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# Development of a high-performance liquid chromatography method for the simultaneous quantification of four organoarsenic compounds in the feeds of swine and chicken

Dongmei Chen, Huahai Zhang, Yanfei Tao, Yulian Wang, Lingli Huang, Zhenli Liu, Yuanhu Pan, Dapeng Peng, Xu Wang, Menghong Dai, Zonghui Yuan*

*National Reference Laboratory of Veterinary Drug Residues/MOA Key Laboratory of Food Safety Evaluation, Huazhong Agricultural University, Wuhan, Hubei 430070, China*

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ABSTRACT

A high-performance liquid chromatography (HPLC) method with UV detection was developed for the simultaneous determination of arsanilic acid, roxarsone, nitarsone, and carbarsone in the feeds of swine and chicken. Feed samples were extracted with methanol/1% acetic acid (90:10, v/v) in an ultrasonic bath and the protein was precipitated with 2% $Cu_2SO_4$. The samples were further purified by solid phase extraction (SPE) on SAX cartridges. Separation was performed on a Zorbax SB-Aq C18 HPLC column using an isocratic procedure with methanol and 1% acetic acid (3:97, v/v) at a flow-rate of 0.7 mL $min^{-1}$, and the UV detector was set at a wavelength of 260 nm. The recoveries of organoarsenic compounds spiked at levels of 2, 20 and 200 μg $g^{-1}$ ranged from 81.2% to 91.3%; the inter-day relative standard deviation values were less than 7.0%. The limits of quantification for four organoarsenic compounds were 1.0–2.0 μg $g^{-1}$. This simple and fast method could be applied to the determination of multi-residues of organic arsenic compounds in animal feeds.

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## 1. Introduction

Roxarsone, arsanilic acid, nitarsone, and carbarsone (Fig. 1) are the best known arsenic compounds. Arsanilic acid and roxarsone are used as animal feed additives for both pig and chicken, whereas nitarsone and carbarsone were used for controlling blackhead disease in turkeys. They are administered orally or mixed with animal feed. However, recent studies have disputed the beneficial effects of these compounds as growth promoters. In addition to these findings, a number of reports have pointed out that these additives can cause toxic effects when used at higher than recommended levels [1,2]. Roxarsone appears to ultimately decompose to water-soluble toxic arsenicals, primarily as inorganic arsenate [3,4]. To ensure confidence in the meat and egg industry and to avoid the misuse of this class of compounds, organoarsenic compounds in animal feeds must be monitored.

This work reported here was primarily concerned with the development of analytical methods capable of separating and determining organoarsenic compounds used in animal feeds. Methods such as HPLC with UV detection [5,6], gas chromatography–mass spectrometry (GC–MS) [7], capillary electrophoresis (CE) [8], liquid chromatography–mass spectrometry (LC–MS) [9,10], and inductively coupled plasma mass spectrometry (ICP-MS) [11–14] have been published describing the analysis of roxarsone or/and arsanilic acid. Only one method has described the simultaneous analysis of arsanilic acid, roxarsone, nitarsone and carbarsone using LC–MS [15]. However, in this work by Pergantis et al., organoarsenic compounds were only determined in standard solution, not in the complex matrices of animal feeds.

At present, no methods have been published for the simultaneous determination arsanilic acid, roxarsone, nitarsone and carbarsone by HPLC with UV detection and only one method for the determination of arsanilic acid and roxarsone in animal feeds using liquid chromatography–hydride generation coupled with atomic fluorescence spectrometry (LC–HG-AFS) [16].

The purpose of this study was to develop a rapid and sensitive HPLC method for simultaneous determination of four organoarsenic compounds in animal feeds. A simple sample preparation method including ultrasonic supported solvent extraction and SPE techniques have been established to decrease the total time of the analysis in this study.

* Corresponding author. Tel.: +86 27 8728 7186; fax: +86 27 8767 2232.
*E-mail address:* yuan5802@mail.hzau.edu.cn (Z. Yuan).

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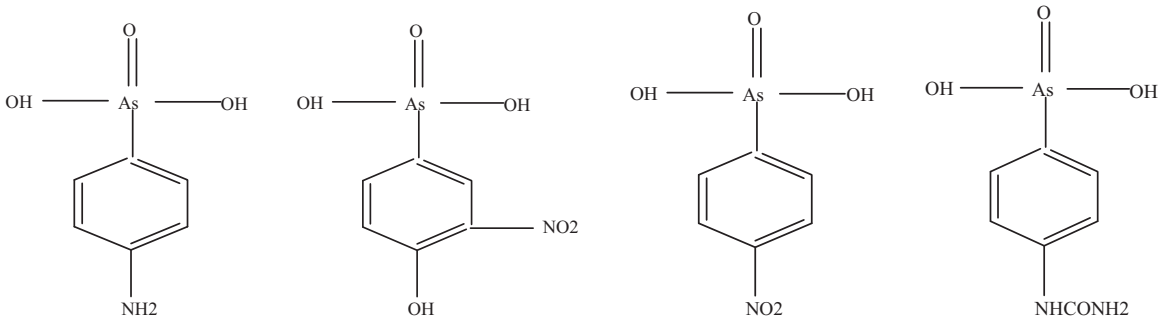


**Fig. 1.** Structures of organoarsenic compounds: arsanilic acid, roxarsone, nitarsone, and carbarsone.

## 2. Experimental

### 2.1. Chemicals and reagents

Analytical standards of arsanilic acid, roxarsone, nitarsone, and carbarson were purchased from Sigma–Aldrich (St. Louis, MO, USA). The four standards were of over 97% purity. Distilled water was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA, USA). Methanol was HPLC-grade and obtained from Fisher (Bar-Bel, France). Other solvents of analytical reagent grade included trichloroacetic acid (TCA), acetic acid, formic acid, $PbAC_2$, and copper sulfate ($Cu_2SO_4$). The cartridge used for SPE was AccuBOND SAX (500 mg, 3 mL, Agilent, Milford, HA, USA). Other cartridges tested were Oasis HLB (60 mg, 3 mL, Waters Corp., Milford, MA, USA), and Oasis MAX (60 mg, 3 mL, Waters Corp.).

The porcine feed samples were supplied by Breeding Swine Testing Centre (Huazhong Agriculture University, Wuhan, China); the chicken feed samples were supplied by Feeds Inspection Point (Huazhong Agriculture University, Wuhan, China).

### 2.2. Standard solutions

Individual stock standard solutions were made by dissolving each pure standard in methanol to obtain 1000 $\mu g\,mL^{-1}$ concentration. Standard diluted solutions were mixed with methanol and 1% acetic acid (3:97, v/v). A 200 $\mu g\,mL^{-1}$ mixed standard fortification solution was prepared by combing 2.0 mL of each stock standard and dilute to 10 mL with methanol. Stock solution was prepared every 3 months and stored in amber vials at or below −20° C. Mixed fortification solution was prepared every 1-month and stored in amber vials at or below −20° C.

### 2.3. Sample preparation

A 5-g finely ground (1 mm) feed sample was transferred into a 50 mL centrifuge tube, spiked with the analytes and 15 mL methanol/1% acetic acid (90:10, v/v) were added to the sample. The mixture was shaken on a vortex system for 5 min, then sonicated in an ultrasonic bath for 10 min at room temperature, and the sample was then centrifuged at 8000 rpm for 10 min. The supernatant was transferred into another 50 mL centrifuge tube. The same extraction procedure was repeated again with 10 mL methanol/1% acetic acid (90:10, v/v). The two supernatant were combined. A 2 mL volume 2% $Cu_2SO_4$ was added to these extracts. After vortex-mixing for 2 min and centrifuging at 5000 rpm for 10 min, the supernatant was transferred and diluted to 30 mL with methanol. Three milliliters of the supernatant were collected and mixed with 6 mL water, which was ready for the clean-up procedure.

The SAX cartridge was pre-conditioned with 3 mL methanol and 3 mL water. All flow rates for conditioning and washing were set at 3 mL $min^{-1}$. The entire extracts were loaded onto the SPE column at flow rates of 1 mL $min^{-1}$. The column was washed with 3 mL water, 3 mL methanol, and 2 mL formic acid/acetonitrile (5:95, v/v), then dried by purging air at the rate of 10 mL $min^{-1}$. The analytes were eluted with 5 mL formic acid/methanol (5:95, v/v) at a flow rate of 1.0 mL $min^{-1}$ into a 10 mL glass tube and evaporated to dryness under a stream of nitrogen at 40 °C. The dry residue was dissolved in 2 mL mobile phase. The flow diagram for sample preparation of organoarsenic compounds residues determination was seen in Fig. 2.

### 2.4. HPLC analysis

HPLC analysis was carried out on a Waters 2695 HPLC system coupled with UV detector. The chromatographic separation was accomplished on a Zorbax SB-Aq C18 column (250 × 4.6 mm, 5 μm) (Agilent Technology, USA) coupled with a 2 mm C18 guard-column at 40° C in a column oven. The mobile phase consisted of 1% acetic acid/methanol (97:3, v/v). A flow-rate and injection volume of 0.7 mL $min^{-1}$ and 50 μL, respectively, were used. The UV detector was set at a wavelength of 260 nm for all the compounds.

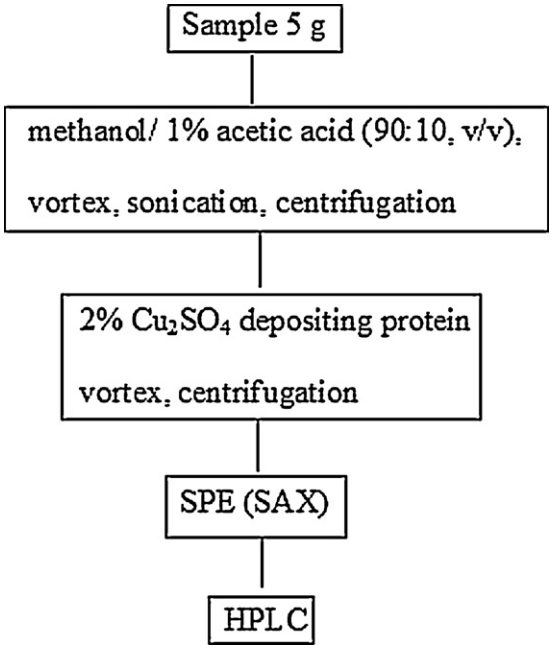


**Fig. 2.** Overview procedure for sample preparation of organoarsenic compounds residues determination.

### 2.5. Method validation

#### 2.5.1. Linearity

External standard calibration method was employed on series of mixed standard solutions of organoarsenic compounds at concentration levels from 0.1 to 50 $\mu g\,mL^{-1}$ and injected in triplicate. The method was further tested by matrix-match calibration curves which were made by fortified with the four compounds at each of six concentrations from 2 to 200 $\mu g\,g^{-1}$. The spiked samples were performed with complete extraction and purification procedure. The calibration curves were calculated using the linear least squares regression analyses of the peak area to concentration ratios.

#### 2.5.2. Accuracy and precision

Samples known to be noncompliant served as blank matrices. Accuracy and precision of the analytical method were calculated by the determination of six replicates of blank samples fortified at three different spiked levels (2, 20, 200 $\mu g\,g^{-1}$) over a period of 3 days. The accuracy of the method was expressed as the mean recoveries of spiked analytes in sample matrix. Intra-assay precision was conducted on the same day. Intermediate precision was determined by repeating the study for three consecutive days.

#### 2.5.3. Limits of detection (LOD) and limits of quantification (LOQ)

The LOD and LOQ were established using spiked feed samples at 8 concentration levels from 0.1 to 5.0 $\mu g\,g^{-1}$. The LOD was calculated by the comparison of the threefold variation of signal to noise ratio (S/N = 3:1) obtained from analysis extract of blank feed samples, while the LOQ was calculated by using a signal-to-noise ration of 10.

#### 2.5.4. Selectivity

The selectivity of a method is important for the differentiation and identification of the existing target analytes in different animal feeds. All the matrices fortified the analytes at 20 $\mu g\,g^{-1}$ concentration were investigated. The analytes were identified by matching peaks retention times with the values of the corresponding standard analyzed under the same experimental conditions.

## 3. Results and discussion

### 3.1. Chromatographic conditions

Acetonitrile and methanol are often used in the mobile phase for reversed-phase (RP) HPLC separation of various compounds. Therefore these two reagents were tested in this study. According to the structure of arsanilic acid, roxarsone, nitarsone and carbarsone, the pH of the mobile phase was a critical factor in achieving the chromatographic separation of the four organoarsenic compounds studied. To achieve optimum LC separation, several mobile phase additives such as acetic acid, ammonium acetate buffer, formic acid and ammonium formate buffer were comprehensively investigated. The maximum sensitivities and satisfactory separation of all analytes were achieved when using 1% acetic acid and methanol as mobile phase. This mobile phase condition led us to look for columns that are stable in acid mobile phase and comparable with high aqueous mobile phases (above 97%) as well. Zorbax SB-Aq C18 column provides excellent stability in low-pH mobile phases and high aqueous mobile phases. SB-Aq column uses a surface chemistry that allows the alkyl bonded phase to remain accessible in highly aqueous mobile phases, and bulky diisopropyl side groups to provide long-term low pH stability. The monitoring wavelength was set at 260 nm which was a compromise among the wavelengths of maximum absorption of the four organoarsenic compounds.

### 3.2. Sample preparation

Different modes of extraction with different solvent systems were used to extract the drugs from porcine feed samples spiked with each individual drug at 20 $\mu g\,g^{-1}$. Based on the structures of the molecules and their solubilities properties, methanol and acetonitrile were chosen as extraction solvents and compared. When acetonitrile was used as the extraction solvent, low recoveries of arsanilic acid and nitarsone were obtained. With methanol almost all of organoarsenic compounds could be extracted, but recovery for nitarsone was lower. Furthermore, acidification of the solvent was investigated in order to evaluate whether the interaction between the drugs and feed matrix could be eliminated. Different concentrations acetic acid and trichloroacetic acid (0.1%, 0.5%, 1%, 2% and 5%) were added to the extract. When 1% acetic acid was added in methanol, all compounds could be extracted with recoveries of more than 80%. However, proteins were found to interfere so $Cu_2SO_4$ and $PbAC_2$ were investigated as protein precipitation agents. Results showed that $Cu_2SO_4$ was more effective. Highest recoveries for all the four compounds in animal feeds were obtained following extraction with 25 mL of methanol/1% acetic acid (95:5, v/v) and deproteinization with 2% $Cu_2SO_4$.

The usual methods for the extraction of residues veterinary drugs from feed samples, such as vigorous shake and vortex with the extraction solvent, require a relatively long time to be accomplished properly. In this study, the time for the extraction procedure was reduced to 10 min with ultrasonic treatment. During the experiments, it was noticed that the mode of the extraction caused different recovery results. Recoveries were compared at different time of 0, 5, 10, 20 and 30 min for ultrasonic extraction. Each of the extraction modes was repeated four times, all of the data were analyzed. It was confirmed that the time of 10 min for ultrasonic extraction was statistically different from other modes and the highest recovery results were obtained with it. Ultrasonic solvent extraction was considered to be a technique capable of reducing operation time. Enhancing the techniques allowed us to reduce the total time for the sample pretreatment to less than 2 h.

The procedure of purification was necessary for animal matrices being rich in protein. According to the properties of the four organoarsenic compounds and the sample/matrix composition, the following cartridges were tested including Oasis HLB, Oasis MAX, and SAX Cartridges. When Oasis HLB and Oasis MAX were used, there were interferences at the same retention time as arsanilic acid. Comparative studies indicated that SAX was superior to the other cartridges in terms of good recovery (>90%) and little matrix interference. During elution steps of SAX, the mixing solution of formic acid and methanol was selected as elution solution for its good extraction efficiency. Finally, a mixture of formic acid and methanol (5:95, v/v) showed a higher elution power to acquire high recoveries and little interference.

### 3.3. Validation of the analytical method

The LOQ of the arsanic, carbarsone, roxarsone, and nitarsone extracted from animal feeds were 1.0 $\mu g\,g^{-1}$, 1.0 $\mu g\,g^{-1}$, 2.0 $\mu g\,g^{-1}$, and 2.0 $\mu g\,g^{-1}$, respectively. The linearity of the standard mixtures was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients being greater than 0.999 for all curves. Matrix matched calibration standard curves were used to quantify the target analytes in animal feeds and to make the method as accurate as possible. The correlation of the coefficient values was above 0.99 for all analytes within the range from 2 to 200 $\mu g\,g^{-1}$. All calibration curves indicated excellent method reliability that could be used to accurately quantify trace amount of these analytes in animal feeds.

**Table 1**
The validation results of organoarsenic compounds in the feeds of swine and chicken.

| Analyte | Spiked concentration ($\mu g\,g^{-1}$) | Average recovery (%, $n = 18$) | | Inter-day RSD (%, $n = 18$) | |
|---|---|---|---|---|---|
| | | Swine feed | Chicken feed | Swine feed | Chicken feed |
| Arsanilic acid | 2 | 85.6 | 83.2 | 5.6 | 4.8 |
| | 20 | 87.8 | 88.6 | 4.8 | 6.2 |
| | 200 | 90.6 | 91.3 | 4.2 | 4.8 |
| Carbarsone | 2 | 81.2 | 84.3 | 6.7 | 5.6 |
| | 20 | 87.7 | 88.2 | 5.0 | 5.7 |
| | 200 | 90.2 | 89.8 | 4.5 | 4.9 |
| Roxarsone | 2 | 83.3 | 84.6 | 5.5 | 5.3 |
| | 20 | 87.7 | 86.0 | 4.8 | 4.7 |
| | 200 | 90.9 | 90.8 | 4.3 | 4.8 |
| Nitarsone | 2 | 84.8 | 82.2 | 6.1 | 5.9 |
| | 20 | 87.1 | 86.1 | 4.8 | 5.3 |
| | 200 | 90.8 | 89.2 | 4.0 | 4.9 |

The results of accuracy and precision data are summarized in Table 1. The mean recoveries were ranged from 81.2% to 91.3%. The intra-day and inter-day values were all below 7.0%, indicating a precise and accurate method for the determination of organoarsenic compounds in animal feeds.

Following a comparison of the background noise from various matrices, the results showed that there were no interference peaks that could be detected at the retention time of these compounds of interest (Fig. 3). It demonstrated that the method could be applied to monitor the residues of the different feed samples.

### 3.4. Analysis of real feed samples

Results of the analysis of the commercial feed samples were also used to validate the method. Twenty commercial feed samples from different factories were analyzed using the described HPLC-

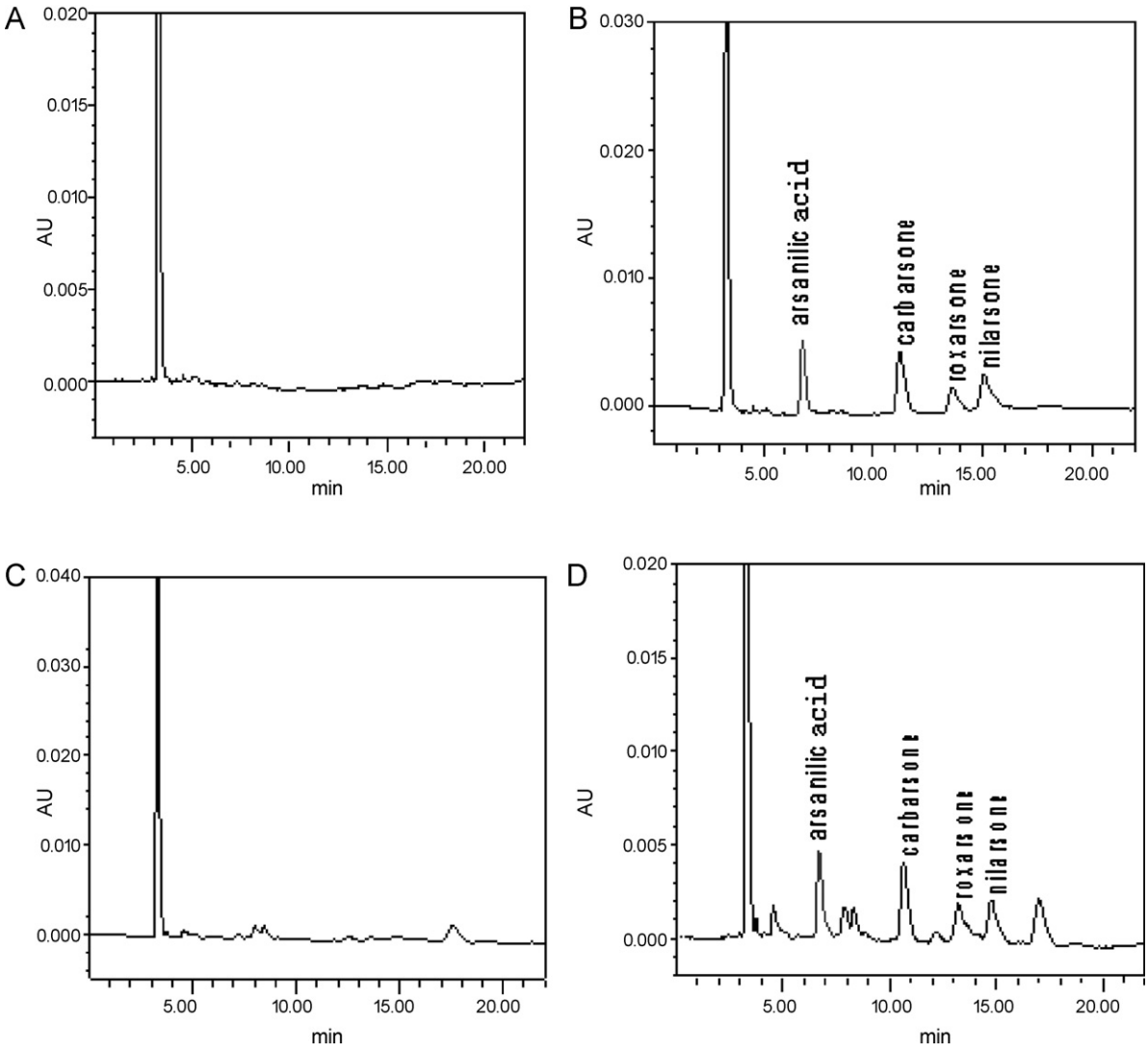


**Fig. 3.** HPLC chromatograms of blank porcine (A), chicken (C) feeds, and spiked porcine (B) and chicken (D) feeds at the concentrations 2 $\mu g\,g^{-1}$ for each of individual drug.

**Table 2**
Actual content of roxarsone in 20 commercial samples of porcine and chicken feeds ($n = 3$).

| Sample no. | Roxarsone concentration ($\mu g\,g^{-1}$) |
|---|---|
| Porcine feed 1 | 51.3 ± 2.7 |
| Porcine feed 2 | 84.2 ± 3.9 |
| Chicken feed 1 | 25.6 ± 1.6 |

UV method. Half of the 20 samples were porcine feed and the other samples were chicken feed. The results are shown in Table 2. Roxarsone has been detected in two porcine feeds and in one chicken feed sample.

## 4. Conclusions

A new analytical method for the simultaneous determination of arsanilic acid, roxarsone, nitarsone, and carbarsone in animal feeds with HPLC-UV was developed. The method included an ultrasonic solvent extraction and a clean-up step by SPE, which made the method selective, reproducible, and fast. The method has been successfully validated in terms of accuracy, precision, sensitivity and selectivity. The results demonstrate that the method could be applied to the testing of the animal feeds for surveillance programs.

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