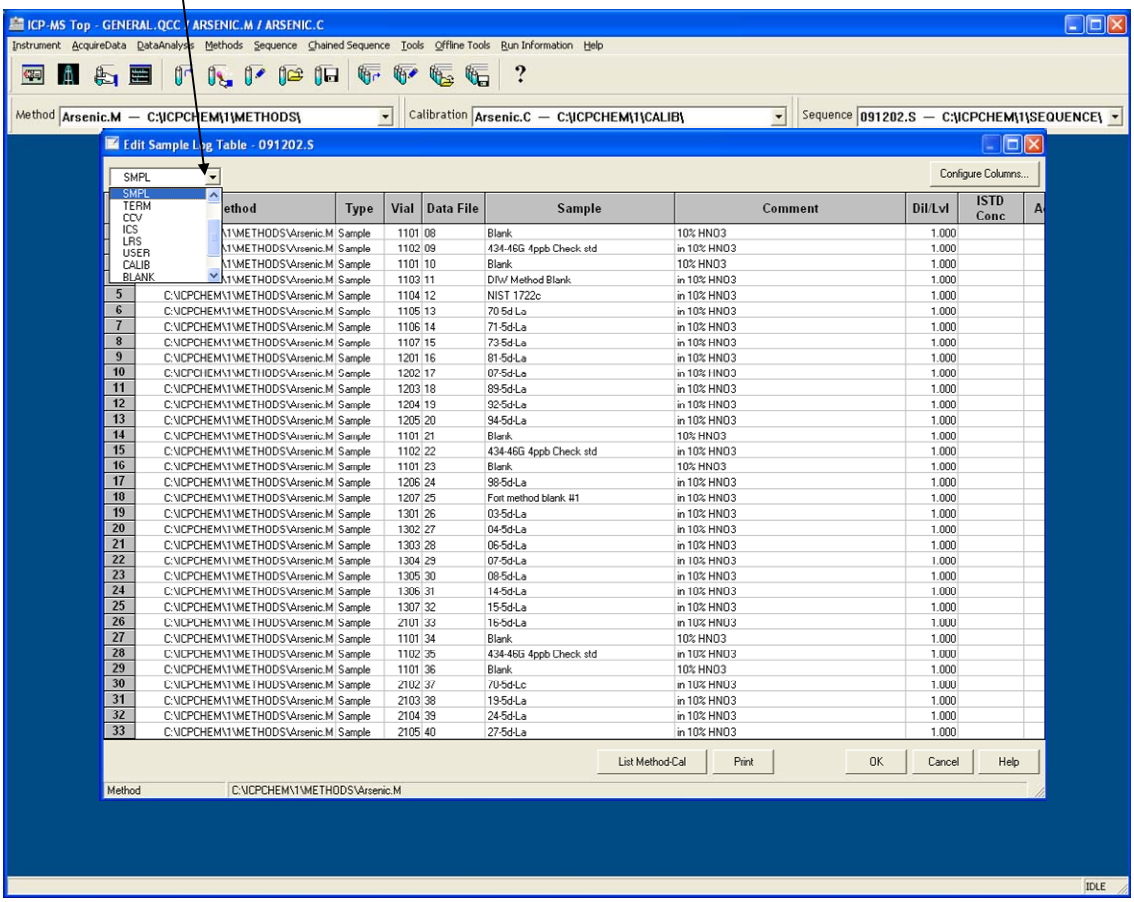
Switch from CALIB to SMPL to Sequence Flow here

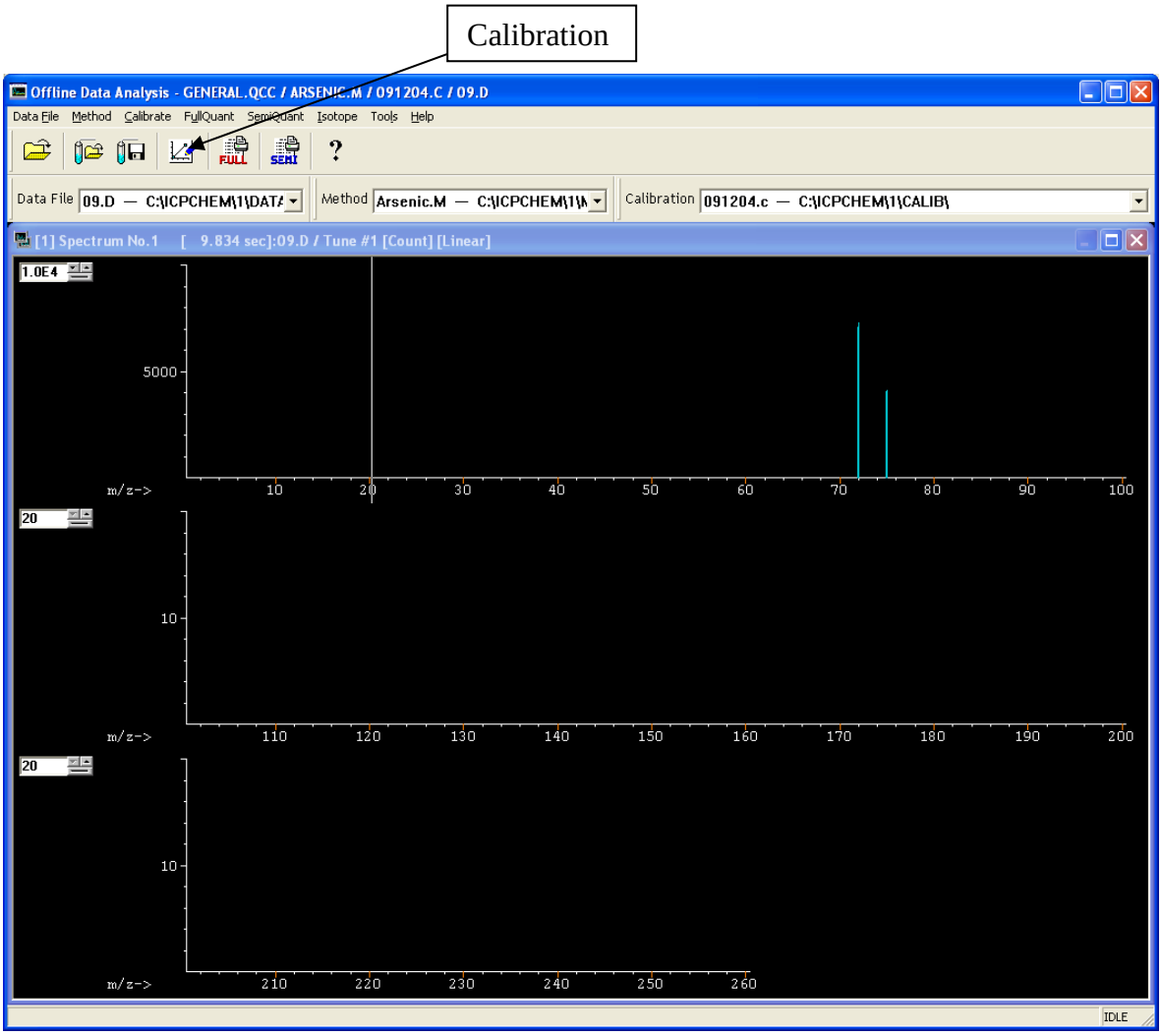


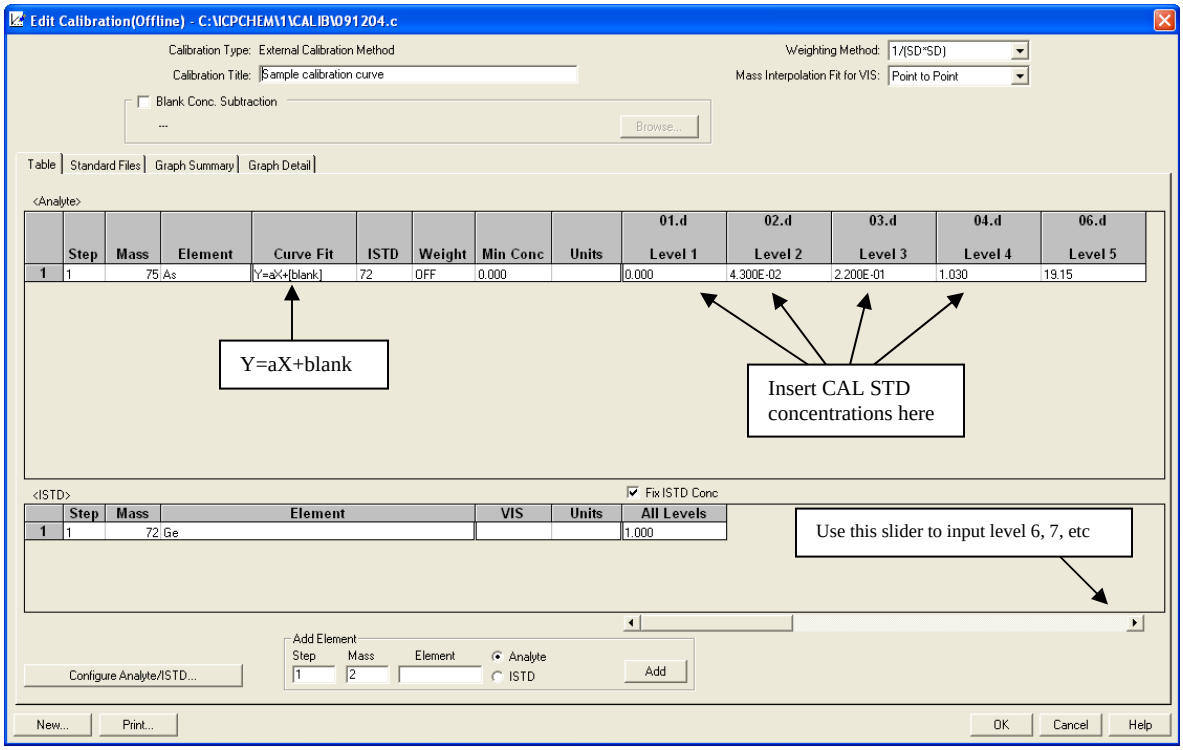
Set up the Sequence Flow to run the CALIB stds first, followed by the SMPL sequence.

Once all the samples have been entered, click the “sequence” dropdown and select “run”. While the sequence is running, samples may be added to or removed from the table.

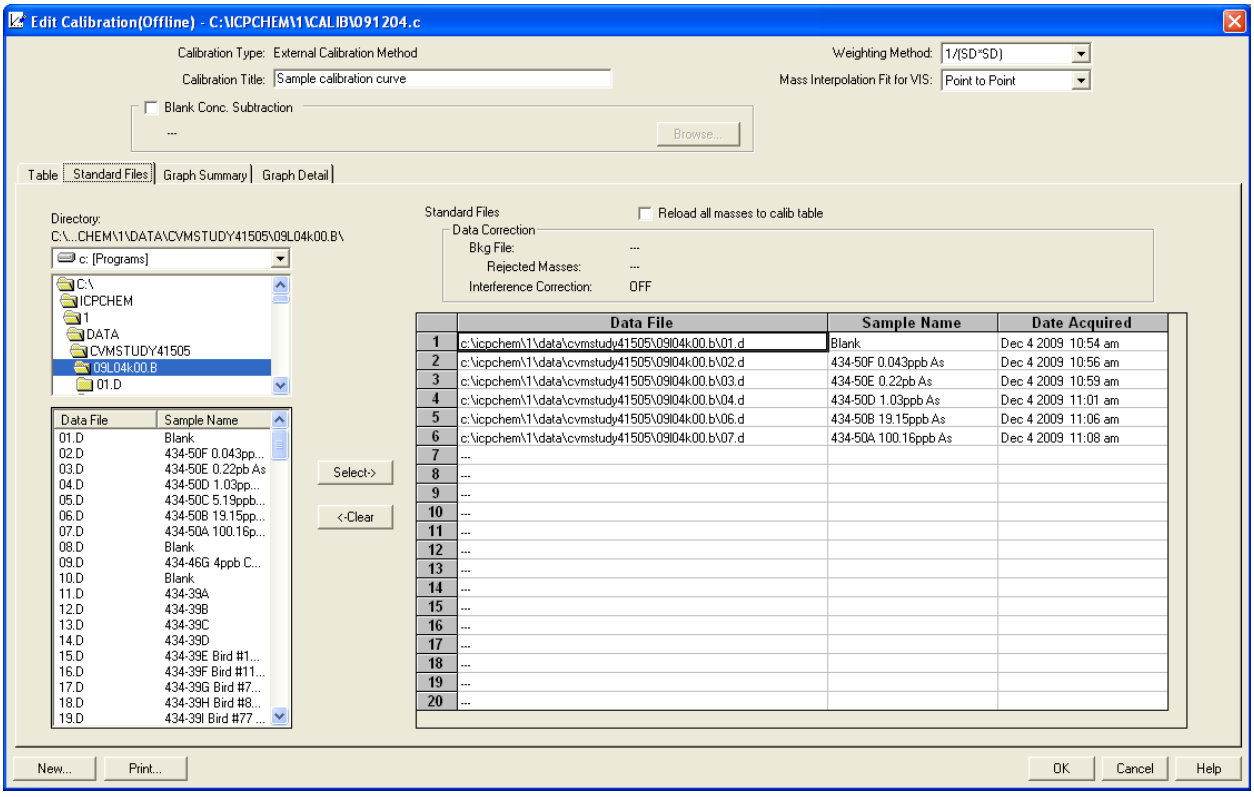
Quantitation

After the sequence has finished, open the offline data analysis program. First, load the Arsenic.m method. Open one of the files from the data set to be processed. Click on the calibration button. Enter the exact concentration of each calibration standard as indicated below.

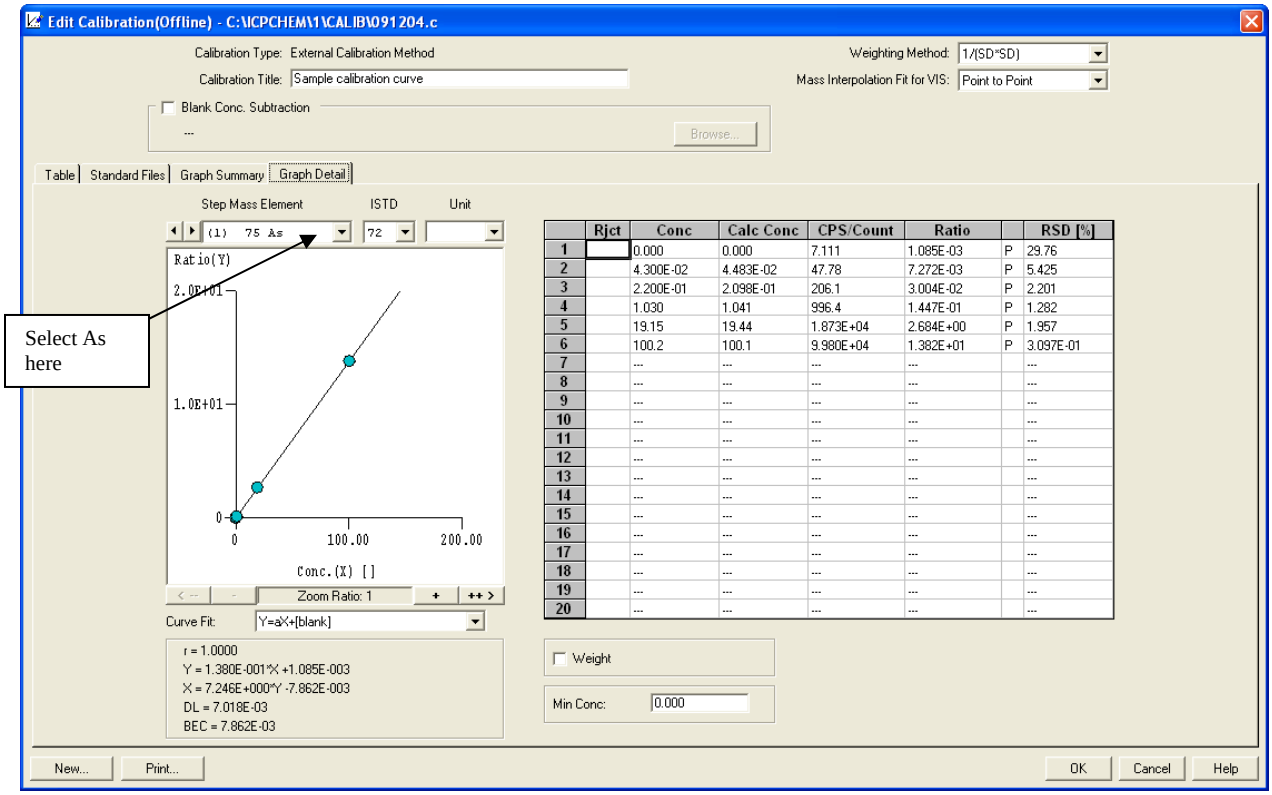




Click the “Standard Files” tab, and select the appropriate data files for each calibration level.

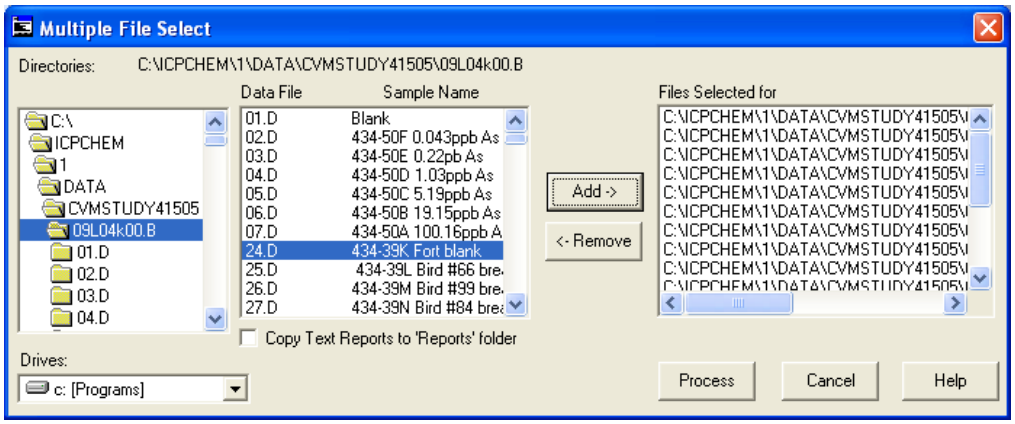


Click the “Graph Detail” tab, select As, and check the calibration plot for linearity. Minimum correlation coefficient for standard curve is 0.995.



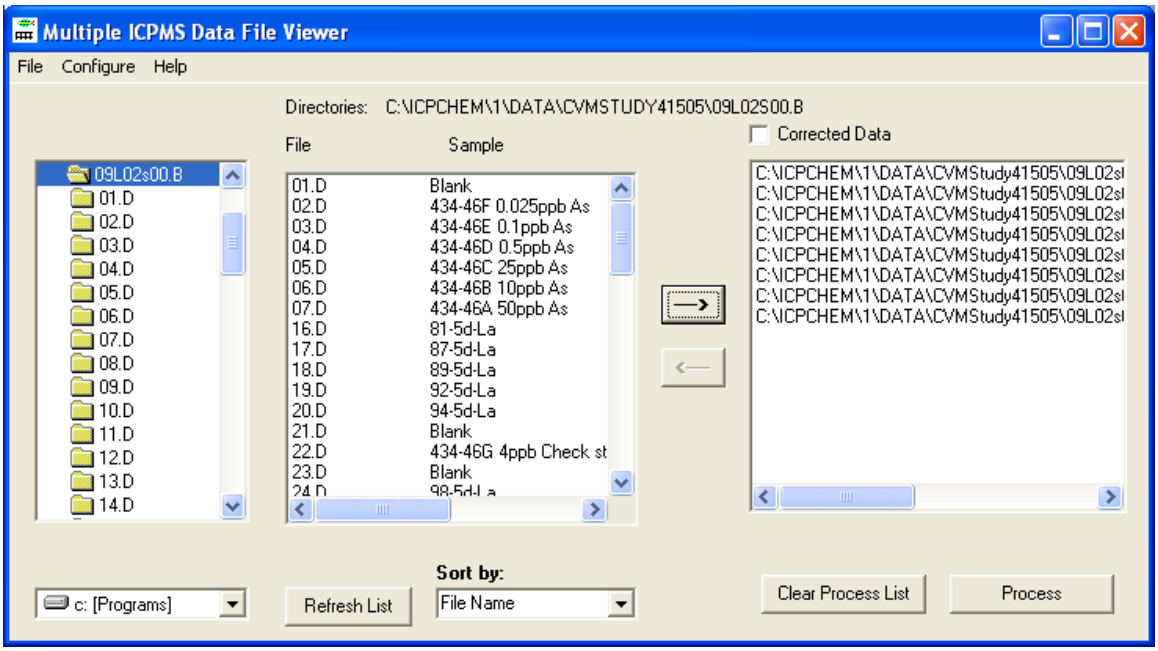
Select As here

Under the “Calibrate” dropdown, save the calibration file. Under the “Tools” dropdown, select “do list”, select option for “fullQ-summary– screen”. Click OK. In the left-most section of the window, open the folder containing the data to be quantitated. In the middle section, select the data files to be processed, and “Add” them over to the right-most window. Click “Process”.

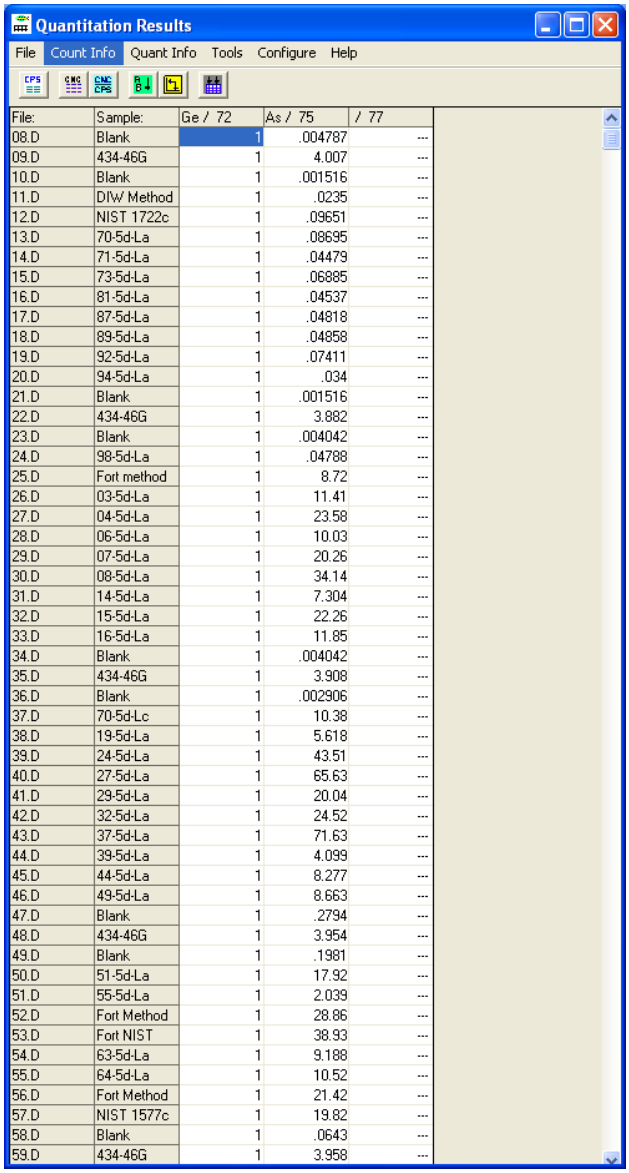


Quantitation results will open for each data file. Once the processing has finished, these can all be closed.

Open FileView32 software. Select the files to be processed (procedure similar to the previous “do list” file selection). Click “Process”.



The resulting data table will contain counts per second data for each mass in each sample. Click the “CMC” button to convert to concentration.



File:	Sample:	Ge / 72	As / 75	/ 77
08.D	Blank	1	.004787	---
09.D	434-46G	1	4.007	---
10.D	Blank	1	.001516	---
11.D	DIW Method	1	.0235	---
12.D	NIST 1722c	1	.09651	---
13.D	70-5d-La	1	.08695	---
14.D	71-5d-La	1	.04479	---
15.D	73-5d-La	1	.06885	---
16.D	81-5d-La	1	.04537	---
17.D	87-5d-La	1	.04818	---
18.D	89-5d-La	1	.04858	---
19.D	92-5d-La	1	.07411	---
20.D	94-5d-La	1	.034	---
21.D	Blank	1	.001516	---
22.D	434-46G	1	3.882	---
23.D	Blank	1	.004042	---
24.D	98-5d-La	1	.04788	---
25.D	Fort method	1	8.72	---
26.D	03-5d-La	1	11.41	---
27.D	04-5d-La	1	23.58	---
28.D	06-5d-La	1	10.03	---
29.D	07-5d-La	1	20.26	---
30.D	08-5d-La	1	34.14	---
31.D	14-5d-La	1	7.304	---
32.D	15-5d-La	1	22.26	---
33.D	16-5d-La	1	11.85	---
34.D	Blank	1	.004042	---
35.D	434-46G	1	3.908	---
36.D	Blank	1	.002906	---
37.D	70-5d-Lc	1	10.38	---
38.D	19-5d-La	1	5.618	---
39.D	24-5d-La	1	43.51	---
40.D	27-5d-La	1	65.63	---
41.D	29-5d-La	1	20.04	---
42.D	32-5d-La	1	24.52	---
43.D	37-5d-La	1	71.63	---
44.D	39-5d-La	1	4.099	---
45.D	44-5d-La	1	8.277	---
46.D	49-5d-La	1	8.663	---
47.D	Blank	1	.2794	---
48.D	434-46G	1	3.954	---
49.D	Blank	1	.1981	---
50.D	51-5d-La	1	17.92	---
51.D	55-5d-La	1	2.039	---
52.D	Fort Method	1	28.86	---
53.D	Fort NIST	1	38.93	---
54.D	63-5d-La	1	9.188	---
55.D	64-5d-La	1	10.52	---
56.D	Fort Method	1	21.42	---
57.D	NIST 1577c	1	19.82	---
58.D	Blank	1	.0643	---
59.D	434-46G	1	3.958	---

Under “Tools” dropdown, this data can be exported to a CSV file, which can then be opened in Excel. Finally, calculate dilution-corrected concentrations, and check recovery of QC samples: Check Solution Recovery must be 100 ± 10%. Control limits for the reference material (True Value Recovery) is 100 ± 20% from certified value. Control limits for the fortified analytical portion (FAP recovery) is 100 ± 30%.

STANDARD OPERATING PROCEDURE

U.S. FOOD AND DRUG ADMINISTRATION
FORENSIC CHEMISTRY CENTER

Agilent 7500ce #2 and #3
Inductively Coupled Plasma Mass Spectrometers

Author(s): Barbara S. Barnes
John R. Urban
Nohora V. Shockey

Version Date: 06/28/2005

Approvals:		Date
Inorganic Branch Director	Karen A. Wolnik	6/29/05
Organic Branch Director	R. Duane Satzger	6/29/2005
Center Director	Fred L. Fricke	6/30/05
QA Director	Robert D. Riley	7/5/2005 Issued

PURPOSE:
To ensure that the Agilent 7500ce Inductively Coupled Plasma Mass Spectrometers (ICP-MS) #2 and #3 are operating within performance standards.

SCOPE:
This procedure applies to two (2) Agilent ICP-MS instruments described under equipment.

RESPONSIBILITY:
The instrument monitors for Agilent 7500ce #2 and #3 are John Urban and Nohora Shockey respectively. The alternates for Agilent 7500ce #2 are Barbara Barnes and Nohora Shockey. The alternates for Agilent 7500ce #3 are John Urban and Barbara Barnes. These individuals are responsible for seeing that the procedures described herein are performed. It is the responsibility of each analyst to verify that the instrument meets the performance standards prior to using it for sample analysis.

DEFINITIONS AND ACRONYMS:
ICP-MS – Inductively Coupled Plasma Mass Spectrometer
EM – Electron Multiplier. When the electron multiplier detector is "tuned" the applied voltages are optimized for maximum sensitivity without reducing the lifetime of the detector.
P/A – Pulse / Analog. The instrument operating software automatically switches the EM between the pulse and analog modes. P/A Factors are determined to assure calibration curve linearity across wide ranges of concentration, when both pulse and analog modes are used for detection.

SAFETY CONSIDERATIONS:
The instrument operates under high voltage and is a source of intense UV light. The instrument has integrated safety interlocks to protect the user from potential exposure which should not be defeated by the user. The instrument uses hydrogen gas (H_2). Caution must be used when hydrogen is in use. Refer to the instrument operating and hardware manuals for proper safety procedures.

The FCC Chemical Hygiene Plan and Material Safety Data Sheets should be consulted for pertinent information on the safe handling of reagents and standards used in conjunction with this equipment. The FCC Hazardous Waste Management Plan should be consulted for proper handling of wastes generated in conjunction with this equipment.

EQUIPMENT:

Agilent 7500ce ICP-MS	#2	#3
Serial Number	JP14101097	JP14101272
FDA Property Number	5105669	5105679

Logbooks: Daily Tuning Logbook, Instrument Maintenance/Repair Logbook

The equipment, logbooks, and manuals are located near instruments #2 and #3 in Rooms 117 and 121 respectively.

REAGENTS:
Reagent water and solutions of trace metals containing acids. Use reagents and acids which are trace metal grade or better.

Instrument Tuning Solution (1 ng/mL each of Li, Co, Y, Ce, Tl in 1% HNO_3 + 0.5% HCl): The instrument tuning solution is used when generating instrument tune reports and measuring stability. The tuning solution is prepared from stock standard solutions.

P/A Standard Solution: The P/A solutions are used to obtain the P/A factors. The P/A solutions are standard solutions that include many of the analytes in the sample to be analyzed, and cover the entire mass range. An optimal P/A tune will generate numerical factors for all masses in the acquisition method; however, it may be sufficient if factors are obtained for fewer masses, if factors are generated at several low, mid, and high masses. A standard containing 100 ng/mL is usually adequate for this purpose; however, additional solutions containing analyte at higher or lower concentration may need to be prepared. Refer to the ChemStation Operator's Manual for additional information.

QC ELEMENTS:
Analytical Limits

Tuning: Examine tuning report(s). The results must meet the limits contained in Attachment A. If not within limits, tune the ion optics and quadrupole analyzer as needed. Repeat the tune procedure.

Stability: The RSD obtained for each of the isotopes analyzed (^{7}Li, ^{89}Y, and ^{205}Tl) from the stability test must be less than 3%.

If any of the analytical limits cannot be met, consult the instrument operating manuals and/or the instrument monitor or alternate(s) for corrective action.

PROCEDURES (PROCESS DETAILS):
Check before each use:

Check the argon supply and replace if necessary. Check the liquid level in the waste container(s); dispose of waste properly as necessary. Check the condition of all glass components, sample tubing, and sampler and skimmer cones; clean or replace as necessary.

Daily with each use:

1. Start the instrument and allow to warm up approximately ½ hour. Load the method to be used for analysis. Load the appropriate tune file depending if the normal mode or the reaction cell modes (H_2 or He) will be used.

2. With the normal tune file loaded, tune EM prior to the first time use on each calendar week. This function is contained in the Autotune menu, which is part of the Tune screen. Save to the appropriate tune file.

3. While aspirating the 1ppb Tuning Solution, generate a tuning report for the normal mode. Compare the results of the test with the limits in Attachment A. If necessary, adjust the ion optics and quadrupole analyzer and generate a second tune report. Record the results on the Tuning Log Sheet.

4. In the normal (non-reaction cell) mode and using the method STABILIT, analyze the 1 ppb tuning solution. When the acquisition is complete, click on the button "Tabulate/Mass" and print the results. The RSD of the 10 replicates should be less than 3% for ^{7}Li, ^{89}Y, and ^{205}Tl. If the RSD is greater than 3% for any of the masses, repeat the acquisition one time. If the RSD's are still greater than 3%, consult the instrument monitor before proceeding with sample analyses. Record the results on the Tuning Log Sheet.

5. Load the tune file for the hydrogen mode (usually file "h2.U"). While aspirating the tuning solution, obtain values for average mean counts (n = 200) at *m/z* 56, 78 and 89. (Note that intensity at masses 56 and 78 are not analyte intensities, but from interfering polyatomic species.) Print the results from the screen and compare with the limits. Record the results on the Tuning Log Sheet. If any of the isotopes monitored do not meet the tuning specifications, adjust the tuning parameters or consult the instrument monitor or alternate for guidance.

6. Repeat, using the helium mode tune file (usually file "he.U") and isotopes 51, 75 and 89. (Note that intensity at masses 51 and 75 are not analyte intensities, but from interfering polyatomic species.) Record the results on the Tuning Log Sheet. If any of the isotopes monitored do not meet the tuning specifications, adjust the tuning parameters or consult the instrument monitor or alternate for guidance.

7. Aspirate the P/A Standard solution and generate a P/A report prior to the first time use on each calendar week. If enough factors are not generated using the P/A solution, repeat the procedure using a solution having the proper concentration of analyte. Be certain that the "Merge in the current data" box is checked when analyzing additional P/A solutions. Print the P/A factor report. Save the tune file. Copy the detector parameters to the hydrogen and helium tune files.

8. When these steps are completed, insert the reports in the appropriate Agilent 7500ce Daily Tuning Log binder.

Instrument Maintenance:

Periodic maintenance must be performed. Refer to Attachments C and D for the maintenance schedule and the maintenance checklist. The Instrument Maintenance

Checklist (Attachment D) must be filled out when doing the “Every Six Months” maintenance (See Attachment C) or whenever significant maintenance (such as changing pump oil or cleaning lenses) or repairs are performed.

RELATED PROCEDURES:
NA

RECORDS GENERATED:
Tuning reports, stability reports, P/A factor tuning report, and instrument maintenance checklist.

REFERENCES:
Agilent 7500 Series ICP-MS Installation Guide, Hardware Guide, and ChemStation Operator’s Manual, located near the instrument.

APPENDIXES (ATTACHMENTS):
Attachment A Daily Tuning Log
Attachment B Sample Tune Report
Attachment C Maintenance Procedures
Attachment D Instrument Maintenance Checklist

CHANGE HISTORY:
06/28/2005
1. Extend the scope of this SOP to Agilent 7500ce #3 ICP-MS
2. Include names of instrument monitor and alternates for Agilent 7500ce #3 ICP-MS.
3. Enter Agilent 7500ce #3 ICP-MS description under equipment.
4. Customize the order in which daily procedures are performed.
5. Change frequency for EM tune.
6. Remove references to the Daily Use Logbook in the “Procedures” section since this book is no longer in use. The Daily Tuning Log serves as the daily use log.
7. Generalize references to the names of the tune files to allow for use of alternate file names.
8. Under “Procedures” and in Attachment A, change the isotopes description to m/z only for the monitored interfering species in the hydrogen and helium modes.
9. Modify limits in Normal Mode (^{7}Li counts) and in Helium mode (counts at m/z 51 and ^{89}Y).
10. Modify limits for oxide and doubly charged in Attachment A.
11. Modify Attachment A to include results of the Stability test.
12. Under Equipment delete information on Recirculator and Autosampler since they are not unique parts of the instrument.

09/21/2005
1. Specify under Procedures the frequency in which P/A factors have to be performed.
2. Add P/A factor Tuning report to the Records Generated section.

Attachment A

Daily Tuning Log

Operator (Initials):_____ Date: _____

EM Tune performed? _____ **P/A Factors adjusted? _____**

Normal Mode

	Specification		Achieved	
Sensitivity (1 ng/mL)	Mean Counts*	% RSD	Mean Counts*	% RSD
^{7}Li	≥ 2500	< 15%		
^{89}Y	≥ 8000			
^{205}Tl	≥ 4000			
Oxide (156/140)	≤ 5%			
Doubly Charged (70/140)	≤ 5%			
Axis ‡	± 0.1 of actual mass		^{7}Li	
			^{89}Y	
			^{205}Tl	
W-10% ‡	0.65 to 0.80		^{7}Li	
			^{89}Y	
			^{205}Tl	
Stability (n=10)	< 3%		^{7}Li	
			^{89}Y	
			^{205}Tl	

Hydrogen Mode

m/z	Specification, Mean Counts*	Achieved, Mean Counts*
56	≤ 1000	
78	≤ 6	
^{89}Y	≥ 3000	

Helium Mode

m/z	Specification, Mean Counts*	Achieved, Mean Counts*
51	≤ 50	
75	≤ 5	
^{89}Y	≥ 1000	

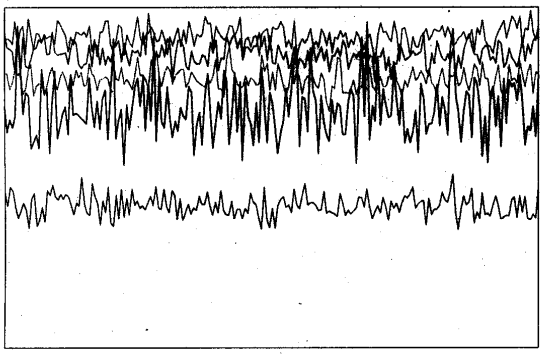
* Mean Counts are the average of 200 - 0.1 second integrations of a 1 ng/mL (ppb) solution
‡ Indicate specification is achieved by checking appropriate box

Comments/Observations:

Attachment B
SAMPLE TUNE REPORT

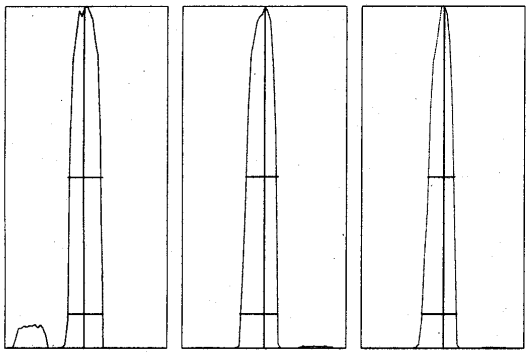
Tune Report

Tune File : Norm.U
Comment : 12/03/04 no changes



Integration Time: 0.1000 sec
Sampling Period: 0.6200 sec
n: 200
Oxide: 156/140 1.43%
Doubly Charged: 70/140 2.10%

m/z	Range	Count	Mean	RSD%	Background
7	5,000	4514.0	4307.7 ✓	3.78	3.10
89	20,000	18912.0	18243.3 ✓	3.17	2.90
205	10,000	8014.0	7869.5 ✓	3.88	4.40
70/140	5	2.4%	2.1% ✓	7.76	
156/140	2	1.6%	1.4% ✓	11.73	



m/z:	7	89	205
Height:	4,299	18,419	7,942
Axis:	7.00 ✓	89.05 ✓	205.05 ✓
W-50%:	0.65	0.60	0.50
W-10%:	0.6500 ✓	0.700 ✓	0.6500 ✓

Integration Time: 0.1000 sec
Acquisition Time: 22.7600 sec

Y axis : Linear

Generated : Dec 03, 2004 14:31:56
Printed : Dec 03, 2004 14:32:01

Attachment C

Maintenance Procedures

Routine maintenance procedures are summarized in Chapter 4 of the Agilent 7500 ICP-MS Hardware Manual, and should be consulted when there are questions about instrument maintenance.

Note that maintenance clocks have been preset in the ChemStation instrument operating software which will remind the user when major routine maintenance is due to be performed (such as checking or changing pump oil, checking lenses, etc.). When a particular procedure has been performed, the maintenance clock may be reset by the monitor or the alternate.

DAILY (OR BEFORE EACH USE): check the argon, hydrogen, and helium gas supplies and filters; check the waste vessels and empty when needed; check the condition of the peristaltic pump tubing; check the condition of the sampling and skimmer cones and their orifices. Clean or replace when necessary.

WEEKLY: Check the condition of the torch, spray chamber, end cap, and nebulizer; clean or replace if necessary. Check the water volume in the recirculator; replenish when needed. Check the oil level in the rotary pump; add oil if the level falls below the minimum.

MONTHLY: Check the level and condition of the oil in the rotary pump. Check the oil mist filter. Change oil and filter if necessary.

EVERY SIX MONTHS: Change the rotary pump oil. Check the condition of the plasma gas, auxiliary gas, and carrier gas tubing. Replace when necessary. Clean the pump strainer and vacuum the condenser fins on the recirculating chiller. Replace the water in the recirculator if algal growth is noticed in the reservoir. The Instrument Maintenance Checklist must be filled out (Attachment D).

YEARLY: Check or replace the oil mist filter of the rotary pump.

WHEN NEEDED: Evaluate and replace the electron multiplier. Clean or replace the ion lenses and the reaction lens assembly. Clean or replace the penning gauge.

Refer to the Hardware Manual for additional guidance.

Attachment D

INSTRUMENT MAINTENANCE CHECKLIST

AGILENT 7500ce # _____

Date _____ Operator's initials _____

Vacuum System

	Check	Clean	Adjust	Replace
Rotary Pump Oil				
Rotary Pump Mist Filters				
O Rings				
Penning Gauge				
Extraction-Omega Lens Assembly				
Octopole Assembly				

RF Generator and Sample Introduction

	Check	Clean	Adjust	Replace
Sampler and Skimmer Cones				
Gas Lines				
RF Contact Strip				
Torch Assembly				
Spray Chamber Cap & O-Ring				
Spray Chamber Drain & O-Ring				
Nebulizer Connector O-Rings				

Miscellaneous

	Check	Clean	Adjust	Replace
Water Lines				
Water Filter (at instrument inlet)				
Gas Filters				
Recirculator Fluid Level				
Recirculator Air Filter				
Recirculator Pump Strainer (Recirc. Inlet)				

Non-Routine Maintenance/Repairs:

A. Title: Speciation of arsenic compounds in liver resulting from roxarsone use in chickens

B. Validation History:

1. Original validation data collected: March through September 2010

2. Independent analyst validation completed: 15 Dec 2010

3. Method Trial completed:

C. Scope: This method is intended to speciate arsenic residues in chicken liver resulting from roxarsone use. In particular, it identifies and estimates roxarsone and its known metabolites, and quantifies the amount of inorganic arsenic (measured as arsenate) present above 0.6 ppb. Inorganic arsenic includes arsenite (AsIII) and arsenate (AsV); organic species such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are also monitored as they may also present toxicity issues. Structures of these compounds are shown in Figure 1.

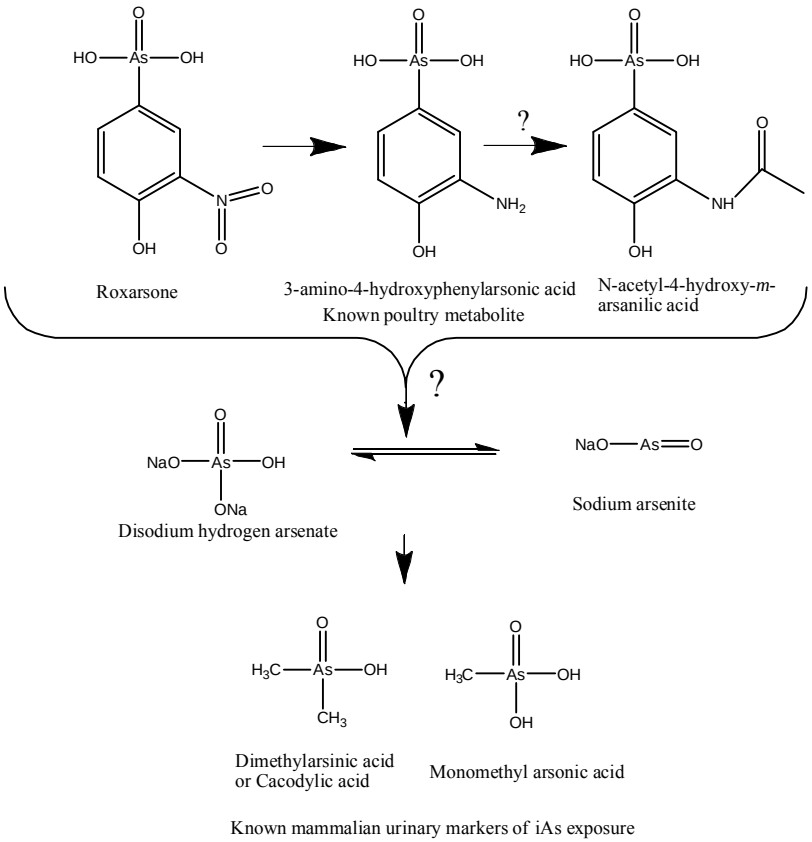


Figure 1. Structures of As compounds included in this speciation.

D. Principles: Tissue is extracted with an aqueous solution of tetramethylammonium hydroxide (TMAH), diluted with water, filtered to remove proteins and other macromolecules, and analyzed by ion chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS). The MS is set to detect As ions (*m/z* 75). Peak identification is by retention time matching with external standards, with standard addition used when necessary. Quantification is by comparison to an external calibration curve.

E. Safety Precautions:

F. Reagents:

1. Water. MilliQ 18MΩ de-ionized water. Used for all water.

2. Methanol (MeOH). LC grade.

3. Nitric acid. Trace metals grade. Used to wash glassware and assist in 3-Amino dissolution.

4. Tetramethylammonium hydroxide (TMAH), 25% w/w aqueous solution. Reagent and Trace Metal grades. Prepare 0.625% solution by diluting 10 mL (reagent grade) to 400 mL with DI water. Store in polypropylene bottle at room temperature. Prepare weekly.

5. Mobile phases.

   a. A-1% MeOH in water. Add 10.0 mL MeOH to a reservoir bottle. Bring to 1000g with water. Stir well. Use within 1 month.

   b. B-100 mM TMAH, 1% MeOH in water. Add 36.46 g 25% Trace Metal grade TMAH, 10.0 mL MeOH, and water to 1000 g to a reservoir bottle. Stir well. Use within 1 month.

6. Standards.

   a. *Roxarsone (4-hydroxy-3-nitrobenzenearsonic acid, CAS 121-19-7)*, >98% pure. (Rox). Acros Organics. Molecular weight = 263.03

   b. *Arsentrioxide (CAS 85586-03-4).* (AsIII). Spex Certiprep Speciation Standards Arsenic +3, 1000 µg/g solution.

   c. *Arsenic acid (CAS 1327-53-3).* (AsV). Spex Certiprep Speciation Standards Arsenic +5, 1000 µg/g solution.

   d. *Dimethylarsinic acid, (CAS 75-60-5),* 98.9% pure. (DMA). ChemService Inc. Molecular weight = 214.03

e. *Monosodium acid methane arsenate sesquihydrate (CAS 2163-80-6),* 98.5% pure. (MMA). ChemService Inc. Molecular weight = 161.95

f. *3-Amino-4-hydroxy-phenylarsonic acid (CAS 2163-77-1),* unknown purity. (3-Amino). Pfaltz & Bauer Rare and Fine Chemicals. Molecular weight = 233.06

g. *N-acetyl-4-hydroxy-m-arsanilic acid ((3-acetamido-4-hydroxyphenyl)arsonic acid, CAS 97-44-9),* unknown purity. (N-Acetyl). Pfaltz & Bauer Rare and Fine Chemicals. Molecular weight = 275.09

G. Equipment:

1. ICP-MS, Agilent 7500ce (upgraded from a 7500c), controlled by an HP Compact dc 7900 computer with Windows XP operating system and ICP-MS ChemStation, vers. B.04.00 instrumental control software. Chromatographic ICP-MS data was processed using PlasmaChrom ICP-MS Chromatographic Software, vers. C.01.00. Most chromatographic processing was done off-line on an IBM ThinkPad T series with operating system Windows XP vers. 5.1.

2. LC, Agilent 1200 Series with Instant Pilot control module (firmware B.02.07 [0001]), binary pump (firmware A.06.10 [005] with resident version A.06.10 [004]), vacuum degasser and refrigerated autosampler (firmware A.06.11 [001] with resident version A.06.10 [004]).

3. Omni-Prep Homogenizer, equipped with hard tissue disposable probes.

4. Single and multi-tube vortex mixers.

5. Centrifuges. Bench top centrifuge capable of 3000 rpm with buckets and carriers for 15mL and 50mL tubes (IEC Centra 8R with 218A rotor or equivalent).

6. Pipettors. Automatic pipettors capable of accurate delivery from 10 µL up to 10.00 mL; repeat pipettor (Eppendorf) with assorted tips.

7. Centrifuge tubes. 15 mL polypropylene with plug-seal caps.

8. Ultrafilters. Centriprep with Ultracel-30 membrane (Millipore Corporation)

9. Autosampler vials and caps. Wash with 2% nitric acid (made from Trace Metal grade) and 4 rinses of water before using to remove trace AsIII and AsV.

10. Analytical column. IonPac AS18, 4.0 x 250 mm (Dionex cat. no. 0060549), equipped with IonPac AG18 guard column (Dionex cat. no. 060551)

H. Procedure:

1. Standard Preparation.

   a. All stock standard solutions are prepared in water except 3-Amino, which required acid to completely dissolve.

      i. Accurately (4 significant digits) weigh 10-30 mg rox std. Dilute with 20.0 mL water, mix well and sonicate. Store refrigerated.

      ii. Dilute 3-Amino (ca 20 mg, accurately weighed) with 20.0 mL water with 1-2 drops nitric acid added. Sonicate and mix well to dissolve. Store refrigerated. Bring to room temperature and mix well before using.

      iii. Dilute N-Acetyl (ca 10 mg, accurately weighed) with 10.0 mL water. Sonicate and mix well to dissolve. Store refrigerated. Bring to room temperature and mix well before using.

      iv. Dilute MMA (ca 10 mg, accurately weighed) with ca 5 g water (accurately weighed) and shake by hand to mix. (ca 1000 ppm)

      v. Dilute MMA (ca 10 mg, accurately weighed) with ca 5 g water (accurately weighed) and shake by hand to mix. (ca 1000 ppm)

   b. For Rox, 3-Amino, N-Acetyl, MMA, and DMA, calculate stock solution concentration in mg of As per mL (or g‡) as follows and verify concentration by total As determination. Use calculated concentration if experimental

      Conc = (purity × weight in mg × MW compound) / (volume in mL‡ × 74.92)

      determination is within 5% of calculation. Otherwise, use experimentally determined concentration.

   c. For compounds with unknown purity (3-Amino and N-Acetyl), determine As concentration experimentally via nitric acid digestion and total As analysis. (For N-Acetyl, purity was determined to be 112%; for 3-Amino it was 78.7%.)

   d. *Working standard solutions.* Prepare in water. Calculate volumes of stock solutions of Rox, 3-Amino, and N-Acetyl which contain 2.00 mg As and dilute to 20.0 mL (g) with water. This is the 100 µg/mL working solution. Dilute this solution 1:100 to make the 1 µg/mL working solution. Calculate weight of MMA and DMA stock solutions which contain 0.1 mg As and dilute this amount to 100g with water to make 1 µg/g working solutions. Prepare AsIII and AsV working solutions by dilution of the Spex Certiprep Arsenic +3 and +5, 1000 µg/g, solutions.

   e. *Calibration standards, mixed solutions at 100, 30, 10, 3, 1, 0.3, 0.1, 0.03 ng each compound as As/mL.* Not stable. Prepare daily in water. 100 ng/mL mixed

standard: Add 200 µL each 1 µg/mL standard to a tube, bringing final volume to 2.00 mL with water. Mix 600 µL of this solution with 1400 µL water to make the 30 ng/mL solution. Prepare remaining standards by serial dilutions (200 µL + 1800 µL) from these two standards.

2. Controls and Fortified Samples (Quality Control Samples)

   a. *Method blank*: 0.5 mL water, 3 mL 0.625% TMAH, then 6.5 mL water.

   b. *Control tissue*: Either tissue from a chicken that has not been treated with Rox, or commercially purchased tissues that have been tested and shown to be free of residues > 0.6 ppb.

   c. *Fortified tissues*: 2000 ppb Rox fortified—add 10 µL of the 100 µg/mL Rox standard to 0.5 ± 0.05 g control tissue. 20, 4, or 2 ppb mix fortified—add 100, 20, or 10 µL 100 ng/mL mixed standard to 0.5 ± 0.05 g control tissue. 1 ppb mix or single analyte fortified—add 50 µL 10 ng/mL mixed or single analyte standard to 0.5 ± 0.05 g control tissue.

   d. *Fortification check samples*: Add the same volume of standard solution used to fortify tissues to a *method blank* sample. Subtract this volume from the amount of water used.

3. Sample Preparation

   a. Weigh 0.4 to 0.55 g tissue into 15 mL centrifuge tube. Record weight to nearest 0.001 g.

   b. Add 3.0 mL 0.625% TMAH.

   c. Homogenize ca 30s on Omni-Prep at 24,000 rpm.

   d. Cap tubes tightly; place on multi-tube vortexer or a shaker/rotator for at least 10 min. Ensure even mixing of all samples.

   e. Add 6.5 mL (minus standard fortification volume for QC samples) water to each sample. Cap tightly and mix well.

   f. Decant into Centriprep devices and centrifuge at 3000 rpm (2000 ×g) as long as needed to get >3 mL filtrate (about an hour).

   g. Transfer a portion of the filtrate solutions to autosampler vials.

4. Instrument Operating Parameters

   a. Set LC parameters as follows:

i. Flow rate: 1 mL/min

ii. Injection volume: 50 µL

iii. Autosampler temperature: 10°C

iv. Needle wash program: 3 sec using Mobile Phase B as the solvent

v. Runtime: 50 min

vi. Gradient: 45% B 0-17 min, linear ramp to 70% B 17- 17.1 min, 70% B 17.1-42 min, linear ramp to 45% B at 42-42.1 min.

b. ICP-MS parameters:

i. The following settings may be used as a starting point. ICP-MS tuning should be checked daily to ensure satisfactory performance. Save tune file as AsChrom.U.

(a) RF power: 1500 W

(b) Carrier gas: 1.1 L min^{-1}

(c) Makeup gas: 0.1 L min^{-1}

(d) Spray chamber temp: 2°C

(e) Nebulizer type: glass concentric

(f) Sampling depth: 8.5 mm

(g) Ions monitored: 75 (As), 77 ($^{40}Ar^{37}Cl$)

(h) Dwell time: 0.8 s (*m/z* 75), 0.2 s (*m/z* 77)

(i) Collision cell: ON, He mode

(j) Collision gas flow: 5.7 mL min^{-1}

ii. Set up a time-resolved method, monitoring ions 75 and 77 for 0.8 and 0.2s, respectively, and 1 replicate per ion. Acquire data for 3000 s (50 min).

5. Procedure for Instrumental Analysis of Samples, Controls and Standards.

a. Turn on chiller and tighten peristaltic pump clamp onto spray chamber drain tubing. Open ICP ChemStation and ignite plasma. Tune according to lab or manufacturer procedure.

b. Start LC. Connect cable to allow communication between ICP and HPLC. Ensure that backpressure is acceptable (2000 psi is normal, >3000 is problematic) and that ICP source is being drained by peristaltic pump (bubbles should be visible in drain tubing).

c. Create/edit the sequence file on the ICP-MS data system. Make sure that the injection list on the LC controller matches the ICP-MS sequence.

d. Inject a standard (100 ng/mL most commonly used) and check peak shape and response.

e. Inject calibration standards set, method blank and fortification check samples, other QC samples, and sample extracts. Inject water blanks as needed. Follow with a repeat injection from one of the standards. Additional standards may be injected throughout the sequence to monitor retention time drift and instrument response.

I. Calculations:

1. Calculate the dilution factor (to be entered in ICP-MS sequence file) for each sample: (9.5+sample wt)/sample wt. Use a dilution factor of 20 for fortified samples, method blanks, and fortification check samples.

2. Process data using ICP-MS Chromatographic software. Create a processing method with DMA, AsIII, MMA, AsV, 3-Amino, N-Acetyl, Rox, and unknown compounds. Use *m/z* 75 as the target ion, signal extraction time abt +/- 2 min, measure response by area, and identify peaks by best RT match. Construct standard curves for each known analyte using Linear Regression with $1/x^2$ weighting. Use Avg of Response Factors for the unknown compounds. Select as the response factor a number close to the slopes of responses for the nearest known analytes. Usually, this number is close to 10000.

3. Open a processing method and save it using the current date or date of analysis as part of the filename.

4. Starting with the calibration standards, open each data file, quantify it ("Quantitate," then "Calculate"), review the peaks, and save the review. For standards, select "Calibrate," "Update," "Update One Level," and replace the response and retention time with the current file's data. Save the method once all levels have been updated. Quantify, review, and save remaining data files from the sequence.

5. Export the quant results from these data files to a single csv file for further processing and summarization. The file will be named QUANTTAB.csv as the software default. Open the csv file in Excel. Add a new worksheet called Conc Summary, copy the concentrations (3rd column of numbers) from each with analysis, and paste it with transposition to a row on the new worksheet. Ensure each row has the correct sample name. Add all species columns to calculate total As species. Save the file, usually as *setname*QUANTTAB.xls, where *setname* follows the ChemStation convention of yyαdd, with α being a single character code for month (A=January, B=February, etc.).

J. System Suitability and Quality Control:

1. Instrument Performance Specifications

   a. All peaks in the initial test standard (step H.5.d.) should be well-resolved except DMA, AsIII, and MMA, and appear similar to Fig. 1. Compounds shown are DMA (2.8 min), AsIII (3.0 min), MMA (3.2 min), AsV (7.0 min), 3-Amino (9 min), N-acetyl (10 min), and Rox (18 min).

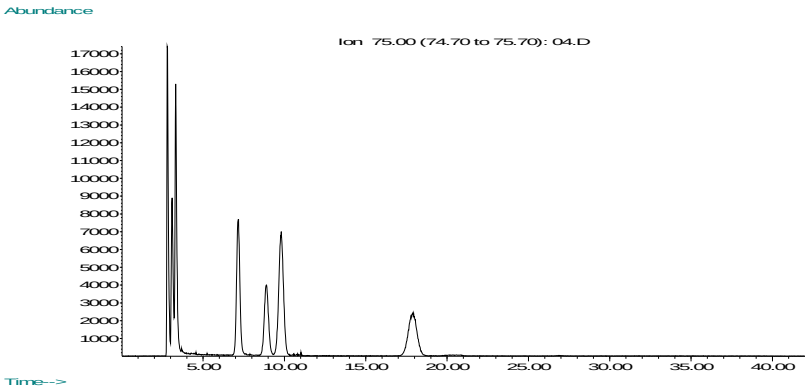


Figure 2. Representative arsenic speciation ion chromatogram. This is a mix of 7 standards prepared in water at 10 ng/mL.

   b. The peak height of AsV should be approximately 10^5 in the 100 ng/mL standard.

   c. All peaks in the next to lowest standard (0.1 ng/mL) should be readily detectable (S/N >3) by visual inspection. AsV should be readily detectable in the lowest standard (0.03 ng/mL).

2. Critical Points and Stopping Points

   a. Peak identity in sample extracts should be verified by individual standard addition if there is suspected retention time shift.

3. Stability of Analyte in Samples and Extracts

a. Stability is to be determined in samples. Literature suggests overall stability is not likely to be a problem in tissues at -80°. Preliminary results suggest that samples should not be repeatedly thawed and refrozen.

b. Extracts in TMAH are not stable. We have observed some conversion of species in standard solutions and extracts after only 3 days at 10°. Extracts analysis should be completed within 48 hours of preparation.

4. Acceptance Tests for Critical Reagents

   a. The method blank should be free of peaks >0.6 ppb (0.03 ng mL^{-1}).

5. Acceptance Criteria for Results

   a. Coefficients of determination (r-square) for DMA, AsV, N-Acetyl, and Rox should exceed 0.99.

K. References:

1. Grant, T. 2004. Assessing The Environmental And Biological Implications Of Various Elements Through Elemental Speciation Using Inductively Coupled Plasma Mass Spectrometry: Chapter 4: Characterization of arsenic species in poultry tissue: Identification of 3-nitro-4-hydoxyphenylarsonic acid. Ph. Dissertation, University of Cincinnati. Inspiration for extraction.

2. Jackson, B. P. and Bertsch, P. M. (2001) "Determination of Arsenic Speciation in Poultry Wastes by IC-ICP-MS." *Environmental Science & Technology* **35**:4868-4873. Inspiration for chromatography.

3. Slingsby, Rosanne W., Al-Horr, Rida, Pohl, Christopher A, and Lee, Joung Hae (May 2007) "Use of Dual-Selectivity IC-ESI-MS for the Separation and Detection of Anionic and Cationic Arsenic Species." *American Laboratory.* Inspiration for choice of IonPac AS18 column.

L. Validation Data:

Accuracy and Precision from Fortified Controls

Fortification Level		DMA	AsIII	MMA	AsV	3-Amino	N-Acetyl	Rox	iAs (III+V)
2 ppm Rox	Recovery							73%	
n = 16	RSD							28%	
20 ppb mix	Recovery	106%	10%	82%	194%	83%	75%	72%	103%
n = 12	RSD	10%	275%	23%	15%	33%	33%	33%	12%
4 ppb mix	Recovery	116%	0%	94%	201%	103%	95%	86%	105%
n = 7	RSD	9%		17%	15%	32%	30%	28%	16%
2 ppb mix	Recovery	117%	2%	86%	185%	86%	81%	70%	103%
n = 10	RSD	7%	316%	20%	18%	32%	29%	33%	15%
1 ppb mix	Recovery	119%	0%	85%	157%	113%	78%	62%	103%
n = 10	RSD	23%		32%	23%	25%	42%	86%	19%
1 ppb AsV	Recovery				102%				93%
n = 5	RSD				13%				17%