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High-Performance Liquid Chromatography−Inductively Coupled Plasma Mass Spectrometry Based Method for the Determination of Organic Arsenic Feed Additives and Speciation of Anionic Arsenics in Animal Feed

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A novel method has been developed to detect two organic arsenic animal feed additives including roxarsone and *p*-arsanilic acid, as well as other arsenic species such as arsenite, dimethylarsinic acid, monomethylarsonic acid, arsenate, and 4-hydroxyphenylarsonic acid, by using high-performance liquid chromatography coupled to an inductively coupled plasma mass spectrometer (HPLC-ICP-MS). The influence of the type and concentrations of ion-pairing reagents on the separation efficiency of the different arsenic compounds was examined. The effects of the mobile phase pH on the retention of arsenic species on the chromatography column were studied. When a gradient elution procedure was used, the best separation of the seven arsenic species could be achieved in <20 min with a mobile phase consisting of 8% methanol and 92% aqueous tetrabutylammonium hydroxide (4 mM, pH 6.25) followed by 92% trifluoroacetic acid aqueous solution (0.1%, pH 2.0). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used as an assistant tool to screen arsenobetain (AsB) in the feed samples by monitoring the reaction at *m*/*z* 179→120. The extractions of arsenic compounds from formula feed samples were studied, and results showed that the extraction with methanol/water (1:1) mixture yielded the most efficient percent compound recovery and the fastest extraction time for all arsenic species. Under optimum conditions, the limits of detection were <1.7 μg of As kg^{-1}, and the recoveries of all seven arsenic species were >78.5% with the relative standard deviation of <10%. The ion-pair reversed phase HPLC-ICP-MS method was then successfully applied to the speciation of arsenic in feedstuff and formula feed samples.

KEYWORDS: Liquid chromatography; roxarsone; *p*-arsanilic acid; arsenic species; inductively coupled plasma mass spectrometry; formula feed; feed stuff

INTRODUCTION

Many countries such as China and the United States add phenylarsonic compounds, including *p*-arsanilic acid (p-ASA) and roxarsone (ROX), to swine and chicken feed as additives to control cecal coccidiosis, increase weight gain, and improve feed conversion and pigmentation (*1*). Variation in the substituent of the aromatic ring (see **Figure 1**) results in differences in the growth-promoting and disease-controlling effects of the compounds. For example, ROX, which contains hydroxyl and a nitro groups, is approved as an additive for poultry, whereas p-ASA, which contains an amino group, is used as an additive for swine (*2*). The general doses of ROX and p-ASA used in breeding poultry and swine are from 50 to 100 g t^{-1} (*3*).

Formula feed is composed of a very complex mixture of corn, mineral elements, and various feed additives. During processing and storage of the feed, the concentrations of p-ASA, ROX, and other arsenic species may change. Therefore, in-depth investigations of which arsenic species are present and at what concentrations can help industries make more accurate assessments of the environmental impact, breeding impact, and health risks (*4*−*6*); the use of such organic arsenic feed additives is of great importance.

To improve our knowledge of ROX, p-ASA, and other arsenic species in formula feed, suitable and accurate analytical methods are needed to determine the species and concentrations of phenylarsonic compounds and other arsenic species. Several methods using chromatographic analytical techniques are available for the speciation of organic arsenic additives in water (*7*, *8*), vegetable samples (*9*), and biological samples (*10*). For example, the determination of p-ASA, 4-nitrophenylarsonic acid (4-NPAA), and *p*-ureidophenylarsonic acid (p-UPAA) commonly uses gas chromatography with a flame ionization detector (GC-FID) (*11*), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) (*12*). Unfortunately, these methods are not suitable for the simultaneous

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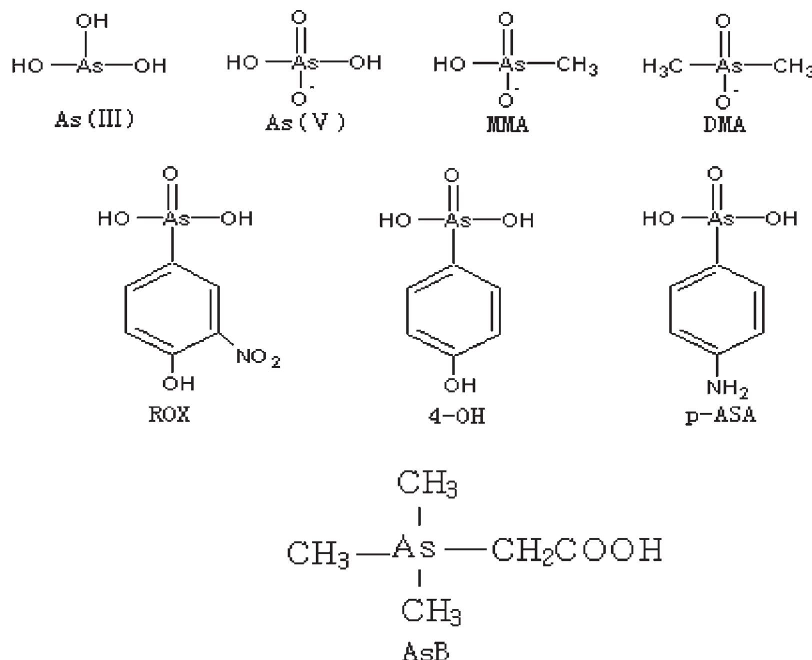

Figure 1. Structures of arsenic compounds examined in this study.

detection of potential metabolites such as arsenate (Aate) and arsenite (Aite). In addition, HPLC coupled to inductively coupled plasa mass spectrometry (ICP-MS) is considered to be an optimal method for element speciation and the determination of ROX as well as other arsenic additive compounds because HPLC-ICP-MS combines the resolving power of HPLC with the specificity and sensitivity of coupled online ICP-MS (*13*, *14*). Another method called narrow-bore HPLC coupled with online ICP-MS is a high-speed (approximately 2 min) separation method for arsenic speciation (*15*), but it is not suitable for detecting ROX. A number of MS methods have been applied to structurally characterize arsenic animal feed additives (*16*). However, none can detect all seven arsenic species including Aite, Aate, dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), p-ASA, ROX, and 4-hydroxyphenylarsonic acid (4-OH) in formula feed efficiently.

In addition to detecting different arsenic species, the extraction procedure is also a crucial step, because no interconversion of species should occur and good extraction efficiency should be achieved (*17*). Most current extraction methods in the literature focus on biological samples (*18*−*20*), paying little attention to animal feed (*3*, *21*).

In this study, we developed an efficient speciation method with ion-pair reversed phase HPLC-ICP-MS with gradient elution for detecting Aite, Aate, DMA, MMA, p-ASA, ROX, and 4-OH in formula feed and feedstuff. The choices of HPLC separation conditions were based on the selection and optimization of ion-pairing reagents, pH, and the compositions of mobile phases as well as flow rates of mobile phases. For ICP-MS detection, we also considered the effects of organic solvent and spectroscopic interferences.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Chromatographic standards of sodium arsenite (Aite), sodium arsenate (Aate), dimethylarsinic acid (DMA), disodium methylarsonate (MMA), arsenobetain (AsB), *p*-arsanilic acid (p-ASA), 4-hydroxyphenylarsonic acid (4-OH), and roxarsone (ROX) (J&K Chemical, Sweden) were prepared in 18.0 MΩ cm deionized water (Millipore, USA). The concentrations of standard storage solution for the seven arsenic species were as follows: Aite, 160 μg mL^{-1}; Aate, 150 μg mL^{-1}; MMA, 20 μg mL^{-1}; DMA, 150 μg mL^{-1}; AsB, 100 μg mL^{-1}; p-ASA, 120 μg mL^{-1}; 4-OH, 400 μg mL^{-1}; and ROX, 150 μg mL^{-1}. The standard storage solutions were stored at 4 °C in the refrigerator, whereas the mixed or single standards of arsenic species were prepared daily in deionized water after appropriate dilutions. These arsenic compounds are listed in **Figure 1**. The certified reference material wheat powder (GBW08503) was from the National Institute of Metrology, People's Republic of China (NIM).

For IP-RP-HPLC separations, tetrabutylammonium hydroxide (TBAH' Fluka, USA) and tetramethylammonium hydroxide (TMAH; Fluka, USA) were used as ion-pairing reagents. Malonic acid (Fisher Scientific, USA) was prepared to saturated aqueous solution and used to adjust the mobile phase pH. An extract of formula feed or feedstuff powder was injected directly onto the HPLC column. All reagents were of analytical reagent grade unless mentioned otherwise. Methanol was purchased from Fisher (USA) and trifluoroacetic acid (TFA, HPLC grade) from Merck-Euro lab (Leuven, Belgium).

Formula feed and formula feed containing p-ASA were purchased from Dabeinong Animal Feed Co. Ltd., Beijing, People's Republic of China. Feedstuff such as corn was obtained from China National Center of Feed Quality Control, Beijing, People's Republic of China.

Apparatus. An Agilent 7500a ICP-MS (Agilent Technologies, USA) were used for elemental detection throughout this study. The quadrupole mass analyzer was operated in the single-ion monitoring mode (m/z 75) for detecting arsenic. Interference from argon chloride (ArCl) was monitored at m/z 35 and 77. A polyfluoroalkoxy (PFA) concentric nebulizer was mounted directly on a water-cooled spray chamber. An HPLC stainless steel column 150 × 4.6 mm ×3.5 μm (Zorbax-SB C18, Agilent Technologies, USA) was connected to the concentric nebulizer via PEEK tubing (30 cm length × 0.13 mm i.d.). An HPLC system (HP1100, Agilent Technologies, USA) was used to separate arsenic species and deliver mobile phase into the ICP-MS at flow rates ranging from 0.7 to 1.0 mL min^{-1}. All chromatographic separations were carried out by gradient elution at room temperature. An autosampler was used to inject samples into the HPLC column. **Table 1** lists the operation parameters for the HPLC and ICP-MS instruments. A Thermo Electron TSQ high-performance liquid chromatograph coupled with a tandem mass spectrometer (Thermo Electron, USA) equipped with an electrospray ionization source (ESI) was used to detect AsB throughout this study. A spray voltage of 4000 V and a capillary temperature of 230 °C were applied to the MS, which operated in positive ion mode. AsB content was determined by selected-reaction monitoring mode set at m/z 179→120. The second MS used argon as collision gas. The Zorbax-SB C18 column was used as

Table 1. Instrument Operation Parameters

Total Arsenic	
ICP-MS	
RF power	1400 W
plasma gas flow rate	15.0 L min^{-1}
argon auxiliary gas flow rate	0 L min^{-1}
argon carrier gas flow rate	1.21 L min^{-1}
chamber temperature	2 °C
sample depth	7.70 mm
sample up rate	0.10 rps
integral time	1s, 3 times
nebulizer	Babington nebulizer

As Speciation					
HPLC					
mobile phase A	methanol				
mobile phase B	4 mM TBAH aqueous solution, pH 6.25				
mobile phase C	0.1% TFA aqueous solution, pH 2.10				
gradient program	time (min)	A (%)	B (%)	C (%)	flow rate (mL/min)
	0−10	8	92	0	0.7
	10−22	8	0	92	1.0
ICP-MS					
RF power	1400 W				
plasma gas flow rate	15.0 L min^{-1}				
argon auxiliary gas flow rate	1.0 L min^{-1}				
argon carrier gas flow rate	1.1 L min^{-1}				
chamber temperature	2 °C				
sample depth	7.20 mm				
nebulizer	PFA concentric nebulizer				

separation column with a mobile phase of methanol/water (1:1) mixture and 0.1% formic acid; the flow rate of mobile phase was 0.2 mL min^{-1}. The elution was isocratic.

Sample Preparation. Formula feed and feedstuff samples were extracted using modified versions of the Vivian (*22*) and Marijn (*23*) procedures. The samples were ground into powder using a grinding machine (ZM200, Retsch Corp., Germany), and about 0.500 g of each sample was weighed into a 10 mL centrifuge tube. To each tube was added 5 mL of a methanol/water mixture (v/v = 1:1). The tubes were sonicated for appropriate times at ambient temperature in an ultrasonic bath (Fungilab, Spain) and then centrifuged for 10 min at 12000 rpm by using the Superspeed centrifuge (Sorvall RC 5C, Thermo Scientific Corp., USA). The extracts were transferred to 25 mL round-bottom flasks. The remaining feed or feedstuff powder in each tube was re-extracted using 5 mL of a methanol/water mixture. The extracts were combined and evaporated to almost dryness by applying the Rotary Evaporator (Laborota4000, Heidolph Instruments Corp., Germany) and subsequently redissolved in 1 mL of deionized water. Finally, the extracts were filtered through a 0.45 μm disposable hydrophobic filter membrane prior to direct injection onto the HPLC-ICP-MS system.

Total Arsenic Determination. The total arsenic concentration in formula feed and feedstuff samples was determined by ICP-MS after mineralization in a muffled furnace for 3 h at 550 °C. The accuracy of the mineralization procedure was checked by analysis against a certified reference material, GBW08503 wheat powder, with a certified value of 0.22 ± 0.02 mg of As kg^{-1}.

RESULTS AND DISCUSSION

Optimization of Separation Condition. Le and Pergantis applied IP-RP-HPLC to separate arsenic species in a variety of matrices (*10*, *24*). They achieved optimum chromatography separations by adjusting mobile phase gradients and mobile phase parameters such as pH, flow rate of mobile phase, organic modifier content, and ion-pairing reagents.

In this study, TMAH was used as the ionic pair reagent at concentrations of 2, 4, 5, and 10 mM at pH 6.00. However, the separation of the mixed arsenic standard solution did not achieve satisfactory results. Aite and DMA eluted in the void volume, and other arsenic species could not be baseline separated on the ZobarxSB C18 column, even with increasing concentrations of TMAH. These chromatograms are not shown here.

Because TMAH failed to yield satisfactory results, TBAH was examined as the ion-pair reagent. Concentrations of TBAH on the separation of arsenic species were investigated, and 4 mM TBAH was selected for further study. Effects of pH on the retention behaviors and separation efficiency of six arsenic species (Aite, Aate, MMA, DMA, p-ASA, and 4-OH) on the ZobarxSB C18 chromatography column were studied carefully. In the pH study, malonic acid was used to adjust the mobile phase pH between 5.50 and 6.75. The resulting chromatograms (**Figure 2**) show that mobile phase pH affects peak resolution, peak shape, and analysis time. The retention times of Aite and 4-OH did not change significantly under mobile phase pH ranging from 5.50 to 6.75, but DMA and Aate retention behaviors changed significantly with increasing pH. One explanation could be that the forms of arsenic species are different under different pH values. The dissociation of Aite, Aate, MMA, DMA, p-ASA, and 4-OH depends on their pK_a value and mobile phase pH, because these compounds possess acidic functional groups. The pK_a of part arsenic compounds were as follows: pK_a[Aite] = 9.2, pK_a[Aate] = 2.20, 6.98, 11.5; pK_a[DMA] = 1.30, 6.30; pK_a[MMA] = 3.41, 8.18; pK_a[p-ASA] = 2, 4.02, 8.92; and pK_a[ROX] = 3.43, 6.38, 9.67. The trends we observed agreed with the findings of Pergantis (*16*).

Under the conditions mentioned above, ROX was not eluted from the ZobarxSB C18 column within 20 min due to the strong interaction between ROX and TBAH within the chromatography column. Therefore, a gradient elution procedure was used with a solution consisting of 4 mM TBAH, 0.1% TFA, and methanol to obtain satisfactory separation efficiency. The resulting chromatogram of arsenic species in **Figure 3** shows the most efficient separation for the seven arsenic species (Aite, DMA, MMA, p-ASA, Aate, 4-OH, and ROX). It is noted that the chromatography

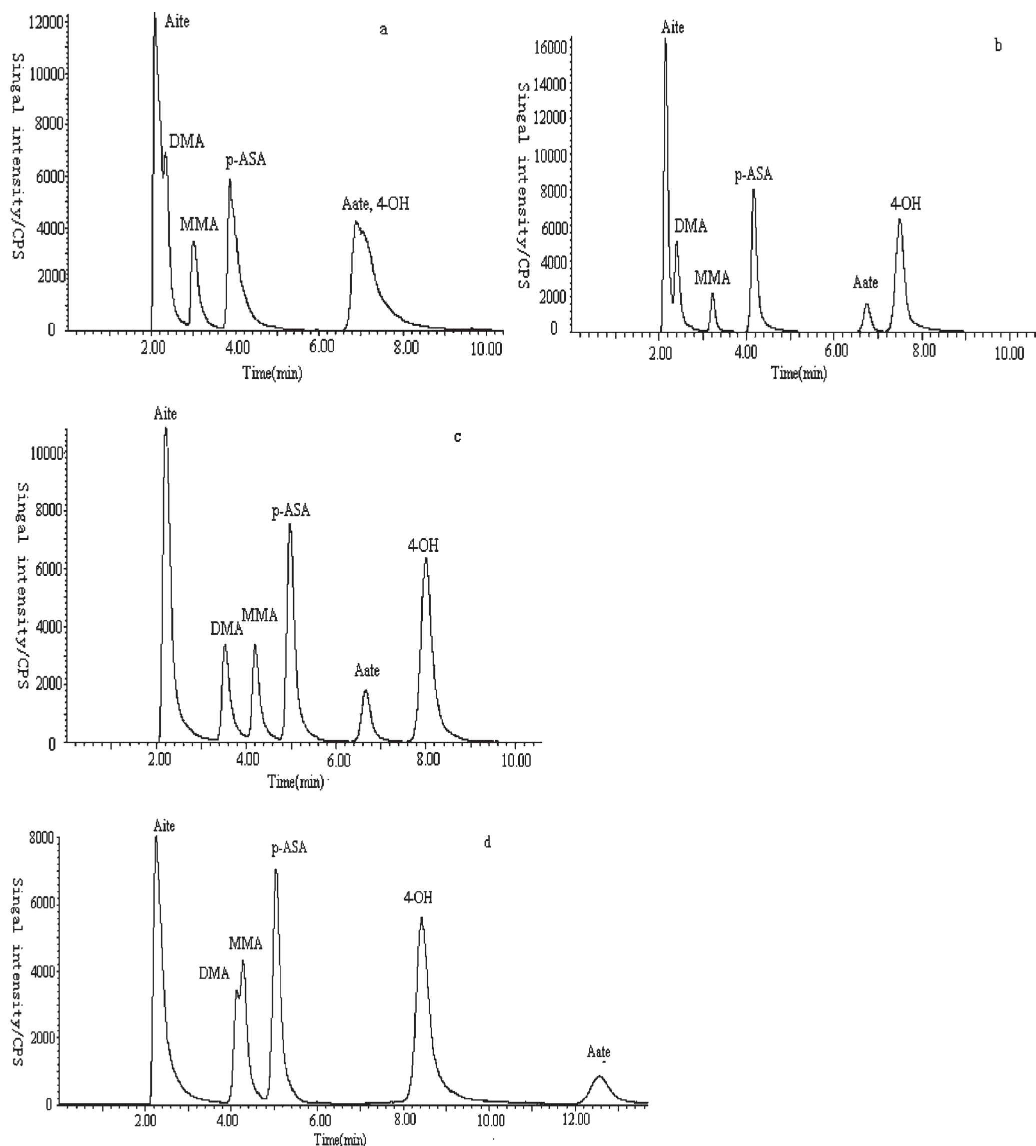

Figure 2. HPLC-ICP-MS chromatogram of six arsenic species obtained using 4 mM TBAH at pH (**a**) 5.50, (**b**) 6.00, (**c**) 6.25, or (**d**) 6.75. Peak assignments: Aite, 0.8 μg mL^{-1}; DMA, 0.3 μg mL^{-1}; MMA, 0.1 μg mL^{-1}; p-ASA, 0.6 μg mL^{-1}; Aate, 0.15 μg mL^{-1}; 4-OH, 0.8 μg mL^{-1}.

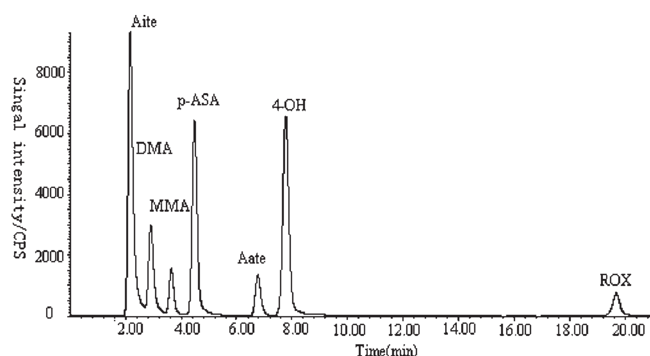

Figure 3. Chromatogram of seven arsenic species obtained using HPLC-ICP-MS: Aite, 0.8 μg mL^{-1}; DMA, 0.3 μg mL^{-1}; MMA, 0.1 μg mL^{-1}; p-ASA, 0.6 μg mL^{-1}; Aate, 0.15 μg mL^{-1}; 4-OH, 0.8 μg mL^{-1}; ROX, 0.15 μg mL^{-1}.

column should be equilibrated about 2 min after sample analysis by using a mobile phase of 4 mM TBAH aqueous solution and methanol (v/v = 92:8). The resolution for each pair of neighboring arsenic compounds was calculated by using the equation $R = 2(t_{R2} - t_{R1})/(W_1 + W_2)$, where t_{R1} and t_{R2} are the retention times for adjacent peaks and W_1 and W_2 correspond to their base peak widths. Under optimum separation conditions, we achieved a resolution of 1.0, which is considered to be acceptable for analytical purposes as it indicates a 98% separation of two neighboring peaks.

In this study, AsB was taken into account because it was identified essentially in marine biota and fish powder, which are used as excellent protein sources in the animal feed. AsB, a cationic arsenic species, could be coeluted with the Aite in the same volume under previous chromatographic separation conditions (see **Figures 3** and **4a**). Therefore, AsB might interfere with the determination of Aite in the feed; thus, LC-MS/MS was used as an assistant tool to screen for AsB in the feed samples by monitoring the reaction at m/z 179→120 (**Figure 4b**,**c**). Its chromatographic characteristics and full MS spectrum agreed with the previous results of Pergantis (*25*).

Optimization of Extraction Condition of Feed Samples. According to Vivian (*22*) and Marijn (*23*), methanol/water (v/v = 1:1) is one of the many common extraction solutions for speciation of arsenics in biological samples. In this study, a methanol/water (1:1) extraction solution was applied to extract the organic arsenic

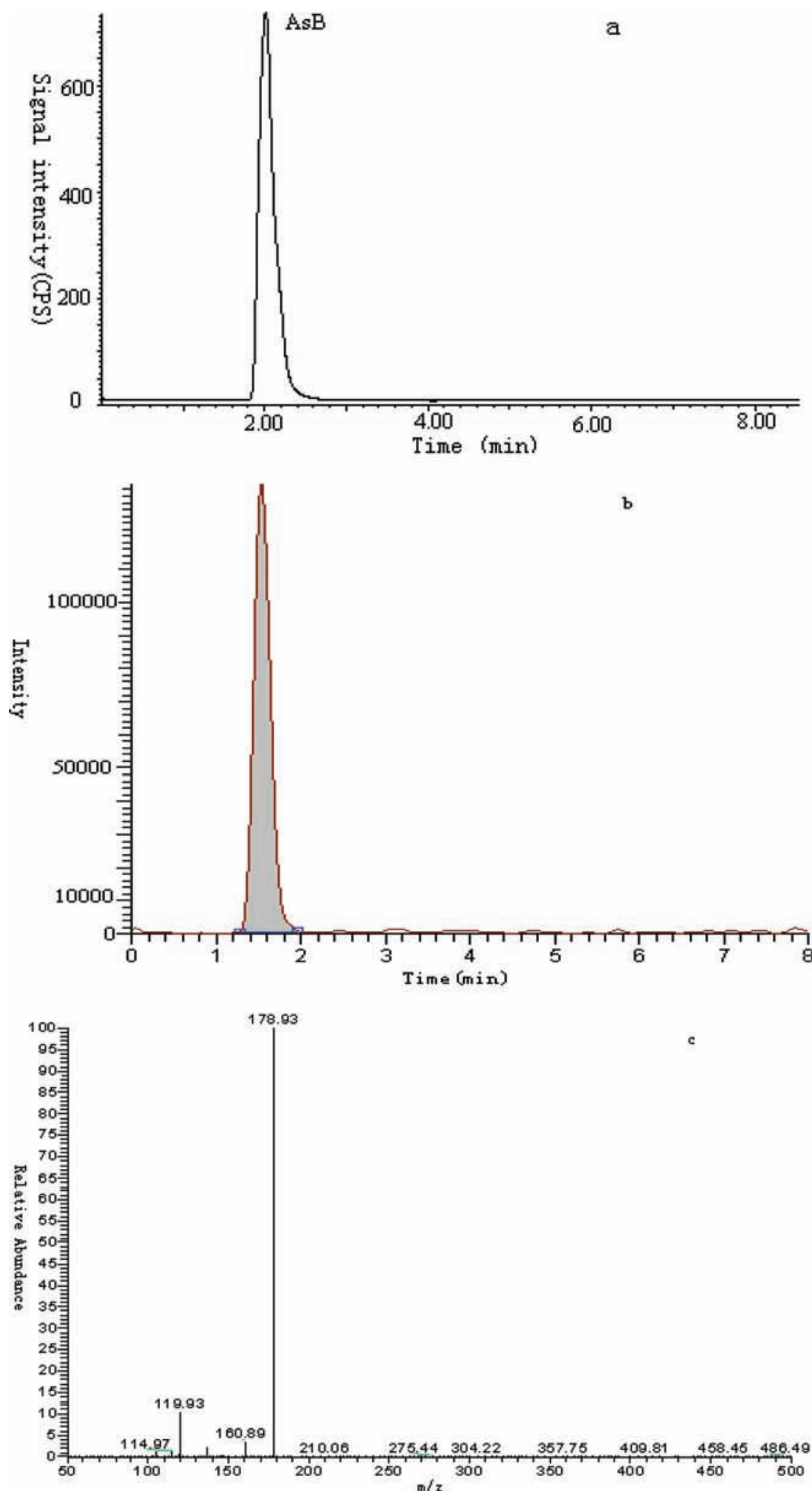

Figure 4. Chromatogram of AsB (0.1 μg mL^{-1}) obtained using (**a**) HPLC-ICP-MS and (**b**) LC-MS/MS; (**c**) full mass spectrum of AsB.

feed additives and other arsenic species in the formula feed. However, arsenic species in formula feed behave differently from those in biological samples, so the extraction time needs to be examined to achieve good recovery. The following procedure was identified as the best preparation of formula feed spiked with seven arsenic species. The appropriate concentration of arsenic species solution was spiked into formula feed samples and blended evenly. The animal feed samples were extracted using a methanol/water (1:1) extraction solution. Results are shown in **Figure 5**. The extraction efficiency could be improved with increased extraction time with the help of sonication. Satisfactory recovery of all arsenic compounds was gained after 40 min. p-ASA, 4-OH, and ROX, which are exogenous arsenic species, were mostly extracted in the first extraction. Aite and Aate, which are endogenous species, need the second extraction for satisfactory recovery. The extraction efficiency was validated by spiking different concentrations of arsenic species in animal feed (**Table 2**) and comparing the sum of the arsenic species extracted in the

formula feed sample with the total mass of arsenic present in the samples (**Table 3**). The results showed there were no significant losses by using the extraction procedure because the sum of arsenic species accounted for > 71.7% of the total arsenics.

Method Validation. The developed method was validated by determining the linearity, limit of detection (LOD), limit of quantification (LOQ), and recovery of arsenic species. All validation data were determined following the separation of each arsenic compound by HPLC using a gradient elution procedure. Seven-point calibration curves for seven arsenic species were obtained by plotting the peak areas against the concentration of arsenic for each species in the range of 0.1−200 μg of As L^{-1}. The calibration curve for each species had good linearity (R > 0.99). It is noted that if the contents of some of the target arsenic species were out of the linear range, the dilution was carried out by using methanol/water mixture (v/v = 1:1) to linear ranges.

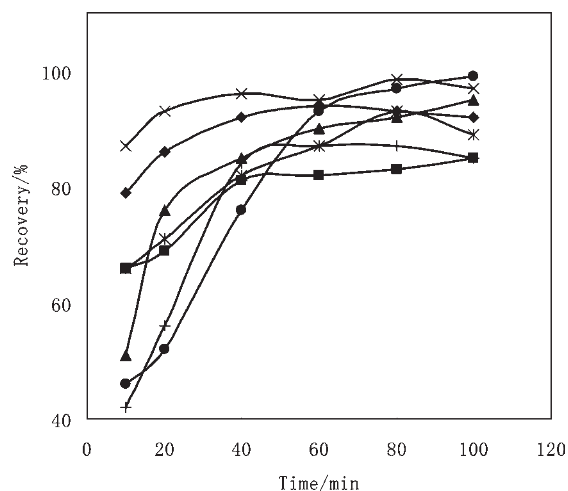

Figure 5. Percent recovery of arsenic species from spiked animal feed samples using ultrasonic generator with methanol/water (1:1) extraction solution: (×) 4-OH; (◆) p-ASA; (▲) ROX; (■) Aite; (∗) Aate; (+) MMA; (●) DMA.

The LOD was calculated as 3 times standard deviation of the background noise of the blank samples at the peaks' retention times (n = 5). The LOQ of the Aite and Aate was calculated as 10 times standard deviation of the background noise of the blank samples; however, the LOQs of MMA, DMA, p-ASA, 4-OH, and ROX were evaluated by analyzing 10 samples containing an arsenic concentration close to the expected LOQ under the same conditions. The LOQ values of arsenic species in this study were confirmed by analyzing six replicates of samples containing all of the arsenic species at the LOQ expected for Aite and Aate (see **Table 2**).

The recovery of arsenic species in the formula feed was studied by spiking with known amounts of seven arsenic species. Under optimized extraction procedures and instrumental operation conditions, the average recoveries of arsenic species were > 78.5% and the RSDs were < 10.0%. The results indicate that the method is accurate for the determination of p-ASA, ROX, and other arsenic species in formula feed.

Table 2 summarizes the linear range, relative coefficient, LOD, and LOQ values for each arsenic compound achieved under the optimum conditions of chromatography. Because of the wide

Table 2. Method Performances Obtained for Arsenic Compounds Using IP-RP-HPLC-ICP-MS[a]

arsenic compound	linear range (μg of As L^{-1})	R^2 (n = 7)	LOD (μg of As kg^{-1})	LOQ (μg of As kg^{-1})	added (μg of As kg^{-1})	mean recovery (%)	RSD (%) (n = 6)
Aite	0.1−100	0.9985	0.4	1.1	60.0	84.6	9.7
					110	82.6	8.1
Aate	0.1−100	0.9992	0.8	2.4	240	79.3	4.9
					600	90.4	5.6
MMA	0.1−100	0.9996	0.6	1.6	1.6	82.5	7.4
					20.0	86.5	4.1
DMA	0.1−100	0.9993	0.4	1.3	1.3	78.7	7.9
					20.0	89.0	5.6
p-ASA	0.5−200	0.9999	0.7	1.9	1.9	85.1	9.0
					20.0	92.7	5.3
4-OH	0.5−200	0.9979	0.9	2.3	2.3	78.5	7.6
					20.0	92.6	6.2
ROX	0.5−200	0.9928	1.7	3.1	3.1	81.1	10.0
					20.0	93.1	6.7

[a] R^2 is the linear correlation coefficient of the calibration curve (n = 7); RSD is relative standard deviation.

Table 3. Analysis of Different Feed Samples (n = 6, Concentration in μg of As g^{-1})[a]

	HPLC-ICP-MS						
sample	Aite	DMA	Aate	p-ASA	sum of species	total As	P (%)
formula feed 1	0.068 ± 0.012		0.276 ± 0.024		0.344 ± 0.035	0.480 ± 0.029	71.7
formula feed 2	0.212 ± 0.035	1.96 ± 0.16	4.01 ± 0.22	32.69 ± 0.70	38.87 ± 2.37	43.70 ± 3.43	88.9
corn	0.234 ± 0.014		0.073 ± 0.006		0.307 ± 0.032	0.340 ± 0.029	90.3

[a] Formula feed 1 is without p-ASA; formula feed 2 contains p-ASA. P is sum of arsenic species as percentages of the total As.

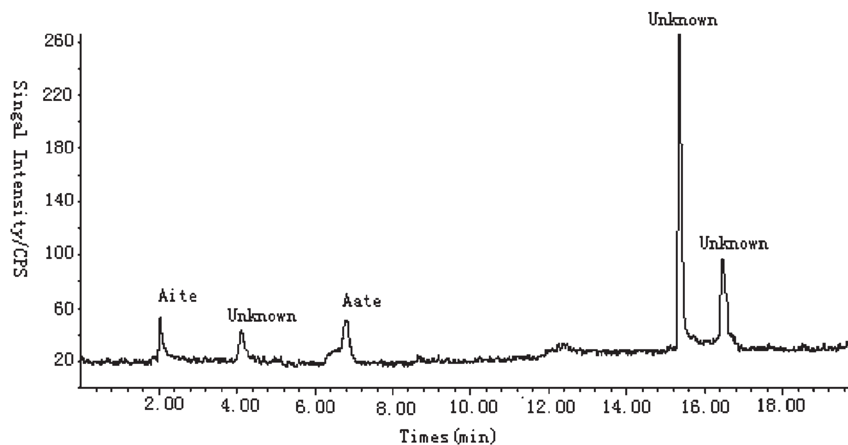

Figure 6. Chromatogram of arsenic species in formular feed 1 obtained by using HPLC-ICP-MS.

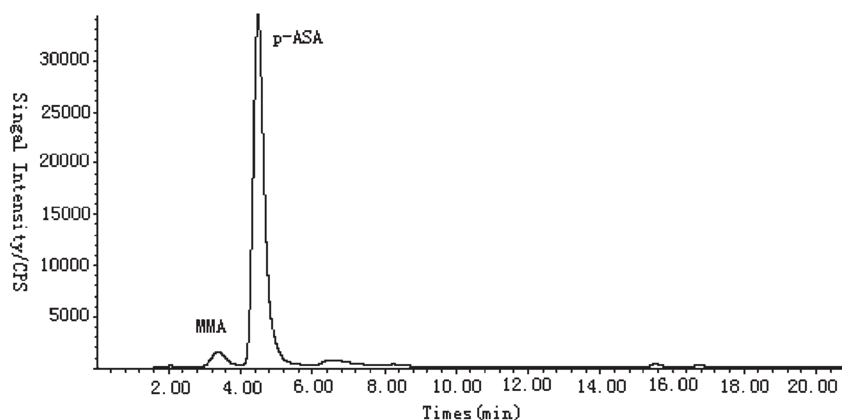

Figure 7. Chromatogram of arsenic species in formular feed 2, which contained p-ASA, with HPLC-ICP-MS detection.

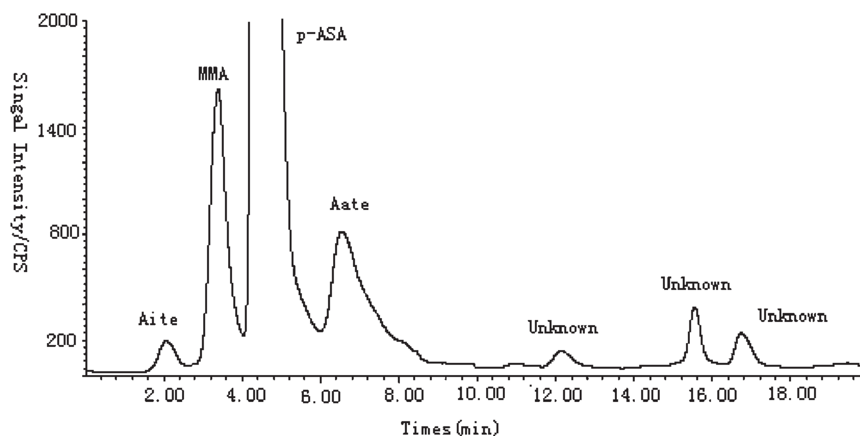

Figure 8. Amplified chromatogram of **Figure 7**.

linear ranges, satisfactory relative coefficients, extremely high sensitivity of the ICP-MS detector, and good recovery of all arsenic compounds, the developed method is suitable for the speciation of arsenics in formula feeds.

Application in Feed Samples. The total arsenics and arsenic speciation in samples, including corn, the formula feed, and the formula feed containing p-ASA were analyzed. The accuracy of the determination procedure for total arsenics was validated by using certified wheat powder with a value of 0.22 ± 0.02 mg of As kg^{-1} (amount found = 0.20 ± 0.04 mg of As kg^{-1} for six samples). The total arsenic concentrations in the samples examined in corn powder ($n = 6$) and animal formula feed ($n = 6$) were 0.34 and 0.48 $\mu g\ g^{-1}$, respectively. On the contrary, the content of total arsenic in the formula feed containing p-ASA ($n = 6$) was 43.7 $\mu g\ g^{-1}$. This value exceeds the controlled amount of total arsenic for formula feed (2.0 $\mu g\ g^{-1}$) in the Chinese feed sanitary standard (*26*).

Arsenic speciation in animal feeds and corn samples was carried out using the HPLC-ICP-MS method developed in this study. As shown in **Table 3**, Aite and Aate are the main species in the corn and blank feed sample (formula feed 1, see **Figure 6**), but the formula feed enriched with p-ASA (formula feed 2) has

complicated and unidentifiable arsenic species profiles (see **Figures 7** and **8**), and the recovery of identified arsenic species against the total arsenics was 88.9%. The extraction solutions were also analyzed by using LC-MS/MS to check the interference of AsB for Aite. No AsB was found in the corn and animal feed samples. The results suggest that the developed method is suitable for the determination of phenylarsonic additives and speciation of arsenic species in animal formula feed and feedstuffs.

Conclusion. A robust method for the simultaneous determination of seven anionic arsenic species in formula feed including Aite, Aate, MMA, DMA, 4-OH, p-ASA, and ROX was developed using HPLC-ICP-MS with gradient elution. The sample preparation and chromatography separation conditions were studied in detail. The proposed method offered a wide linear range and high sensitivity and was applied successfully to evaluate arsenic species and concentration in feed samples and feedstuffs. The results of sample analysis indicate that the present method is a suitable and accurate analytical method for the quantitative and qualitative determination of phenylarsonic compounds and other arsenic species in animal feeds.

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