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Occurrence of Arsenic Impurities in Organoarsenics and Animal Feeds

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ABSTRACT: Organoarsenics are widely used as excellent feed additives in animal production in the world. Roxarsone (ROX) and arsanilic acid (ASA) are two organoarsenics permitted to be used in China. We collected 146 animal feed samples to investigate the appearance of ROX, ASA, and potential metabolites, including 3-amino-4-hydroxyphenylarsonic acid (3-A-HPA), 4-hydroxyphenylarsonic acid (4-HPA), As^V, As^{III}, monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) in feeds. The stability of ROX in both ROX additives and animal feeds was also examined. The results show that 25.4% of the 146 animal feeds contained organoarsenics, with average contents of ROX and ASA as 7.0 and 21.2 mg of As/kg, respectively. Unexpectedly, As^{III} and MMA frequently occurred as As impurities in feeds bearing organoarsenics, with higher contents than organoarsenics in some samples. 3-A-HPA, 4-HPA, and DMA were not detected in all samples. ROX and As impurities in both ROX additives and feeds stayed unchanged in the shelf life. It suggests that As impurities in animal feeds bearing organoarsenics should generate from the use of organoarsenics containing As impurities. This constitutes the first report of As impurities in organoarsenics.

KEYWORDS: *Animal feed, roxarsone, arsanilic acid, arsenic species, organoarsenics*

INTRODUCTION

Organoarsenics, such as roxarsone (3-nitro-4-hydroxylphenylarsenonic acid, ROX), arsenilic acid (4-aminobenzenearsonic acid, ASA), and other phenylarsonic acids, have been recognized as safe and excellent feed additives since the 1960s[1−5] and widely used to control disease, promote growth, and enhance feed efficiency in food animal production for decades.[6,7] However, the environmental and public health risks associated with organoarsenic use in animal feeds, such as food contamination by arsenicals in animal feeds, exposure to arsenic (As) residues in poultry products, As in animal waste, etc., have been reported by Silbergeld and Nachman.[7] Pfizer, Inc. (New York, NY), the world famous pharmaceutical company, announced they would voluntarily suspend the sale of ROX in June 2011 because the chicken fed with ROX was detected with trace inorganic As as issued by the Food and Drug Administration (FDA) of the U.S.A.[8] Although approximately 70% of the poultry production systems use ROX in the U.S.A.,[6] information is not available on actual or total amount of organoarsenics in animal production because feed formulation is considered business secret in the U.S.A. and other countries.[7] In China, ROX and ASA are two organoarsenics permitted to be used in animal production;[9] however, their actual use is also not clear because of the same reason.

Although ROX is low in toxicity to animals, it is finally degraded to more potent inorganic forms when the poultry litter is composted or applied to soil.[10,11] ROX and its metabolites, such as 3-amino-4-hydroxyphenylarsonic acid (3-A-HPA), 4-hydroxyphenylarsonic acid (4-HPA), arsenate (As^V), arsenite (As^{III}), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and some unknown As compounds, are commonly detected in animal manures.[12−16] However, all of the previous studies cannot demonstrate whether all of the As compounds, except ROX, in animal manures result from the degradation of ROX itself. Is it possible that some species or amount of As compounds in animal manures generate from the As compounds in animal feeds bearing organoarsenics directly?

In this work, we gathered 146 samples of chicken and pig complete feeds (for feeding directly with no need to add any other supplements) sold in Guangdong province, the major animal production region of China, to determine the As species, including ROX, ASA, 3-A-HPA, 4-HPA, As^V, As^{III}, DMA, and MMA. The stability of ROX in both commercial ROX additives and animal feeds was also investigated. Our purpose was to reveal the actual use of organoarsenics in animal feeds and try to draw more attention to the potential As health hazard associated with organoarsenics.

MATERIALS AND METHODS

Sample Collection and Preparation of Animal Feeds. A total of 146 samples of animal complete feeds, including 70 chicken and 76 pig feeds, were collected randomly at the feed stores in Guangdong province. All of the feeds were produced by 40 feed factories in Guangdong, Jiangxi, and Hunan provinces, in southern China, in 2010. Generally, one feed factory produces a series of pig and/or chicken feeds. While sampling, we collected all chicken and pig feeds produced by one factory and sold at one feed store. The feed samples were immediately lyophilized (ALPHA 1-4/LD-Plus, Christ) after delivery to the lab and then pulverized to a fine powder for total As and As speciation analysis.

Estimation of ROX Stability. Two commercial ROX additives, purchased in two medicine stores for animals in May and July 2010 in

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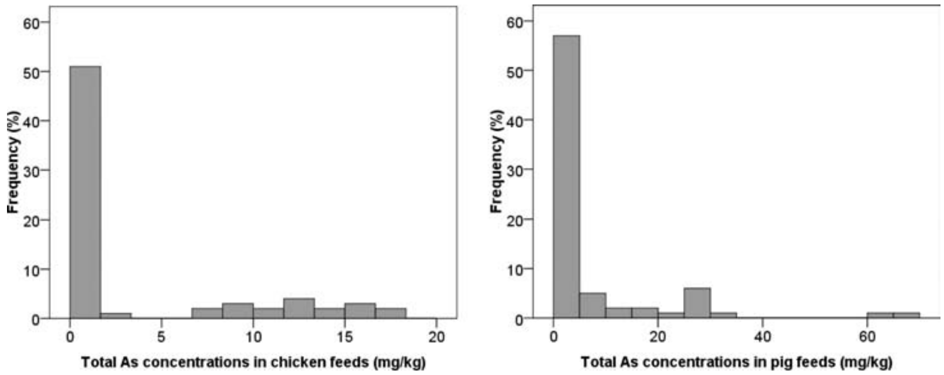

Figure 1. Frequency analysis of total As contents in chicken and pig feeds.

Guangzhou, Guangdong province, were placed into culture dishes, kept at room temperature (18.9−37.1 °C) in the lab, and sampled at 0, 5, 10, 15, 20, 25, 30, and 40 days for the earlier ROX additive and at 0, 5, 10, 15, 20, 25, and 45 days for the latter ROX additive. All of the ROX samples were prepared to determine the As forms.

Six 40 kg bags of chicken complete feeds amended with ROX additives were gathered at the manufacturing lines in two feed factories located in Huizhou, Guangdong province, in June and September 2010. Three bags of feed were collected in each factory. Both feeds were stored at room temperature (20.0−37.1 °C) after delivery to the lab. Because the quality assurance for animal feeds is commonly 1 month at room temperature in southern China, feed samples were collected at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 days during storage for the earlier feed and at 0, 5, 10, 15, 20, 25, 30, and 40 days for the latter feed. All of the samples were prepared for the As speciation analysis.

Chemical Analysis. Approximately 0.01 g of commercial ROX additive was dissolved with a certain amount of ultrapure water and poured into a 100 mL volumetric flask, and then ultrapure water was added to the fixed volume. The solution was diluted 100 times for As speciation analysis using a liquid chromatograph coupled with hydride generation atomic fluorescence spectrometry (LC−HG−AFS). The LC (20AT, Shimadzu, Japan) was equipped with a reversed C_{18} column (Phenomenex ODS3, 250 × 4.6 mm inner diameter, 5 μm). The mobile phase used in the chromatographic separation included mobile A, NaH_2PO_4 (10 mM/L) + tetrabutylammonium bromide (0.5 mM/L) + CH_3OH (3%, v/v), and mobile B, NaH_2PO_4 (50 mM/L) + tetrabutylammonium bromide (0.5 mM/L) + CH_3OH (5%, v/v). The scheme of gradient elution was 0−11 min for 100% mobile A, 11−20 min for 100% mobile B, and 20−30 min for 100% mobile A, at a flow rate of 1.0 mL/min. The determination of As speciation was performed by HG−AFS (8× dual channel system/SA-10 speciation analysis, Jitian, Beijing, China). The analyte was carried by 7.0% HCl, mixed with 2.0% $K_2S_2O_8$, reacted with 2% KBH_4, and then introduced into the atomizer by carrier gas as 400 mL/min argon.

The As species in feeds were extracted and determined using the similar methods in our previous study.[17] The detailed procedure was presented as follows: Approximately 0.5 g of feed was placed into a 50 mL Eppendorf vessel, and then 10 mL of mixture of 9:1 $NH_4H_2PO_4$/H_3PO_4 was added to the vessel. All of the mixture was digested at 55 °C for 10 h, then ultrasonated for 20 min, and centrifuged at 4000 rpm for 10 min, and the supernatant was collected. The residue was extracted twice using the same procedure. The three supernatants were mixed and filtered through a 0.22 μm membrane for LC−HG−AFS analysis.

Standards and Reagents. The reference materials of ROX (97.5%) and ASA (99%) were purchased from Dr. Ehrenstorfer Gmbh (Germany). 3-A-HPA (99%) was purchased from Sigma-Aldrich (St. Louis, MO). 4-HPA (98%) was purchased from TCI Tokyo Kasei (Japan). The standard stock solutions of As^V ($Na_2HAsO_4 \cdot 12H_2O$, 17.5 ± 0.4 mg/L), As^{III} (Na_3AsO_3, 75.7 ± 1.2 mg/L), MMA ($CH_4AsNaO_3 \cdot 1.5H_2O$, 25.1 ± 0.8 mg/L), and DMA ($C_2H_6AsNaO_2 \cdot 2H_2O$, 52.9 ± 1.8 mg/L) were bought from the Chinese National Standard Materials Center and stored in the dark at −4 °C. Prior to use, the four stock solutions were diluted to 100, 75.5, 50.2, and 105.8 μg/L, respectively. High-performance liquid chromatography (HPLC)-grade methanol (Burdick and Jackson, Morristown, NJ) and analytical-grade reagents were used in all experiments. Ultrapure water was prepared with Millipore Milli-Q Academic.

The recoveries of standard addition for ROX, ASA, 3-A-HPA, 4-HPA, As^V, As^{III}, MMA, and DMA in animal feeds were 88.5 ± 2.0, 84.9 ± 3.4, 54.4 ± 6.9, 92.2 ± 8.6, 91.5 ± 2.5, 93.4 ± 1.3, 85.0 ± 3.4, and 91.9 ± 2.4%, respectively. The detection limits for ROX, 3-A-HPA, 4-HPA, ASA, As^V, As^{III}, MMA, and DMA were 9.5, 12.1, 3.8, 8.4, 4.7, 1.8, 1.9, and 3.6 μg/L, respectively. The contents of As species in all samples were reported as elemental As concentrations without specific explanation. All data were the means of three replications and expressed as the mean ± standard deviation.

■ RESULTS

Total As in Animal Feeds. The total As contents in chicken feeds varied from 0.1 to 17.7 mg/kg and averaged 3.6 mg/kg. Pig feeds contained 0.1−67.8 mg of As/kg, with a mean of 6.5 mg of As/kg. The average As content in pig feeds was higher than that in chicken feeds, which might explain the fact that pig manures contained higher As than chicken manures in Guangdong province as documented in our previous study.[18] Moreover, the means of total As contents in both chicken and pig feeds in this work were considerably higher than those reported in England and Wales.[19] The frequency analysis of total As contents in both feeds showed that 72.9% of the chicken feeds and 67.1% of the pig feeds contained less than 2 mg of As/kg (Figure 1), which is the total As limit issued by the Chinese Hygienical Standard for Feeds (HSF) when no organoarsenics are used in animal feeds.[9] The use limits for organoarsenics are 50 mg of ROX/kg and 100 mg of ASA/kg (namely, 14.2 and 34.5 mg of As/kg, respectively) in animal feeds in China. It was assumed that even organoarsenics were added in the feeds, only 8.6% of the chicken feeds and 15.8% of the pig feeds had total As contents greater than 14.2 mg/kg, and 2.6% of the pig feeds had total As contents greater than 34.5 mg/kg.

Organoarsenics and As Impurities in Animal Feeds. The detection of organoarsenics in animal feeds was shown in Table 1. A total of 21 feed samples, accounting for 14.4% of all feed samples, contained ROX at a range of 0.7−17.7 mg/kg (equaling 2.4−62.1 mg of ROX/kg) and with an average of 7.0 mg/kg. For the 21 samples bearing ROX, only 1 sample contained ROX but without any other As species. A total of 71.4% of the 21 feed samples contained As^V, and a total of 95.2% of samples had As^{III}, suggesting that most of the samples included two inorganic As impurities simultaneously. Particularly, the contents of As^{III} in feeds bearing ROX varied from 0.2 to 16.3 mg/kg and averaged 4.9 mg/kg. Eight samples, 40% of the samples bearing ROX, contained As^{III} higher than ROX. The

Table 1. As Species in Animal Feeds ($n = 146$)

As compound detectable	sample number	detection rate (%)	content (mg/kg)	mean (mg/kg)
ROX	21	14.4	0.7−17.7	7.0
+−[a]	1	0.7 (5.0[b])	17.7	17.7
+$\mathrm{As^{V}}$	15	10.3 (71.4)	0.1−3.3	1.3
+$\mathrm{As^{III}}$	20	13.7 (95.2)	0.2−16.3	4.9
+DMA	0			
+MMA	0			
+3-A-HPA	0			
+4-HPA	0			
sum of As species			0.3−25.1	12.5
total As			0.4−24.6	12.5
ASA	16	11.0	0.4−64.3	21.2
+−[a]	3	2.1 (18.8)	7.9−8.2	8.1
+$\mathrm{As^{V}}$	2	1.4 (12.5)	0.7−2.0	1.3
+$\mathrm{As^{III}}$	11	7.5 (68.8)	0.2−5.1	1.9
+DMA	0			
+MMA	9	6.2 (56.3)	0.7−1.6	1.1
sum of As species			4.7−65.9	23.4
total As			4.3−67.8	23.8
$\mathrm{As^{V}}$, $\mathrm{As^{III}}$, and $\mathrm{As^{V}}$ + $\mathrm{As^{III}}$	68	46.6	0.0−6.7	1.0
sum of As species			0.1−7.3	0.8
total As			0.1−11.9	0.9
none[c]	41			

[a]− refers to no other As species, except ROX or ASA, being detectable in feed samples. [b]Numbers in parentheses are percentages of samples containing organoarsenics and As impurities, accounting for all of the samples bearing ROX or ASA. [c]None refers to no As compound being detectable in feed samples.

contents of $\mathrm{As^{V}}$ ranged from 0.1 to 3.3 mg/kg, with a mean of 1.3 mg/kg in samples bearing ROX. The sums of inorganic As compounds in 20 samples bearing ROX were in the range of 0.3−16.5 mg/kg, with an average of 5.9 mg/kg. 3-A-HPA, 4-HPA, DMA, and MMA, the potential metabolites of ROX, could not be detected in all samples bearing ROX. Considering that the primary ingredients of animal feeds, such as maize, wheat, soybean meal, and other supplements, might naturally contain low concentrations of As compounds (≤2 mg/kg as permitted by the Chinese HSF), we suggested that $\mathrm{As^{III}}$ was the most commonly occurring As impurity in feeds bearing ROX. The sums of all As species in samples bearing ROX were 0.3−25.2 mg/kg, with an average of 12.5 mg/kg.

ASA was detectable in 16 samples (Table 1), 11.0% of all animal feeds. ASA contents ranged from 0.4 to 64.3 mg/kg (equaling 1.2−186.4 mg of ASA/kg), with an average of 21.2 mg/kg. Among the 16 samples containing ASA, three samples could not be determined with any other As species. A total of 12.5% of the 16 samples contained $\mathrm{As^{V}}$ in the range of 0.7−2.0 mg/kg and with a mean of 1.3 mg/kg, and a total of 68.8% of samples had $\mathrm{As^{III}}$ at concentrations from 0.2 to 5.1 mg/kg and with an average of 1.9 mg/kg. There were four samples, 30.8% of the samples containing ASA, bearing $\mathrm{As^{III}}$ higher than ASA. Surprisingly, 56.3% of the 16 samples contained low levels of MMA (0.7−1.6 mg/kg). Additionally, DMA was not detectable in all samples bearing ASA. It indicated that the most commonly detectable As impurities in feed samples bearing ASA were $\mathrm{As^{III}}$ and MMA. The average content of the sum of all As species in samples containing ASA was 23.4 mg/kg, much higher than that (12.5 mg/kg) in samples bearing ROX, which should be related to the higher use limit of ASA than ROX in feeds permitted by the government.

Additionally, 68 feed samples, 46.6% of the 146 feed samples, were detected with one or two inorganic As species and without any organic As forms, demonstrating that MMA was the As impurity accompanying ASA in samples bearing ASA. Inorganic As species in these samples varied from 0.0 to 6.7 mg/kg and averaged 1.0 mg/kg. The inorganic As species in these feeds should be introduced by the raw materials of feeds, such as maize, wheat, soybean meal, and other supplements. In view of this, the very low total As contents in animal feeds reported in England and Wales[19] implies that organoarsenics might not be used in those feeds. In total, 41 samples, 28.1% of the 146 feed samples, could not be determined with any of the above As species.

Organoarsenics in Chicken and Pig Feeds. Organoarsenics could be found in 17 chicken feeds, accounting for 24.3% of all 70 chicken feeds, while 16 samples had ROX and only 1 sample contained ASA (Table 2). There were 20 pig feeds containing organoarsenics, with 25.0% of these samples determined with ROX and 75.0% containing ASA. Although ROX and ASA are allowed to be used in both chicken and pig feeds, the above results showed that ROX was primarily used in chicken diets and ASA was mainly in pig feeds, which might be ascribed to the fact that ROX functions better than ASA in growth promotion and coccidiosis control in chicken.[20] Animal manure is commonly applied as a fertilizer, which will lead to environmental and public health risks of As contamination via the way of organoarsenics → animal feed → animal manure → soil → crop. It has been recently reported that the primary As compounds in vegetables fertilized with chicken manures bearing ROX and its metabolites are DMA and $\mathrm{As^{III}}$,[15,21] indicating that the potential risk associated with the use of organoarsenics in animal production is considerably enhanced in the food chain.

Stability of ROX in Commercial ROX Additives. The high $\mathrm{As^{III}}$ impurity in both chicken and pig feeds bearing organoarsenics intrigued us about their origins. Therefore, two commercial ROX additives were purchased to examine the stability of As compounds at room temperature. ROX contents in the first ROX additive varied from 94.8 ± 0.2 to 95.1 ± 0.3% during 40 days of storage (Figure 2). Surprisingly, the second ROX additive contained not only ROX but also a similar content of $\mathrm{As^{III}}$. ROX contents remained in the range from 20.2 ± 0.3 to 21.7 ± 0.3%, and $\mathrm{As^{III}}$ stayed at concentrations from 20.9 ± 0.3 to 21.9 ± 0.3% during 45 days of storage. It demonstrated that the

Table 2. Occurrence of Organoarsenics in Chicken and Pig Feeds

		organoarsenic-detectable sample		ROX-detectable sample		ASA-detectable sample	
feed type	sample number	number	percentage	number	percentage	number	percentage
chicken feed	70	17	24.3	16	22.9 (94.1[a])	1	1.4 (5.9)
pig feed	76	20	26.3	5	6.6 (25.0)	15	19.7 (75.0)

[a]Numbers in parentheses refer to the percentages of samples containing ROX or ASA, accounting for the total samples bearing organoarsenics.

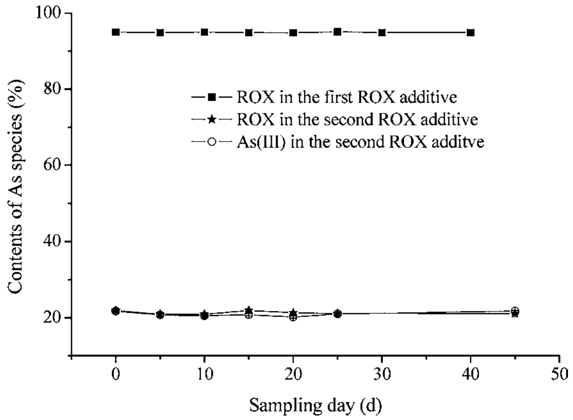

Figure 2. Changing trend of As species in two commercial ROX additives stored at room temperature (18.9−37.1 °C). The first ROX additive contained ROX, and the second ROX additive contained ROX and As^{III}. The contents of ROX and As^{III} were presented as ROX and As, respectively. Bars are standard errors of means (n = 3).

commercial ROX additives might contain high As^{III} impurity themselves; moreover, both ROX and As^{III} impurity in the additives could stay unchanged for at least 40 days at room temperature.

Stability of ROX in Animal Feeds. The changing trends of As compounds in two commercial chicken complete feeds were illustrated in Figure 3. As compounds in the first chicken feed were initially comprised of ROX (7.6 ± 0.1 mg/kg), As^{III} (8.7 ± 0.3 mg/kg), and low levels of As^{V} (1.0 ± 0.1 mg/kg) (Figure 3) and remained almost constant in the shelf life of the feed. ROX, the only As compound in the second chicken feed, stayed unchanged within 40 days. The above indicated that ROX and the As^{III} impurity in chicken feeds remained stable during the quality assurance period of chicken feeds.

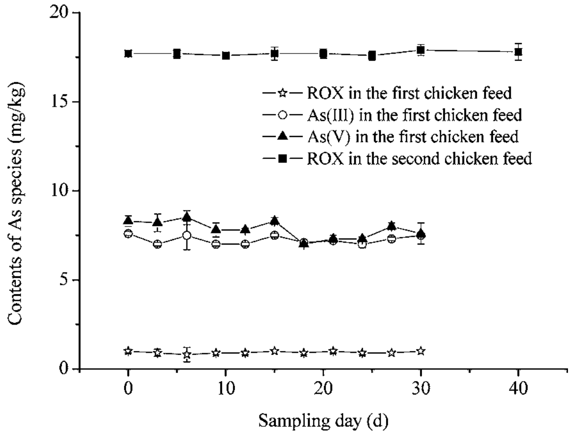

Figure 3. Changing trend of As species in two chicken complete feeds stored at room temperature (20.0−37.1 °C). The first feed contained ROX and As impurities, including As^{V} and As^{III}, and the second feed contained ROX but without As impurities. The contents of all As species were presented as elemental As. Bars are standard errors of means (n = 3).

■ DISCUSSION

Our investigation of As species in 146 animal feeds showed that some feed samples had very low contents of organoarsenics, which should be contaminated by the residual organoarsenics in the production line of feeds. A total of 25.4% of all of the feed samples contained ROX or ASA, and quite a few of them were determined with high levels of As^{III} and low contents of MMA. The ROX additive is added and physically mixed evenly with the feeds before packaging in the feed production line. ROX and As^{III} impurity in commercial ROX additives could stay unchanged at room temperature for at least 30 days (Figure 2), suggesting that abiotic degradation of ROX did not occur in normal conditions. It is documented that any reductive degradation of ROX must first involve the reduction of the nitro group by microbes[16] and the biotransformation of ROX is only gained through the introduction of poultry litter containing microorganisms capable of ROX biotransformation.[22] However, 3-A-HPA and 4-HPA, the potential metabolites of ROX,[16,23] could not be detected in all feed samples bearing ROX. Moreover, ROX and As^{III} impurity in chicken feeds stayed unchanged at room temperature during the shelf life (Figure 3), indicating that biodegradation of ROX did not occur during the production and storage of feeds as well. Consequently, we suggested that high levels of As^{III} impurity in animal feeds bearing ROX should result from the use of ROX additives bearing high As^{III} impurity.

At present, the major ROX production techniques used in China include (1) reaction of anilinie and arsenic trioxide,[24] (2) reaction of *para*-hydroxyanilinie and sodium arsenite,[25] and (3) condensation of 3-nitro-4-hydroxy-anilinie and arsenic trioxide.[26] ASA is generated from (1) synthesis of anilinie and arsenic acid,[26,27] (2) complexation of anilinie and arsenic trioxide,[28] and (3) synthesis of *para*-nitroaniline and sodium arsenite.[29] It is reported that even the patented ASA production method[27] is subjected to low productivity and substantive byproducts of As forms.[29] The high levels of As^{III} impurity in organoarsenics maybe result from the excessive As^{III} during organoarsenics production, and low concentrations of MMA in ASA additives might be the byproduct of ASA complexation. However, the detailed production schemes for organoarsenic additives are commercial secrets, and the actual sources of As impurities in organoarsenics are worth further elucidation.

Traditionally, inorganic As compounds are regarded as more toxic to organisms than organic forms.[30] However, more and more medical studies show that the trivalent methylarsenicals and in some cases the pentavalent compounds are more potent than the inorganic forms for animals and human beings.[31−34] The traditional concept that inorganic forms are more toxic has been strongly questioned.[33] Therefore, the As impurities in organoarsenics should be of great concern.

As impurities, including As^{III} and MMA in organoarsenics, might counteract the effect of organoarsenics on animal growth and disease control and even impair the animal health directly. More importantly, the As impurities in commercial organoarsenic additives and animal feeds significantly enhance the potential As health hazard to workers involved in the production and retail of organoarsenic additives and animal feeds. Consequently, the safety and rationality of worldwide use of organoarsenics in animal production should be questionable.

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Notes

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■ ABBREVIATIONS USED

ROX, roxarsone; ASA, arsanilic acid; 3-A-HPA, 3-amino-4-hydroxyphenylarsonic acid; 4-HPA, 4-hydroxyphenylarsonic acid; MMA, monomethylarsonic acid; DMA, dimethylarsinic acid

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper published January 7, 2013 with an error in the legend of Figure 2. The correct version published January 16, 2013.