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Development of an Ion Chromatography−Inductively Coupled Plasma−Mass Spectrometry Method To Determine Inorganic Arsenic in Liver from Chickens Treated with Roxarsone

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ABSTRACT: Roxarsone, (4-hydroxy-3-nitrophenyl)arsonic acid, is an arsenic-containing compound that has been approved as a feed additive for poultry and swine since the 1940s; however, little information is available regarding residual arsenic species present in edible tissues. We developed a novel method for the extraction and quantification of arsenic species in chicken liver. A strongly basic solution solubilized the liver, and ultrafiltration removed macromolecules and particulate material. Ion chromatography separated the species [arsenite, arsenate, monomethylarsonic acid, dimethylarsinic acid, (4-hydroxy-3-aminophenyl)arsonic acid, (4-hydroxy-3-acetaminophenyl)arsonic acid, and roxarsone] in the extracts, which were then detected by inductively coupled plasma−mass spectrometry. The extraction oxidized most arsenite to arsenate. For fortification concentrations at 2 μg kg^{-1} and above, recoveries ranged from 70 to 120%, with relative standard deviations from 7 to 34%. We detected roxarsone, its 3-amino and 3-acetamido metabolites, inorganic arsenic, and additional unknown arsenic species in livers from roxarsone-treated chickens. Both the originating laboratory and a second laboratory validated the method.

KEYWORDS: *Arsenic speciation, roxarsone, IC−ICP−MS, chicken liver*

INTRODUCTION

The arsenic-containing drug (4-hydroxy-3-nitrophenyl)arsonic acid or roxarsone (Rox) has been used for decades as a feed additive for chickens, turkeys, and swine to control disease, improve weight gain and feed efficiency, and improve meat pigmentation.[1] The approved conditions of use mandate a 5-day withdrawal period from the medicated feed before animals are slaughtered, and limits are in place for total residues of combined arsenic (As) in meat from Rox-treated animals [0.5 ppm (mg kg^{-1}) As in muscle tissue and eggs and 2 ppm As in liver and kidney].[2] These requirements place upper limits on the total arsenic allowable in edible tissues and were established with the understanding at the time that organic arsenicals were primarily excreted unchanged and that the residue remaining in tissues was organic arsenic.

Toxicities of individual arsenic species range from virtually nontoxic, e.g., arsenobetaine (AsB), to known human carcinogens arsenite (As^{III}) and arsenate (As^{V}).[3] Early work on the stability and metabolism of phenylarsonic acids demonstrated that organic arsenicals are relatively stable in poultry, with the majority of the drug excreted unchanged.[4−8] Moody and Williams reported only one metabolite, 3-amino-4-hydroxyphenylarsonic acid, in excreta and intestinal contents from hens fed Rox.[5] The authors reported that there was no evidence to show conversion of Rox to inorganic arsenic. However, the methodology used in these early studies was not capable of detecting low concentrations of inorganic arsenic.

The development of arsenic speciation using liquid chromatography, specifically ion chromatography, coupled to sensitive, element-specific detectors, such as inductively coupled plasma−mass spectrometry (LC−ICP−MS or IC−ICP−MS) or hydride generation with atomic fluorescence spectrometry (LC−HG−AFS) has made it possible to re-examine the fate of these arsenic-containing drugs in poultry. Dean et al.[9] used LC−ICP−MS to analyze for Rox in breast and leg tissue from chickens fed a Rox-supplemented diet. They found Rox in some of the leg tissues from treated birds with no withdrawal time but did not detect Rox in breast muscle samples with or without a 7-day withdrawal period prior to slaughter. They did not report any arsenic species other than Rox. The reported quantification limit in chicken was 25 μg kg^{-1} Rox (6.9 μg kg^{-1} as As), while fortification recoveries ranged from 85 to 103%. Grant used IC−ICP−MS to identify arsenic species in chicken liver obtained from a local market.[10] The arsenic species were extracted using tetramethylammonium hydroxide (TMAH), and Rox was identified as one of the major species detected along with minor amounts of As^{V}. Sánchez-Rodas et al.[11] reported the presence of nitarsone (4-nitrophenylarsonic acid) and AsB in two different samples of chicken breast tissue obtained from a local market using LC−

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HG−AFS. Both LC−ICP−MS and LC−HG−AFS have been used in studies investigating arsenic speciation in chicken tissue from birds given a known amount of inorganic arsenic[12] or in freeze-dried chicken meat candidate reference material.[13] Arsenic species reported in these studies included As^{III}, AsB, and dimethylarsinic acid (DMA).

Methods for the determination of arsenic species that include Rox have been reported for non-tissue samples, including poultry litter and manure.[14−17] These studies and subsequent work[18−21] suggest that, under some environmental conditions, Rox can transform into much more toxic inorganic arsenic (iAs). The goal of this project was to develop and validate a method for the extraction and quantification of arsenic species in edible chicken tissues, with special emphasis on the detection of iAs.

MATERIALS AND METHODS

Safety. Inorganic arsenic is a carcinogen, and care must be taken to avoid exposure. Typical labwear (lab coat, safety glasses, and gloves) and use of a fume hood will provide adequate protection. Waste should be considered hazardous and disposed of accordingly.

Chemicals and Reagents. All solutions were prepared using water, which was deionized to resistance >18 MΩ·cm with a Milli-Q Academic system (Millipore, Bedford, MA). Tetramethylammonium hydroxide [TMAH, electronics grade, 25% (w/w) in water] was from Alfa Aesar (Ward Hill, MA). Electronics-grade methanol and trace-metal-grade nitric acid were from Thermo Fisher Scientific (Pittsburgh, PA). A standard solution of arsenic (10 μg mL^{-1} in 2% HNO_3) was purchased from High Purity Standards (Charleston, SC). As^{III} and As^{V} were purchased as 1000 mg L^{-1} solutions from Spex Certiprep (Metuchen, NJ). Monosodium acid methane arsonate (MMA, 98.5% purity) and DMA (98.9% purity) were from Chem Service (West Chester, PA). Roxarsone (4-hydroxy-3-nitrobenzenearsonic acid, >98% pure) was from Acros Organics (now part of Thermo Fisher Scientific). 3-Amino-4-hydroxy-phenylarsonic acid (3-amino) and *N*-acetyl-4-hydroxy-*m*-arsanilic acid ((3-acetamido-4-hydroxyphenyl)arsonic acid) (*N*-acetyl) were both from Pfaltz and Bauer Rare and Fine Chemicals and provided without certificates of analysis or purity information.

Standard Reference Materials (SRMs). SRM 1577c bovine liver was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD) and used as a quality control for total As analysis. SRM 1577c was included with each batch of liver samples digested for total As analysis. Accuracy within 20% of the certified total As content (19.6 μg kg^{-1}) was required for acceptance of the data set. NIST SRM 2669 Arsenic Species in Frozen Human Urine, which is certified for As^{III}, As^{V}, DMA, MMA, arsenobetaine, arsenocholine, and trimethylarsine oxide (TMAO), was used to demonstrate the accuracy of identification and quantification of individual arsenic species. This material was diluted 5-fold with water and syringe-filtered prior to analysis by IC−ICP−MS. However, it was not taken through the same extraction and filtration process as the liver samples.

Standard Solutions. All concentrations are expressed as the amount of As per either volume or weight unit. All stock standard solutions were prepared in water at concentrations between 100 and 1000 μg mL^{-1}, except 3-amino, which required approximately 2 drops of nitric acid/20 mL to aid in dissolution. Stock solutions were sonicated to ensure complete dissolution of Rox, 3-amino, and *N*-acetyl. Concentrations of all stock solutions were verified by microwave digestion and total As determination by ICP-MS. Stock solutions were diluted with water to individual analyte working solutions of 100 and 1.00 μg mL^{-1}. These were stored at 4 °C. A mixed standard solution consisting of 100 ng mL^{-1} of each analyte in water was prepared daily from the 1.00 μg mL^{-1} solutions. The 100 ng mL^{-1} mixed standard solution was diluted serially to provide additional chromatographic calibrants at 10, 3, 1, 0.3, 0.1, and 0.03 ng mL^{-1}.

Animal Treatment. Tissues used in method validation contained incurred residues of Rox from broiler chickens fed Rox-medicated feed according to label directions (50 mg of Rox/kg of feed) and withdrawn from medicated feed for 0, 3, or 5 days before slaughter. Birds were euthanized by CO_2 asphyxiation. The Center for Veterinary Medicine Institutional Animal Care and Use Committee approved the animal portion of this work under OR Study 275.30.

Sample Treatment. A container of chicken livers was purchased from a local store for use in method development and validation. Visible fat and connective tissues were removed, and the livers were homogenized in a blender. The slurry was transferred to 50 mL polypropylene tubes and stored in a −80 °C freezer. As needed, a tube was thawed and divided into aliquots (0.5 ± 0.05 g), which were stored for up to 1 month in a −20 °C freezer. This batch of liver had no As species present above the lower limit of quantification in multiple analyses and was used as control liver. The 0.5-g control liver aliquots were also used to prepare fortified samples for speciation by adding 10−100 μL aqueous Rox, As^{V}, or mixed standard solutions to the liver immediately before extraction. Livers from chickens raised in-house were collected immediately after sacrifice, placed in Whirl-Pak plastic bags, and stored whole at −80 °C to minimize enzymatic activity and potential residue change. Portions of the livers were transferred to −20 or −30 °C freezers for up to 1 month prior to analysis. When a liver portion was removed from the freezer, 0.5-g subsamples were removed quickly, and the remainder was returned to the freezer before the liver thawed completely. Connective tissue, blood clots, and fat were avoided when taking subsamples.

Total As Determination. A MARSXpress microwave system (CEM, Matthews, NC) was used to digest samples prior to total As determination using ICP−MS (Agilent 7500ce). Table 1 contains operating parameters for the microwave digestion and ICP−MS. Liver portions (~0.5 g) were accurately weighed and transferred to Teflon digestion vessels. After the addition of 7 mL of nitric acid, the vessels were capped and heated to 200 °C for 20 min. Cooled digests were

Table 1. Microwave, ICP−MS, and LC Parameters

Microwave	
power	1600 W
ramp	20 min to 200 °C
hold	200 °C for 20 min
vessels	40 Teflon Xpress vessels, 55 mL capacity
ICP−MS	
RF power	1500 W
carrier gas	1.1 L min^{-1}
makeup gas	0.1 L min^{-1}
spray chamber temperature	2 °C
nebulizer type	glass concentric
sampling depth	8.5 mm
ions monitored	75 (As), 77 ($^{40}Ar^{37}Cl$)
dwell time	0.8 s (*m*/*z* 75), 0.2 s (*m*/*z* 77)
collision gas	He
collision gas flow	5.7 mL min^{-1}
internal standard	Ge (100 ng g^{-1}) (not used during chromatography)
LC	
column	Dionex IonPac AS18 (4 × 250 mm) with guard AG18 (4 × 50 mm)
mobile phase A	100 mM TMAH with 1% (v/v) methanol
mobile phase B	1% (v/v) methanol in water
elution gradient	0−17 min of 45% A, 17.1−42 min of 70% A, and 42.1−50 min of 45% A
flow rate	1 mL min^{-1}
injection volume	50 μL
autosampler temperature	10 °C

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transferred to polypropylene tubes and diluted to 50 g with water. Agilent 7500ce ICP−MS (Agilent, Palo Alto, CA) was equipped with an ASX500 autosampler. Quantification used an external calibration curve. To minimize the polyatomic interference from $^{40}Ar^{35}Cl^{+}$ on $^{75}As^{+}$, the ICP−MS was operated in helium collision mode. The instrument was tuned daily to ensure sufficiently low levels of oxides, doubly-charged ions, and ArCl interference. Sample was introduced at 0.3 mL min^{-1} and mixed with a germanium internal standard solution in a Teflon tee. Method blanks, fortified method blanks, NIST SRM 1577c, fortified NIST SRM 1577c, and at least one fortified tissue digest were analyzed with each batch of samples. Additionally, a check standard solution was analyzed after every 10 tissue digest samples.

Speciation Sample Processing. Portions (0.4−0.55 g) of liver were accurately weighed in polypropylene centrifuge tubes and homogenized 30−90 s in 3.0 mL of 0.625% TMAH in water using an Omni-Prep multi-sample homogenizer equipped with hard tissue disposable probes and set at 24 000 rpm (Omni International, www.omni-inc.com). The homogenates were vortex-mixed an additional 15−30 min, diluted with 6.5 mL water, remixed briefly, and then transferred to Centriprep Ultracel YM-30 centrifugal ultrafilters (Millipore Corp.). Homogenates were centrifuged at 2000*g* for at least 1 h to obtain >3 mL filtrate. A portion of the filtrate was transferred to nitric acid-washed glass autosampler vials for analysis.

IC−ICP−MS. Table 1 shows the operating parameters for the LC system used for IC. Speciation analysis by IC was performed using an Agilent 1200 LC system (Agilent, Palo Alto, CA), consisting of a vacuum degassing unit, binary pump, and temperature-controlled autosampler. The outlet of the column was connected directly to the concentric nebulizer with ∼30 cm Teflon tubing. The chromatographic conditions were initially optimized on the basis of the separation of seven species: As^{III}, As^{V}, DMA, MMA, Rox, 3-amino, and *N*-acetyl. Separation of species was achieved with a 250 × 4 mm IonPac AS18 anion-exchange column equipped with a 50 × 4 mm IonPac AG18 guard column (Dionex, Sunnyvale, CA), using the conditions shown in Table 1. Chemstation Plasma Chromatographic software was used to quantify known species based on external calibration. Calibration slopes were nearly constant across known species, allowing for the estimation of unknown species concentrations based on the responses of the known species eluting closest to them.

Method Evaluation. We assessed linearity by analyses of mixed standard solutions from 0.03 to 100 ng mL^{-1} in water. Standard curves used peak area as the response, ignored the origin, and were weighted by $1/x^2$. We used multiple methods to estimate limits of detection (LODs). These included visual inspection of chromatograms, calculation of 3× the standard deviations of 10 replicates of low concentration aqueous standards and fortified liver extracts, and calculations of (3.28 × standard error of the intercept)/slope. Method lower limits of quantification (LOQs) were lowest fortification level that gave acceptable accuracy and precision.

We tested accuracy of the chromatographic portion of the method by analysis of an SRM. We evaluated the extraction procedure multiple ways. We looked at accuracy from control liver fortified with either As^{V} at a very low concentration or a mixture of species at a range of concentrations. We tested control liver fortified with Rox only at a very high concentration to better emulate tissue from Rox-treated chickens and to assure that the extraction procedure did not itself degrade Rox to iAs. We analyzed Rox-incurred liver and compared the sum of the As species to the total As.

The final overall method was evaluated by both the originating laboratory and a second laboratory through the analysis of control chicken liver samples, samples fortified with concentrations of mixed standards ranging from 1 to 20 $\mu g\ kg^{-1}$, samples fortified with just Rox at 2000 $\mu g\ kg^{-1}$, samples fortified with just As^{V}, and replicate analyses of liver samples from treated chickens.

RESULTS AND DISCUSSION

Method Optimization. *Chromatography.* We used a standard mix containing seven species (As^{III}, As^{V}, MMA, DMA, 3-amino, *N*-acetyl, and Rox) (shown in Figure 1) to optimize

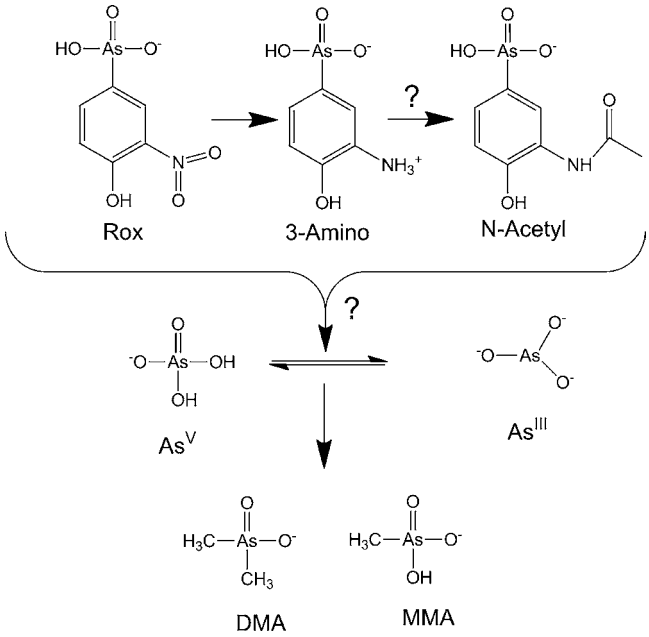

Figure 1. Structures of arsenic compounds included in this method. The arrows illustrate known and potential metabolic pathways. Moody and Williams[5] demonstrated that Rox is reduced to 3-amino in hens. Subsequent acetylation is possible. As^{V} and As^{III} are readily interchangeable. Several species, including humans, will methylate As^{III} to MMA and DMA.[27]

chromatography. We selected these standards because of their commercial availability and potential as Rox metabolites. On the basis of its widespread use in arsenic speciation, we tried separation of the standard mix using a PRP-X100 anion-exchange column. Yao et al.[22] recently reported using this column for speciation analyses including Rox. However, we confirmed Grant's observation that Rox is strongly retained on this column,[10] making it unsuitable for this study. Separation on a Dionex AS7 column, which was reported by Grant,[10] Jackson et al.,[15] Polatajko and Szpunar,[13] and Bednar et al.,[23] appeared promising. The AS7 column combined with 10 mM nitric acid mobile phase worked well with standard solutions. However, with water/methanol extracts of both chicken tissue and feed, the MMA peak retention time (RT) was variable and sometimes split. We also observed this behavior with neutralized TMAH extracts. The mobile phase pH was near the pK_{a1} of MMA, 3.6, which may have caused this peak splitting. Finally, when neutralized TMAH extracts of Rox-incurred liver were injected, we observed a very large unretained peak and discontinued work with this column.

Jackson and colleagues[15,24] reported good separation of As^{III}, As^{V}, DMA, MMA, *p*-arsanilic acid, and Rox (five of the seven species used in the current work) using a Dionex AS16 column with NaOH mobile phase. The authors achieved good separation of six species in less than 10 min. However, we found that 3-amino and *N*-acetyl were not well-separated and the As^{V} peak shape was poor. The use of a strongly basic mobile phase remained intriguing because of its compatibility with the strongly basic TMAH liver extracts that Grant[10] had used. Indeed, Jackson et al.[15,24] mention that similar separations could be achieved using TMAH in place of NaOH.

We evaluated a similar column, the Dionex AS18, using a TMAH mobile phase. The AS18 separated the seven species in the standard mix reasonably well. Although it did not provide

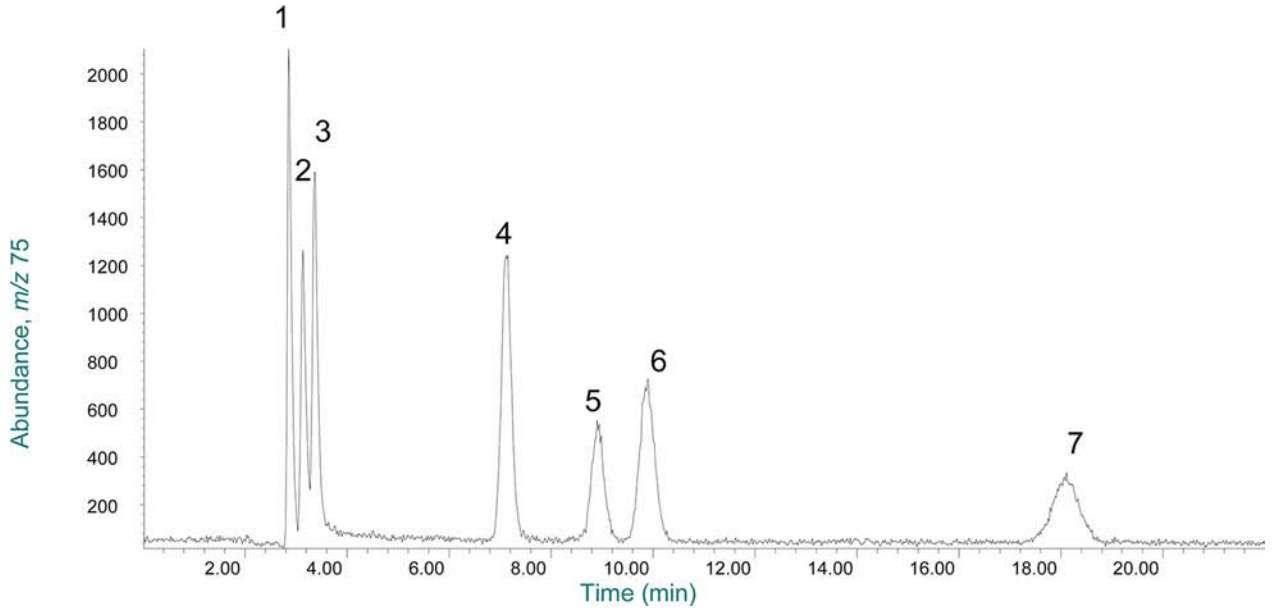

Figure 2. Isocratic chromatography of a 1 ng mL^{-1} mixed standard in water, AS18 250 × 4 mm with AG18 guard cartridge, mobile phase 45 mM TMAH, 1% MeOH. Peaks: (1) DMA, (2) As^{III}, (3) MMA, (4) As^{V}, (5) 3-amino, (6) *N*-acetyl, and (7) Rox.

baseline resolution of the first three peaks (DMA, As^{III}, and MMA), As^{V} was well-resolved from interferences (Figure 2). However, after several injections of Rox-incurred liver extracts, we noted that strongly retained broad peaks were eluting in subsequent runs. To remedy this, we added a gradient step from 45 to 70 mM TMAH after the Rox peak. The optimized elution program (shown in Table 1) eliminated carryover peaks. Starting at a lower ionic strength with a gradient step after MMA elution allowed for baseline resolution of all seven peaks but caused a chromatography artifact signal at the RT of As^{V}. Because quantification of As^{V} was our primary concern, we kept the initial portion of the chromatography at a constant ionic strength and only increased ionic strength after most compounds of interest had eluted. We observed little As^{III} in the extracts because the TMAH extraction solution oxidized most As^{III} to As^{V}; therefore, the lack of complete baseline resolution had minimal impact. Figure 3 shows typical chromatograms for a standard, control liver extract, and incurred liver extract using this extended gradient chromatography. The control liver extract is shown at ~10× the scale of the other two chromatograms. It resembles both water blank and reagent blank analyses. It has the ubiquitous trace amount of As^{V} present; the apparent peak at 25 min is an artifact of the gradient change.

Sample Preparation. We initially tried water followed by methanol as an extraction scheme based on previous studies.[11,12] This appeared to work for speciating trace iAs in the control feed using the AS7 column with nitric acid mobile phase; however, it did not extract residues in early experiments with Rox-incurred muscle, and as noted above, the peak for MMA split in extracts.

We next tried a TMAH extraction similar to the one used by Grant.[10] TMAH is strongly basic. Liver samples dissolved nearly completely in 0.625% TMAH, while muscle samples tended to become intractable gels. Because total As concentrations were much higher in liver than in muscle from treated chickens (data not shown), we focused our efforts on liver samples. The AS18 separation with TMAH mobile phase appeared to be compatible with the TMAH extraction. Unfortunately, liver extracts quickly clogged the column. To reduce this likelihood, we evaluated several extract cleanup processes. Acid precipitation/neutralization adversely affected the chromatography. Simple high-speed centrifugation was insufficient, and the extracts would not pass through the several kinds of syringe filters tried. We tested Millipore Ultracel centrifugal filter units (30 000 nominal molecular weight cutoff). Ultrafiltered extracts had the benefit of being pH-matched with the mobile phase, simplifying sample preparation, and minimizing extract matrix effects on analyte RT. Columns lasted much longer using ultrafiltered extracts. However, ultrafiltration did reduce recoveries by about 30%, particularly of the phenylarsonic acids Rox, 3-amino, and *N*-acetyl, if centrifugation time was too short.

Contamination Control. We needed to carefully control reagents and glassware to reduce background levels of As^{V}. All reagents had to be trace-metal- or electronics-grade. Off-the-shelf borosilicate glass autosampler vials introduced nearly 0.1 ng mL^{-1} As^{V}, equivalent to 2 μg kg^{-1} in liver. Polypropylene autosampler vials were not better. Overnight soaking of glass vials and their caps in 2% trace-metal-grade nitric acid reduced background concentrations of As^{V} to less than the lowest calibrant (0.03 ng mL^{-1}). Both polypropylene centrifuge tubes and ultrafilters were tested to ensure that they did not contribute significantly to background As^{V}. We included water blanks and reagent blanks with each batch of samples analyzed to assure that background As^{V} remained below 0.03 ng mL^{-1}.

Method Performance Characteristics. Chromatographic response was linear from 0.03 to 100 ng mL^{-1} for all seven compounds, with comparable slopes for most of the seven standards. We achieved best fits for all compounds with $1/x^2$ weighting. The chromatographic calibration residuals were generally less than 5% of the nominal value for most compounds. 3-Amino seemed to be the least stable compound and had some residuals as high as 20%.

We estimated concentration LODs by four procedures: (1) visual inspection of chromatograms, (2) calibration curves (3.28 × standard error of the intercept/slope, average of 10−11 curves), (3) 3.6 × standard deviation of 10 replicate injections

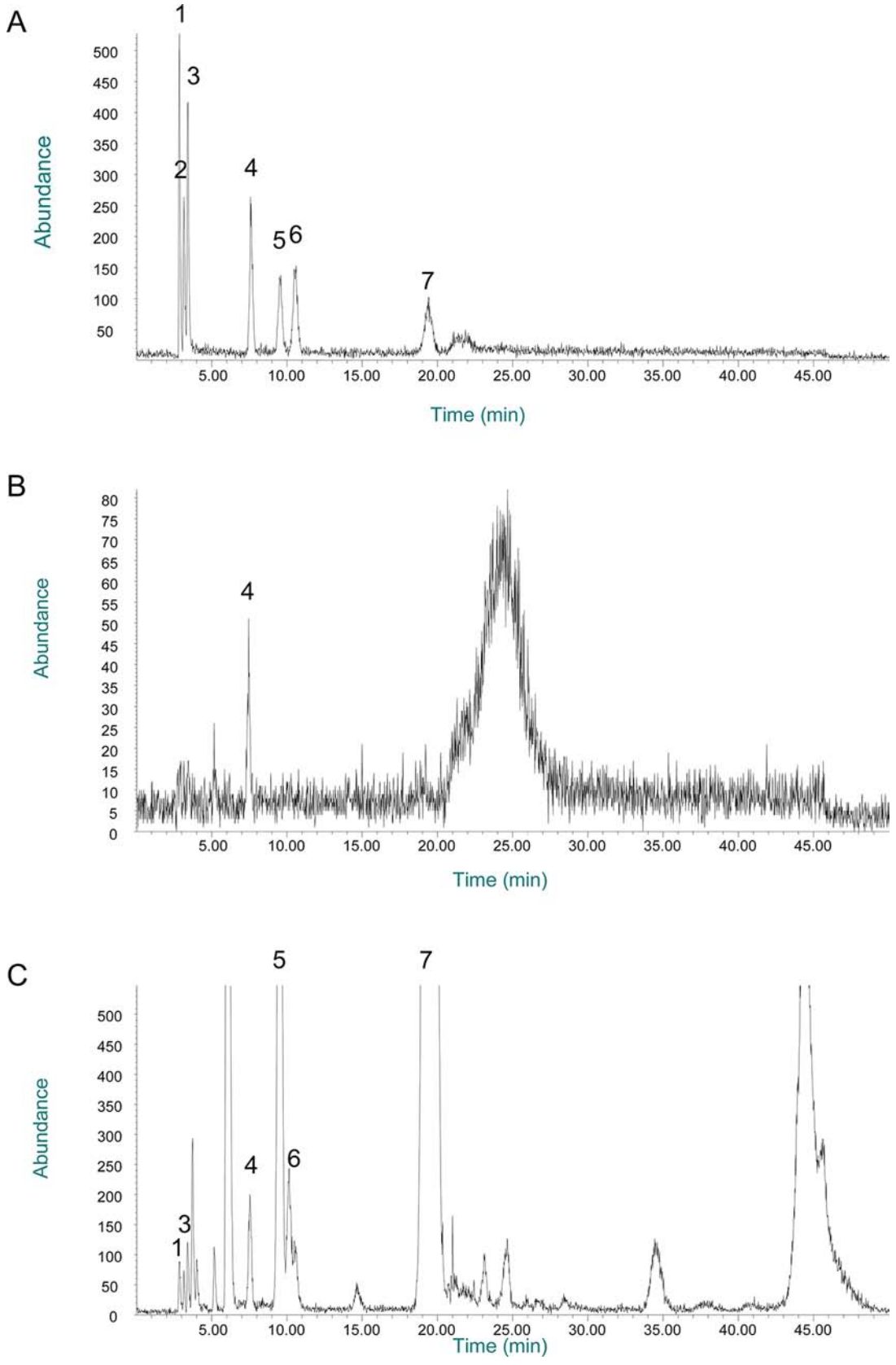

Figure 3. Gradient chromatography, with conditions as in Table 1. (A) Mixed standard (0.3 ng mL^{-1}) in water (equivalent to 6 $\mu g\ kg^{-1}$ in liver). (B) Control liver extract. (C) Liver extract from a bird treated with Rox. The *y* scale in panel B is a 10-fold zoom compared to panels A and C; the apparently large peak in the middle is an artifact of the gradient. The other peak labels are the same as in Figure 2.

of a 0.2 ng mL^{-1} standard mix, and (4) for As^{V} only, 3.6 × standard deviation of 10 replicates of 1 $\mu g\ kg^{-1}$ fortified liver analyses. Chromatographic limits in ng mL^{-1} convert to liver limits in $\mu g\ kg^{-1}$ by multiplying by 20, the extract dilution

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Table 2. Validation Results for the Speciation Method: Fortified Liver Accuracy and Precision[a]

fortification concentration		DMA	As^{III}	MMA	As^{V}	iAs	3-amino	*N*-acetyl	Rox
2 mg kg^{-1} Rox	average found concentration	0.3			0.9	*0.9*	2.7		1490
n = 15	accuracy (%)								74
	RSD (%)	97			103	*103*	173		28
20 μg kg^{-1} mix	average found concentration	21.3	2.2	16.4	38.6	*40.9*	16.7	15.1	14.7
n = 11	accuracy (%)	106	11	82	193	*102*	83	75	74
	RSD (%)	10	262	24	16	*13*	34	34	34
4 μg kg^{-1} mix	average found concentration	4.6	0.0	3.8	8.0	*8.0*	4.1	3.8	3.4
n = 7	accuracy (%)	116	0	94	201	*100*	103	95	86
	RSD (%)	9		17	15	*15*	32	30	28
2 μg kg^{-1} mix	average found concentration	2.3	0.04	1.7	3.6	*3.6*	1.7	1.6	1.4
n = 9	accuracy (%)	117	2	86	180	*91*	86	82	70
	RSD (%)	7	300	21	18	*18*	32	29	33
1 μg kg^{-1} mix	average found concentration	1.2	0.00	0.8	1.6	*1.6*	1.1	0.8	0.6
n = 10	accuracy (%)	119	0	85	157	*78*	113	78	62
	RSD (%)	23		32	23	*23*	25	42	86
1 μg kg^{-1} As^{V}	average found concentration	0.3			1.0				
n = 5	accuracy (%)				102				
	RSD (%)	16			13				

[a]Analyses were conducted across multiple days and sets, except for 1 μg kg^{-1} As^{V}, which was all in one set. Found concentration units are micrograms of As per kilogram of wet weight liver. The values for iAs are the sum of As^{III} and As^{V}.

factor. Nearly all chromatograms, including water blanks, exhibited a peak for As^{V}. In water blanks, reagent blanks, and control liver, this peak was comparable to the calibration curve y intercepts. Blanks and control liver did not exhibit any visually detectable peaks for the other six analytes. All analytes, except Rox, were always visually detectable (peak height ≥ 3× noise) in the 0.03 ng mL^{-1} calibrant, suggesting LODs for these compounds of ≤0.6 μg kg^{-1}. The LOD for Rox was at or above 0.6 μg kg^{-1}, as we did not detect Rox in one of the eleven 0.03 ng mL^{-1} calibrants.

The average LODs calculated from standard error of intercepts from calibration curves (n = 10 or 11) ranged from a low of 0.07 μg kg^{-1} for DMA to a high of 0.18 μg kg^{-1} for 3-amino. The calculated LOD for As^{V} was 0.15 μg kg^{-1}. This method of calculating noise and the resulting limits likely underestimated them, because the curves were weighted $1/x^2$. Early in method development, we estimated noise by calculating the standard deviation of 10 replicate injections of a 0.2 ng mL^{-1} mixed standard and multiplying by 3.8. The resulting LODs ranged from 0.4 μg kg^{-1} (*N*-acetyl) to 1.6 μg kg^{-1} (As^{III}) liver equivalent, with the LOD for As^{V} at 1.1 μg kg^{-1}. This estimation used a test concentration nearly 10× the eventual lowest calibrant and, therefore, likely overestimated LODs of the final method. We repeated this experiment but using extracts from 10 liver samples fortified with 1 μg kg^{-1} As^{V}. This resulted in an LOD for As^{V} of 0.3 μg kg^{-1}.

We used Center for Veterinary Medicine (CVM) criteria for acceptable determination performance. Guidance for Industry (GFI) 3, part IV,[25] states that accuracy should be 80−110% and intralaboratory precision [relative standard deviation (RSD)] should be ≤10% for marker residues at 0.1 ppm and above. For concentrations less than 0.1 ppm, the criteria relax to an accuracy of 60−110% and a precision of ≤20%. GFI 208[26] recommends tighter accuracy limits, 70−110% for concentrations between 10 and 100 μg kg^{-1}, but relaxes criteria to 60−120% for concentrations from 1 to <10 μg kg^{-1} and to 50−120% for concentrations below 1 μg kg^{-1}. It also provides separate criteria for within-run precision and between-run precision. The criteria for between-run precision are RSDs of 16% for concentrations ≥100 μg kg^{-1}, 23% for concentrations ≥10 and <100 μg kg^{-1}, 32% for concentrations ≥1 and <10 μg kg^{-1}, and 45% for concentrations <1 μg kg^{-1}.

Table 2 shows the accuracy and precision of analyses conducted on fortified liver samples. All but the last group, 1 μg kg^{-1} As^{V}, comprise analyses over 2 or more days. The 1 μg kg^{-1} As^{V} data are from a single run. DMA and MMA meet accuracy criteria and meet or nearly meet precision criteria in GFI 208 at all concentrations tested, consistent with an LOQ of 1 μg kg^{-1}.

This method is clearly not suitable for individual speciation of As^{III} and As^{V}. As^{III} and As^{V} readily interconvert via oxidation and reduction, and the extraction procedure oxidizes most As^{III} to As^{V}. This is evident in Table 2, where recovery for As^{III} is quite low, while recovery of As^{V} approaches 200% in all of the mixed standard fortified samples. If one sums As^{III} and As^{V} values in the mixed fortified samples, the accuracy for inorganic arsenic (iAs) determination ranges from 78 to 102% across all concentrations tested, with RSDs from 13 to 23%. These results also meet CVM criteria for quantitative determination with an LOQ at 1 μg kg^{-1}.

The method is not quantitative for Rox or its two metabolites 3-amino and *N*-acetyl because of high variability. The accuracy of the Rox determination was also too low (∼70%) for a residue whose concentration is ≥0.1 ppm (CVM criteria of 80−110% accuracy and <10% RSD). Because the determination of iAs was the most crucial measurement and the method was optimized for this measurement, we found less than ideal performance characteristics for 3-amino, *N*-acetyl, and Rox acceptable. Concentrations of 3-amino, *N*-acetyl, and Rox

found using this speciation method should be considered estimates only. MMA and DMA could be determined at 1 μg kg^{-1}.

Analysis of liver fortified with Rox only at 2 mg kg^{-1} had <1 μg kg^{-1} iAs present. This was comparable to control liver values and showed that the strong extraction conditions did not degrade Rox to iAs.

We wished to test the method with an SRM. Most available reference materials certified for arsenic species are seafood or urine matrices and are certified for only a few species, such as CRM 18 (urine certified for AsB and DMA) and BCR 627 (tuna certified for AsB). We opted for the reference material certified for the greatest number of species included in our standard mix, regardless of matrix. Therefore, we used NIST SRM 2669 arsenic species in frozen human urine, which is certified for seven arsenic species (As^{III}, As^{V}, DMA, MMA, arsenobetaine, arsenocholine, and trimethylarsine oxide), to evaluate the accuracy of species identification and quantification. The SRM was thawed, diluted 5-fold with water, and chromatographed on the AS18 column using isocratic conditions of 45 mM TMAH and 1% MeOH, because we expected no species in this matrix that would bind tightly to the column. The results are in Table 3. AsB elutes in the void volume near DMA on this system, as does TMAO. As^{III} recovery was high, and As^{V} was low, possibly reflecting reduction during sample storage, preparation, or chromatography, but the sum of As^{III} and As^{V} was acceptable at 114% of the combined certified values. The total found species was 105% of the sum of the certified values.

Table 3. Analysis of NIST SRM 2699 Level II (n = 5)

species	certified value (μg/L)	found concentration (μg/L)	accuracy (%)
AsB	1.43 ± 0.08		
DMA	25.3 ± 0.7		
TMAO	1.94 ± 0.27		
AsB + DMA + TMAO	28.7 ± 1.0	28.5 ± 0.4	99
As^{III}	5.03 ± 0.31	7.6 ± 0.3	151
MMA	7.18 ± 0.56	7.4 ± 0.1	104
AsC	3.74 ± 0.35	4.4 ± 0.2	118
unknown		0.5	
As^{V}	6.16 ± 0.95	5.2 ± 0.1	85
total inorganic (As^{III} + As^{V})	11.2 ± 1.3	12.8 ± 0.3	114
sum	50.7 ± 6.3	53.1 ± 0.6	105

We selected a liver from a treated bird for replicate analyses to evaluate precision in a "real" sample. Five replicate portions of liver from the same bird were extracted and analyzed on a single day. The results are in Table 4 and are consistent with precision results in the fortified samples.

The incurred liver had numerous peaks that did not correspond to any standards used (Figure 3). These included a pair of small peaks that eluted right after MMA, a large peak at around 5.7 min, a pair of peaks right after 3-amino, one of which was probably *N*-acetyl, and several late eluting peaks. We tested a number of species that were not included in our standard mix, including AsB (RT = 2.8 min), TMAO (RT = 2.8 min), 4-arsanilic acid (RT = 3.6 min), tetramethylarsonium ion (RT = 3.9 min), arsenocholine (RT = 3.92 min), and nitarsone (4-nitrobenzenearsonic acid, RT = 7.6 min). None of these compounds was a RT match with any of the unknowns mentioned above, although arsenobetaine and trimethylarsine oxide both partially coeluted with DMA. Most of these coeluting species are of little concern, because species that eluted in less than 4 min were present only at very low concentrations (usually <2 μg kg^{-1}) in treated bird livers. The other unidentified peaks are of interest in that they represent possible Rox metabolites and because they make up a significant proportion (10−80%) of the chromatographed As.

As^{V} is clearly one of the smallest peaks in a liver extract chromatogram from a treated bird (Figure 3C). To demonstrate that this peak is truly As^{V}, we conducted two additional experiments. First, using the method of standard addition, we took portions of an extract from a putative As^{V}-containing liver, added amounts of As^{V} standard equivalent to roughly 4×, 2×, and 1× the estimated As^{V} concentration, and analyzed them. The resulting As^{V} peak was still a single, symmetrical peak, with area increased as expected, as shown in Figure 4. As^{V} 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 RTs over the course of this experiment. The amount of As^{V} in the original liver sample was quantified as 4.4 μg kg^{-1} by external calibration and as 5.0 μg kg^{-1} by standard addition.

We also chromatographed the extracts from this 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 20 mM ammonium carbonate at pH 9. As^{III} elutes before DMA in this system, and As^{V} elutes at 30 min

Table 4. Intraday Precision with an Incurred Rox Liver Sample[a]

sample	DMA	As^{III}	MMA	unknown 3.5	unknown 4.5	unknown 5.6	As^{V}	iAs	3-amino	*N*-acetyl	unknown 13	Rox	unknown 21	unknown 32	unknown 36
treated 11a	1.2	1.1	1.8	5.0	1.7	62	4.5	5.6	65	9.7	2.1	543	8.0	8.4	121
treated 11b	1.3	1.0	1.4	5.4	1.2	63	3.2	4.2	44	7.9	nd[b]	377	4.9	7.0	81
treated 11c	1.3	1.3	1.8	6.0	1.9	60	4.7	6.0	71	10.1	2.2	605	7.6	10.9	122
treated 11d	1.1	1.2	1.4	3.7	1.8	42	4.6	5.7	41	6.9	2.0	328	6.1	8.4	96
treated 11e	1.2	0.9	1.6	4.7	1.8	51	4.2	5.2	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	5.3	54	8.4	1.7	446	6.4	8.8	104
standard deviation	0.1	0.1	0.2	0.9	0.3	8.8	0.6	0.7	13	1.4	1.0	121	1.3	1.4	17.8
RSD (%)	8	12	13	18	17	16	14	13	24	17	57	27	21	16	17

[a]Five extractions (a−e) from one liver (11) were analyzed in the same set. Concentration units are micrograms of As per kilogram of wet weight liver. The lowest calibrant with this set was equivalent to 0.6 μg kg^{-1} in liver. Unknown species are identified by their approximate RT in minutes. [b]nd = not detected, used 0.0 in calculating average and standard deviation.

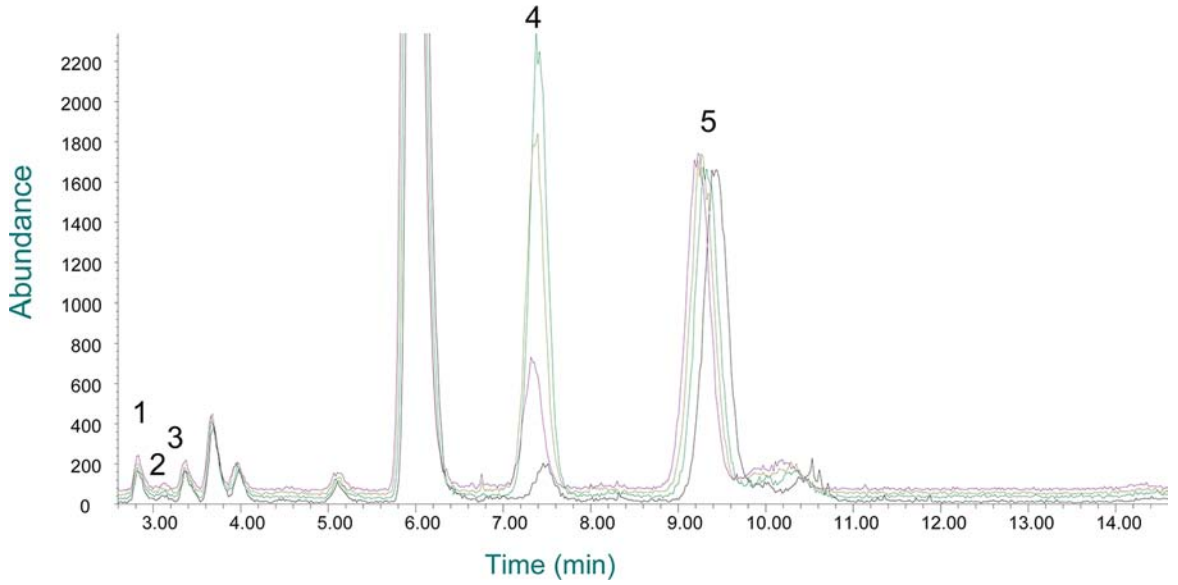

Figure 4. Chromatograms of an incurred liver extract and three concentrations of As^V standard addition. The liver As^V was determined to be 4.3 μg kg^{-1}; As^V standard was added at concentrations equivalent to 5, 10, and 20 μg kg^{-1}. Chromatography was the same as in Table 1. Peaks were labeled the same as in Figure 2.

Table 5. Accuracy and Precision Reported by the FCC from Fortified Control Liver Samples

fortification concentration		DMA	As^{III}	MMA	As^V	(iAs)	3-amino	*N*-acetyl	Rox
2 mg kg^{-1} Rox	accuracy (%)								98
n = 5	RSD (%)								9
20 μg kg^{-1} mix	accuracy (%)	116	14	89	237	*120*	93	104	105
n = 5	RSD (%)	8	0	8	8	*8*	11	7	8
2 μg kg^{-1} mix	accuracy (%)	134		80	210	*105*	88	98	90
n = 5	RSD (%)	7		6	8	*8*	12	8	15
2 μg kg^{-1} As^V	accuracy (%)				92	*92*			
n = 5	RSD (%)				6	*6*			

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 was limited on the PRP to avoid interference in subsequent runs. The amount of As^V found, 4.4 ± 0.7 μg kg^{-1}, agrees very well with the 4.2 ± 0.6 μg kg^{-1} found on the AS18 analyses in Table 4. Together, these two experiments provide strong evidence that the peak that we identified as As^V truly is As^V.

We evaluated the overall extraction efficiency by comparing the sum of species to the total As concentration in livers from treated birds. We estimated the concentration of the unknown peaks by reference to the nearest standard peaks; i.e., for the peak eluting at 5.7 min, we applied a response factor equal to the average of the slopes for MMA and As^V in that set. The species sums in general do not add up to the total As concentrations. 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 (11 and 27). Of the total As concentrations of 1760 and 2940 μg kg^{-1}, 93 and 108% were extracted, respectively, as demonstrated by nitric acid digestion of the TMAH extracts. This indicated nearly quantitative extraction of As compounds from the liver. We found 67 and 69% of the total liver As in the digested ultrafiltrates. This loss through ultrafiltration was consistent with the Rox loss seen in fortified experiments (Table 2). However, the sum of the species found for these two samples, including estimated concentrations of the unknown As-containing metabolites, added to only 53 and 32%, respectively, 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 to the value obtained from the new guard cartridge resin. Used resin had approximately 2 mg kg^{-1} As at the inlet end of the column and 0.5 mg kg^{-1} at the outlet end of the same column, while new guard cartridge resin had only 0.02 mg kg^{-1} As. Clearly, some As compound(s) bound very tightly to the AS18 column under the conditions that we used. The low and variable mass balances suggest that we do not have a full accounting of all of the As species that may exist in livers from Rox-treated birds and that the numbers found by this method should be treated as minimum numbers. Actual concentrations of some species may be higher, and other uneluted unknown species may be present.

As a final step in method validation, CVM asked the FDA's Forensic Chemistry Center (FCC) in Cincinnati, OH, to

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Table 6. FCC Precision with Two Incurred Liver Samples[a]

	DMA	As^{III}	MMA	unknown 3.5	unknown 3.8	unknown 4.8	unknown 5.7	unknown 6.3	As^{V}	3-amino	*N*-acetyl	unknown 9.4	unknown 14	Rox	unknown 25	unknown 27	unknown 31	unknown 37
treated 12a	1.1	1.32	2.2	3.5	1.8	1.8	35.4	<0.6	18.7	273	9.2	18.4	4.7	1104	34.1	14.5	9.3	22.3
treated 12b	1.3	1.35	2.2	5.2	2.7	1.8	40.9	<0.6	23.0	428	14.0	20.9	4.4	1697	26.5	15.9	8.4	21.4
treated 12c	0.8	1.39	1.9	3.4	2.0	2.3	29.3	<0.6	21.8	259	9.3	22.7	4.8	1012	59.2	14.6	13.0	29.9
treated 12d	1.2	1.36	1.3	3.0	1.7	1.8	29.7	<0.6	18.8	220	8.5	18.7	4.4	725	35.5	10.9	10.2	18.1
treated 12e	1.3	1.03	1.4	4.1	2.1	1.9	38.1	<0.6	17.3	224	8.7	12.0	4.5	1005	30.3	8.5	7.1	22.0
average	1.1	1.3	1.8	3.9	2.1	1.9	34.7		19.9	281	10.0	18.5	4.6	1109	37.1	12.9	9.6	22.7
RSD (%)	17	12	24	22	19	11	15		12	30	23	22	5	32	35	24	23	19
treated 62a	<0.6	<0.6	1.0	9.7	1.9	0.7	29.9	<0.6	10.6	185.8	6.3	67.1	7.4	343	56.3	8.8	28.1	62.0
treated 62b	<0.6	11.13	0.7	5.2	1.3	0.8	28.7	<0.6	5.5	78.7	3.2	44.4	5.8	176	44.6	7.1	72.3	51.8
treated 62b	<0.6	<0.6	0.6	13.0	2.5	0.9	36.8	<0.6	10.2	91.7	4.9	47.6	6.3	240	13.5	4.1	26.4	44.7
treated 62b	0.7	<0.6	1.4	12.8	2.4	<0.6	45.3	<0.6	10.7	234.9	9.3	52.6	5.5	549	43.5	8.1	18.7	45.8
treated 62b	0.7	0.71	1.3	12.7	3.2	<0.6	40.8	<0.6	14.5	145.6	7.1	59.3	6.9	383	25.5	8.8	28.8	46.8
average			1.0	10.7	2.2	0.8	36.3		10.3	147	6.2	54.2	6.4	338	36.7	7.4	34.8	50.2
RSD (%)			36	31	32	11	20		31	44	37	17	12	42	46	27	61	14

[a]CVM weighed five 0.5 g pieces from livers from two treated birds and then sent the samples to FCC in randomized and blinded numbered tubes. FCC sent the results back to CVM, who unblinded, sorted, and calculated averages and RSDs. Concentration units are micrograms of As per kilogram of wet weight liver. The lowest calibrant with this set was equivalent to 0.6 μg kg^{-1} in liver. Unknown species are identified by their approximate RT in minutes.

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conduct a peer validation of the speciation method. CVM provided a method standard operating procedure, sufficient control liver for fortification studies, and five preweighed aliquots of two different incurred livers in 10 randomized and blinded tubes. FCC established system suitability and conducted method familiarization. They then performed the validation analyses in two sets, with approximately equal numbers of control, fortified, and unknown randomized incurred samples in each set. Their only modification to the method that was provided by CVM was to adjust the mobile phase gradient step slightly to optimize for their instrument and to increase the ultrafilter centrifugation time to 2 h. The increased centrifugation time resulted in higher recoveries and better precision for Rox, 3-amino, and *N*-acetyl in fortified samples (Table 5), although precision results with incurred samples were not as good (Table 6) and were comparable to those achieved by CVM/CFSAN (Table 4).

We believe that this method is suitable for identifying whether carcinogenic inorganic arsenic is present in liver from birds fed Rox-medicated feed. The method has limitations in quantifying other arsenic species. It does not provide quantitative recovery of all arsenic in liver, and it has poor precision for the phenylarsonic acid compounds. Several significant metabolites remain unidentified. However, the method is rugged enough to be used by other laboratories to determine inorganic arsenic in chicken liver.

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Notes

The authors declare no competing financial interest.

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