Supporting Information

Arsenic metabolites, including N-acetyl-4-hydroxy-m-arsanilic acid, in chicken litter from a Roxarsone-feeding study involving 1600 chickens

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Table S1. Gradient elution conditions for HPLC separation[a]

Time (min)	A	B	flow rate (mL/min)
0.00	0	100%	2.4
3.00	100%	0	2.4
11.00	100%	0	2.4
14.00	0	100%	2.4
15.00	0	100%	2.4

[a] The program started with a linear gradient from 100% mobile phase B to 100% mobile phase A during the first 3 min. The composition remained at 100% A from 3 min to 11 min. From 11 min to 14 min, the mobile phase returned from 100% A to 100% B in a linear gradient, and remained at 100% B for another minute (14-15 min). The flow rate was kept at 2.4 mL/min for the entire 15 min.

Table S2. Chemical structures and pK_a values of arsenic species studied

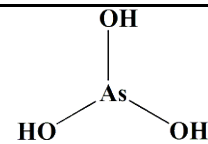
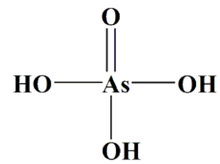
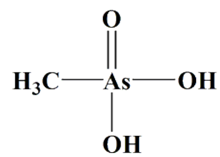
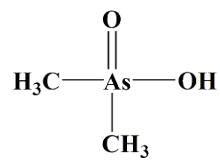
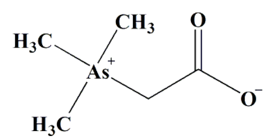
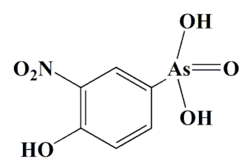
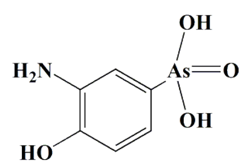
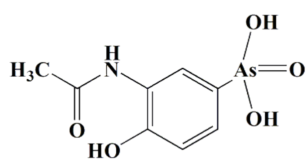
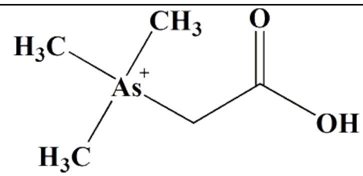
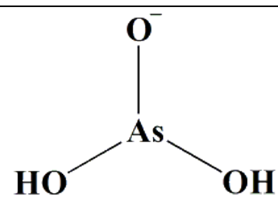
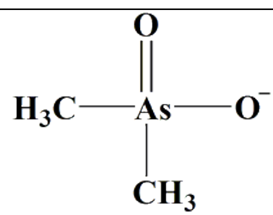
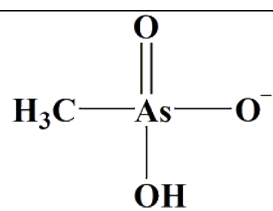
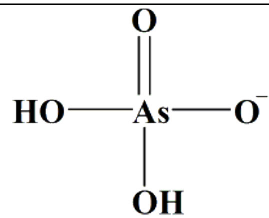
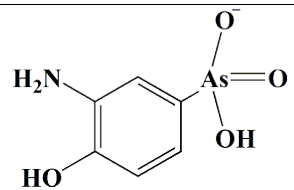
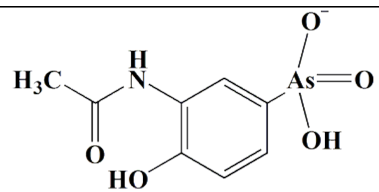
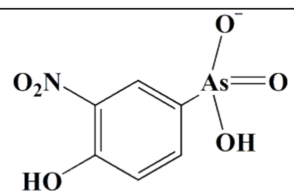
Name	**abbreviation**	**chemical structure**	**pK_a values**
arsenite	As^{III}		9.2, 12.1, 13.4
arsenate	As^{V}		2.3, 6.8, 11.6
monomethylarsonic acid	MMA^{V}		3.6, 8.2
dimethylarsinic acid	DMA^{V}		6.2
arsenobetaine	AsB		2.2
3-nitro-4-hydroxy-phenylarsonic acid	ROX		3.5, 5.7, 9.1
3-amino-4-hydroxy-phenylarsonic acid	3-AHPAA		-
N-acetyl-4-hydroxy-m-arsanilic acid	N-AHAA		-

Table S3. Selected operating parameters of the 5500 QTRAP ESIMS

Parameter	**Value in positive ionization mode**	**Value in negative ionization mode**
Curtain Gas (CUR)	30 psi	30 psi
Collision Gas (CAD)	High	High
Ionspray Voltage (IS)	4500 V	-4500 V
Temperature (TEM)	600 ℃	600 ℃
Ion Source Gas 1 (GS1)	50 psi	50 psi
Ion Source Gas 2 (GS2)	50 psi	50 psi
Entrance Potential (EP)	10 V	-10 V
Dwell Time for Each Transition	150 ms	150 ms

Table S4. MRM parameters for arsenic speciation using HPLC-ESIMS

Arsenic Species	Polarity	Molecular ion structure	Molecular ion (m/z)	Characteristic fragments	Fragment structure	DP (V)	CE (V)	CXP (V)
AsB	Pos		179	105	$(CH_3)_2As^+$	71	37	9
				120	$(CH_3)_3As^+$	71	28	11
As^{III}	Neg		125	107	AsO_2^-	-10	-18	-15
DMA^V	Neg		137	107	AsO_2^-	-70	-30	-11
				122	$CH_3AsO_2^-$	-70	-18	-13
MMA^V	Neg		139	107	AsO_2^-	-40	-40	-43
				124	AsO_3H^-	-40	-24	-7

Arsenic Species	Polarity	Molecular ion structure	Molecular ion (m/z)	Characteristic fragments	Fragment structure	DP (V)	CE (V)	CXP (V)
As^V	Neg		141	107	AsO_2^-	-15	-58	-13
				123	AsO_3^-	-15	-20	-7
3-AHPAA	Neg		232	107	AsO_2^-	-20	-64	-11
				123	AsO_3^-	-20	-28	-25
N-AHAA	Neg		274	107	AsO_2^-	-45	-72	-13
				123	AsO_3^-	-45	-36	-11
ROX	Neg		262	107	AsO_2^-	-30	-94	-15
				123	AsO_3^-	-30	-38	-11

Note: DP: Declustering Potential; CE: Collision Energy; CXP: Cell Exit Potential.

Table S5. Summary of probability values for 3-way analysis of variance (ANOVA) of various species of arsenic in broiler chicken litter. Sources of variation included ROX treatment (control or Roxarsone-fed), strain (Ross 308 and Cobb 500), and chicken age (sample collection day).

	Arsenic species							
Source	3-AHPAA	As^{III}	AsB	As^{V}	DMA^{V}	MMA^{V}	N-AHAA	ROX
	Probability							
Treatment (T)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Strain (S)	0.0014	0.0196	0.6235	0.9260	0.5047	0.0320	0.2130	<0.0001
Age (A)	<0.0001	0.0521	<0.0001	0.0259	<0.0001	0.0006	<0.0001	<0.0001
T x S	0.0019	0.0126	0.0051	0.5871	0.9359	0.0007	0.0481	<0.0001
T x A	<0.0001	0.1384	<0.0001	0.0756	0.1495	0.0082	<0.0001	<0.0001
S x A	0.2923	0.4616	0.5867	0.3319	0.5905	0.0095	0.0900	<0.0001
T x S x A	0.2961	0.4856	0.6926	0.2273	0.3374	0.0504	0.0292	<0.0001

Identification and quantitation of arsenic species in chicken litter

Chemical structures of all arsenic species involved in this study are listed in Supporting Information Table S2. ROX and six other arsenic standards (As^{III}, As^{V}, MMA^{V}, DMA^{V}, 3-AHPAA, and AsB) were selected for initial chromatography, due to their potential as biotransformation products of ROX. AsB is a non-toxic arsenic species that commonly exists in fish and seafood which are significant sources of dietary arsenic intake for farmed poultry. Baseline resolution of these seven arsenic species in a standard mixture was achieved on the PRP-X110S anion exchange column within 10 min (Figure S1a). The elution order was as follows: AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, and ROX. Figure S1b shows that the method was also able to separate all arsenic-containing compounds in the litter extract. The retention time match suggests the presence of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, ROX, and an unknown species of interest in the poultry litter.

To further demonstrate the true identities of these seven arsenic species, we first spiked the litter extract with each of the arsenic standards and analyzed the spiked samples using HPLC-ICPMS. A typical chromatogram of the 3-AHPAA spiking experiment is presented in Figure S2a. The resulting peak of suspected 3-AHPAA in the spiked litter extract was symmetrical, with height increasing as expected. Similar results were obtained for AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, and ROX spikings (data not shown). As illustrated in Figure S1 ans S2a, the peak of interest eluted between 3-AHPAA and ROX. Plausible arsenic standards with structures related to 3-AHPAA and ROX were therefore spiked into the litter extract, and we found that this peak had a retention time match with N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA) (Figure S2b).

The identities of all detected arsenicals including the putative N-AHAA in the extract of litter samples were further confirmed by HPLC-ESIMS. After optimizing operating parameters (Table S3, Supporting Information) and MRM transition conditions (Table S4, Supporting Information) of individual arsenicals, we performed HPLC-ESIMS analyses on the extracts of litter samples. As shown in Figure 1a of the main manuscript, all arsenic-containing compounds except As^{III} were detected in MRM mode, and their retention times agreed well with those obtained from HPLC-ICPMS (Figure ab). Thus, the presence of AsB, DMA^V, MMA^V, As^V, 3-AHPAA, and ROX as well as N-AHAA in poultry litter was verified. The undetectability of As^{III} in litter extracts by the QTRAP mass spectrometer could be due to its inherently low concentration and poor ionization efficiency. Typical MRM chromatograms of N-AHAA in litter extracts are given in Figure S3 (Supporting Information). MS/MS fragmentations of the suspected arsenic species illustrate two MRM transitions, *m/z* 274 to 123 and *m/z* 274 to 107, which are characteristic of N-AHAA (Table S4, Supporting Information).

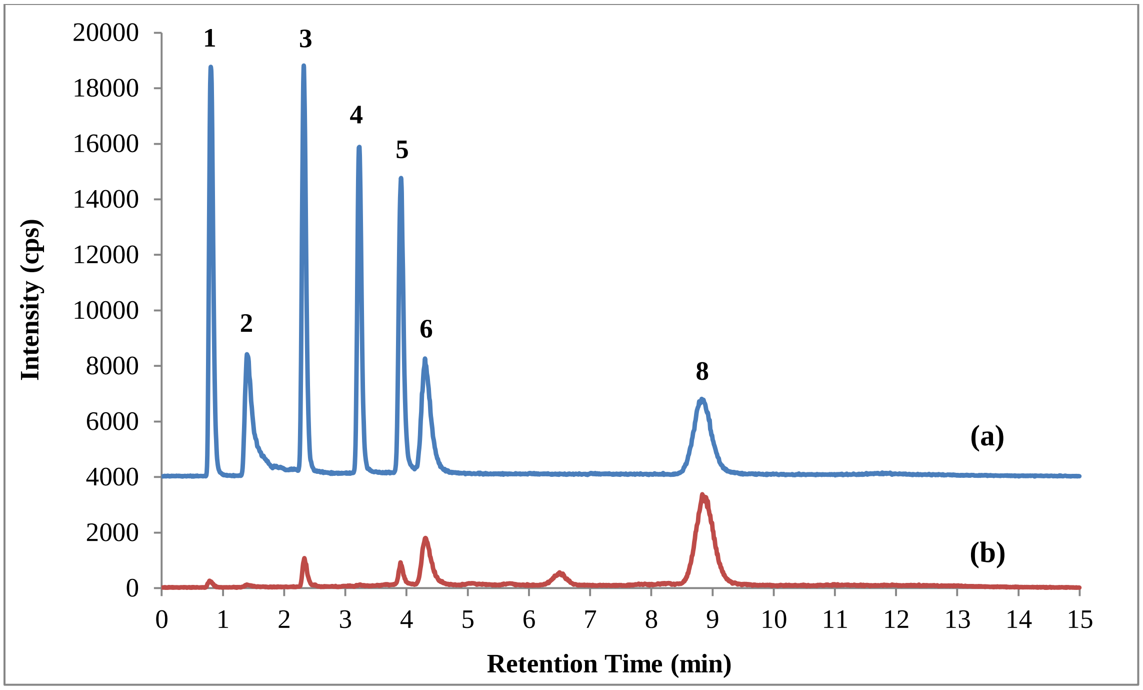


Figure S1. Chromatograms from HPLC-ICPMS analyses of (a) a standard mixture of seven arsenic species at 10 µg As/L each and (b) a litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (8) ROX.

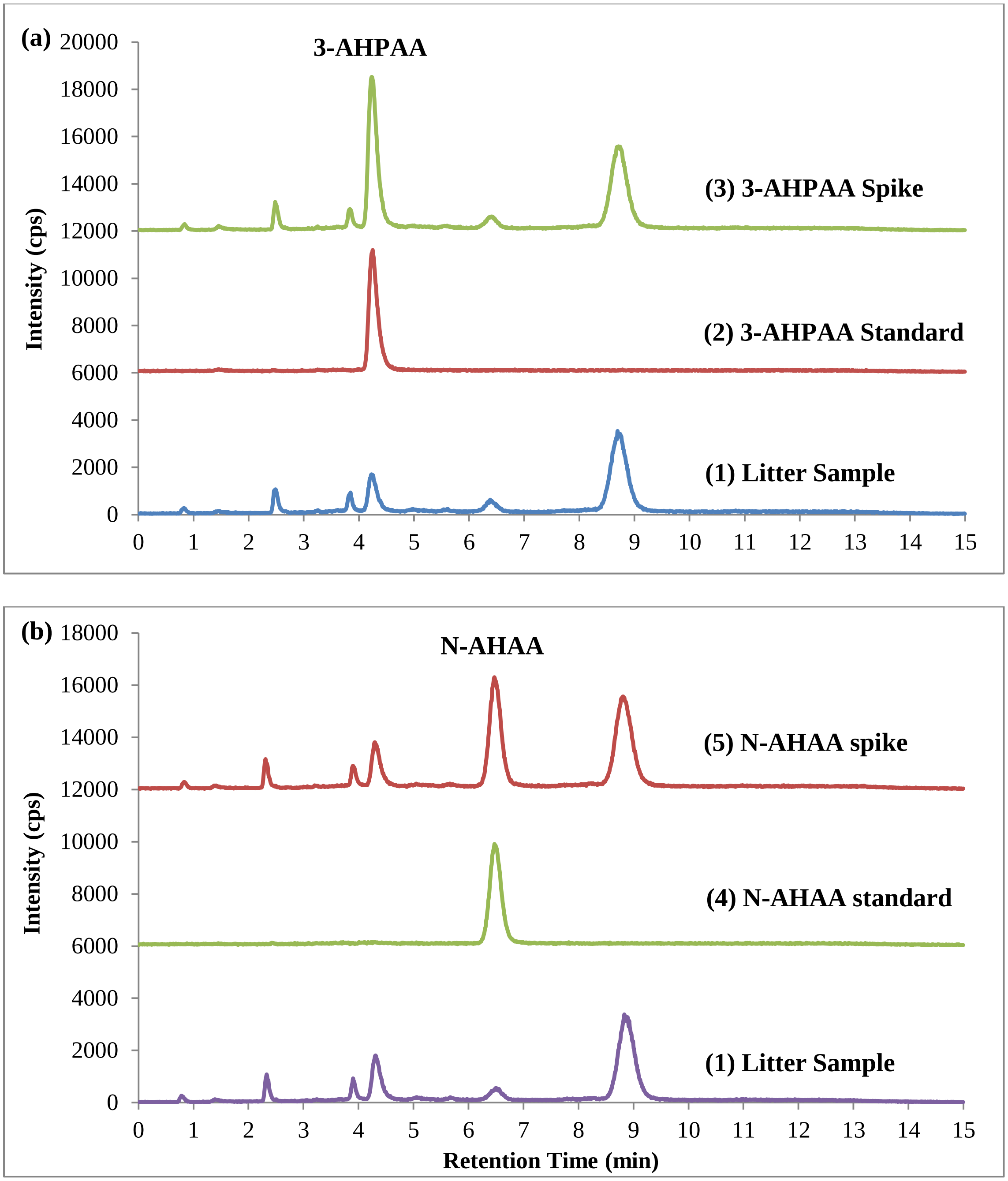


Figure S2. Chromatograms obtained from HPLC-ICPMS analyses of (1) a litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308; (2) 3-AHPAA standard; (3) the litter sample spiked with the 3-AHPAA standard; (4) N-AHAA standard; and (5) the litter sample spiked with the N-AHAA standard. (a) Analyses of the litter sample and the litter sample after spiking with 3-AHPAA. (b) Analyses of the litter sample and the litter sample after spiking with N-AHAA.

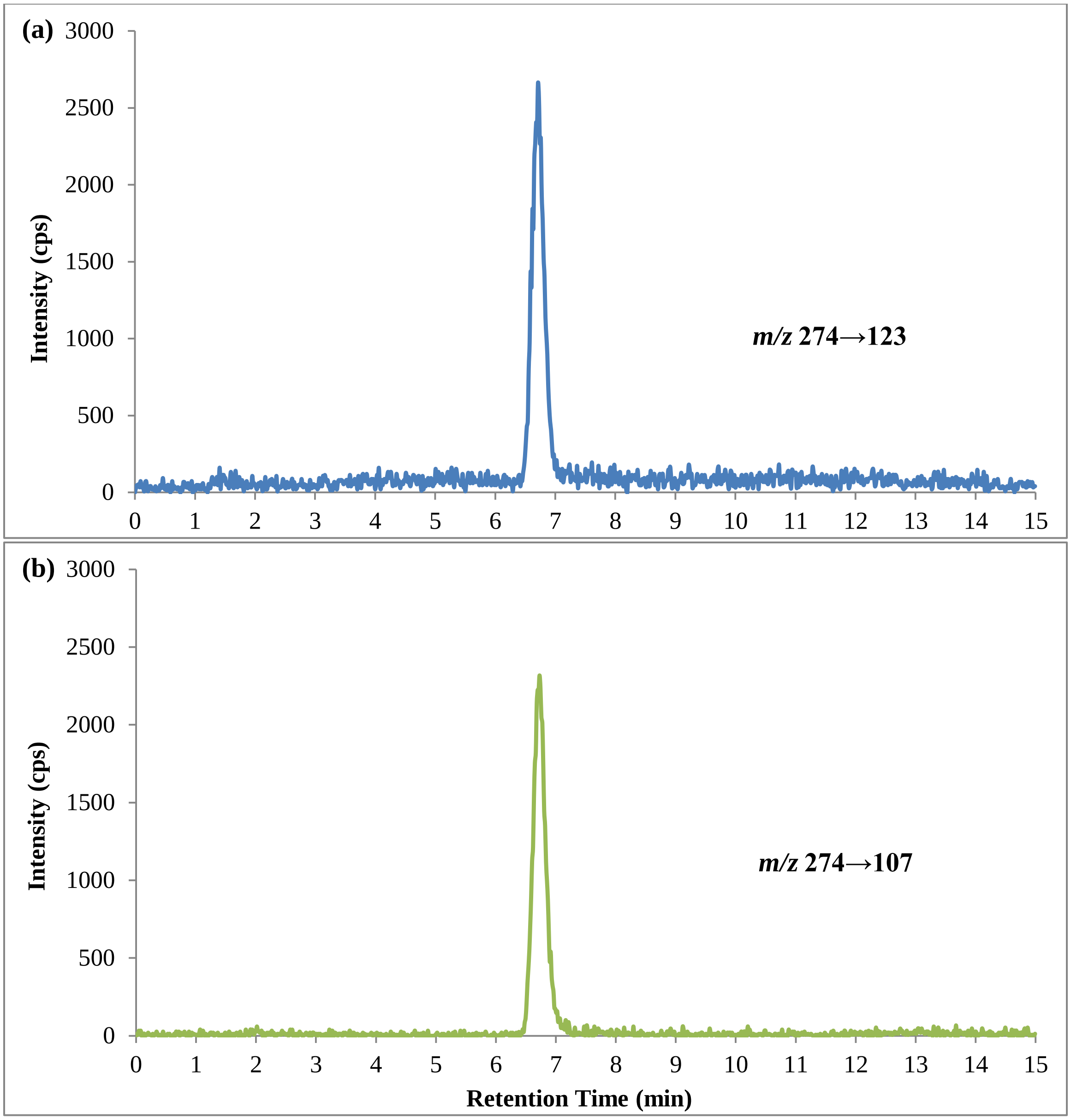


Figure S3. Chromatograms obtained from MRM (HPLC-ESIMS) analyses of a litter sample collected on day 28 from ROX-fed chickens (pen #24, Cobb 500) based on MRM transitions of 274/123 and 274/107, characteristic of N-AHAA. (a) MRM transition 274/123 and (b) MRM transition 274/107.

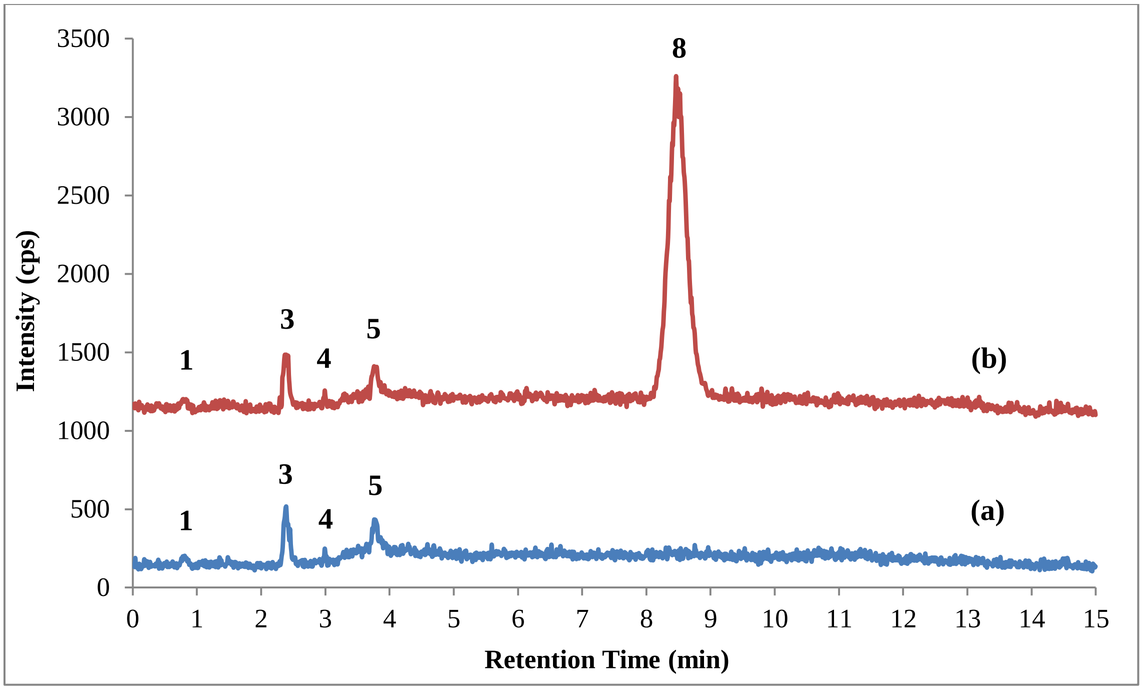


Figure S4. HPLC-ICPMS chromatograms from the analyses of (a) a litter sample collected on day 14 from control chickens (pen #17, Ross 308) and (b) the same litter sample spiked with ROX standard and stored at -20 ^{o}C for 2 months. Peaks: (1) AsB, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (8) ROX.

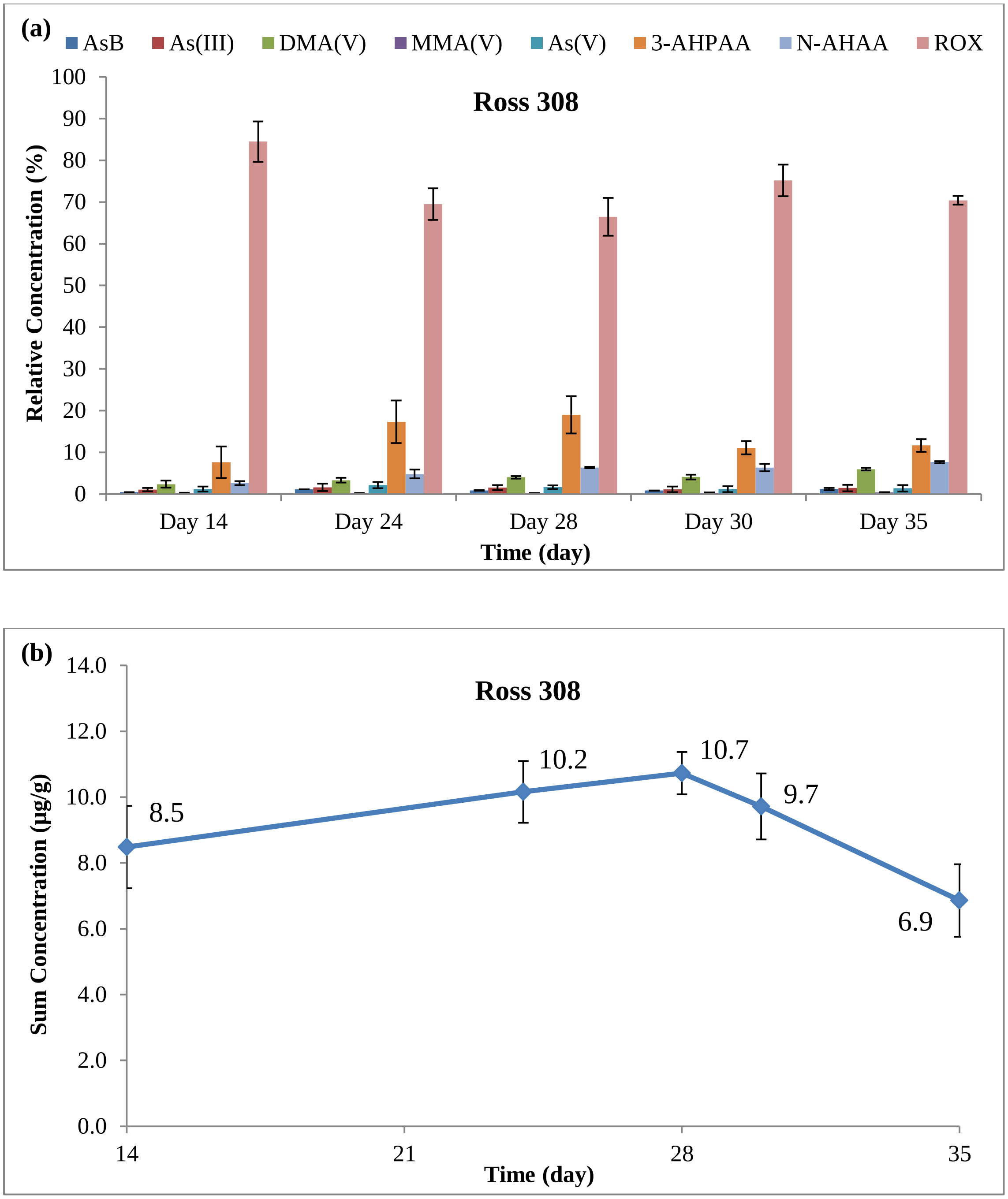


Figure S5. (a) Relative concentrations of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX in the litter samples of ROX-fed chickens from strain Ross 308. (b) The sum of arsenic in the litter samples collected on different days from ROX-fed chickens (strain Ross 308). The maximum sum of arsenic appeared on day 28 just prior to ROX being withdrawn from the feed.