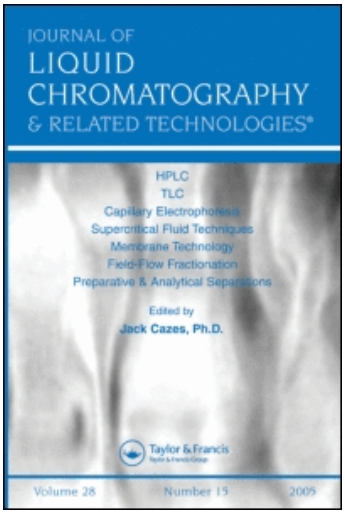

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DETERMINATION OF ARSANILIC ACID IN LIVESTOCK FEEDS BY HPLC USING AN ANION EXCHANGE COLUMN AND ULTRAVIOLET DETECTION

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DETERMINATION OF ARSANILIC ACID IN LIVESTOCK FEEDS BY HPLC USING AN ANION EXCHANGE COLUMN AND ULTRAVIOLET DETECTION

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□ *A liquid chromatography method was developed for the determination of arsanilic acid in livestock feeds. A variety of livestock feeds typical of those used in commercial practice were obtained from a local feed mill. The samples included complete swine and poultry feeds, swine and poultry concentrates (high in protein), and a swine premix (high in vitamins and minerals). The feeds were spiked with various levels of arsanilic acid. The first step in determining arsanilic acid content was to extract the arsanilic acid from the feed. Ground complete feeds and concentrates were extracted with 25 mmol/L sodium hydroxide in a water bath at 50° C. For the vitamin-mineral premix, extraction was conducted at ambient temperature since contamination by organic matter was lower for these samples. The analysis of arsanilic acid was performed by means of high performance liquid chromatography using an anion exchange column and ultraviolet detection at 244 nm. The mobile phase consisted of 40 mmol/L sodium dihydrogen phosphate-methanol (960+ 40, v/v) and the running time for an analysis was about 12 minutes. Average spike recoveries for samples prepared at spike levels of 10, 50, 100, 150, 300, and 1250 mg/kg were 92.8, 92.4, 96.1, 94.7, 98.3, and 99.6%, respectively. The limits of detection and quantification were 0.05 and 0.4 mg/kg, respectively. One advantage of the method is that it determines the arsanilic acid content of feeds directly, without conversion to arsenic.*

Keywords anion exchange, arsanilic acid, livestock feeds, ultraviolet detection

INTRODUCTION

Arsanilic acid [(4-aminophenyl) arsonic acid] is approved for use in diets fed to pigs and poultry in the United States, Canada, China, and many other countries. It has long been used in veterinary medicine as a chemotherapeutic agent because it inhibits the growth of some microorganisms. It is also

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used by the feed industry for the purpose of promoting growth, increasing feed efficiency, and controlling disease. For growth promotion, arsanilic acid is generally incorporated into swine feeds at levels between 50 and 100 mg/kg and in poultry feeds at 100 mg/kg. For disease control, the medicated dose of arsanilic acid is 250 to 400 ppm in feeds for swine dysentery, and the therapy period is 5 or 6 days.[1] The recommended withdrawal period is 5 to 6 days.

In the past, analysis of arsanilic acid in livestock feeds has been conducted using colorimetric assay,[2] thin-layer chromatography,[3] low pressure liquid chromatography, mass spectrometry,[4] photo-oxidation with a fluorimetric detector,[5] and a long path length absorbance spectrophotometric method.[6] Unfortunately, these procedures are time consuming and some procedures cannot separate arsanilic acid from other arsenic containing compounds. In addition, some of the methods are only applicable in the absence of sulfonamides and arsanilic acid is commonly fed in combination with these antibiotics.

A search of the literature failed to discover any published methods related to the use of high performance liquid chromatography for the determination of arsanilic acid in livestock feeds. High performance liquid chromatography has several advantages compared with other methods due to its high specificity, as well as its rapid quantification and analysis time. The use of rapid analysis procedures can improve laboratory efficiency. Therefore, the objective of the present work was to develop a simple, sensitive, and rapid assay, using high performance liquid chromatography for the determination of arsanilic acid in livestock feeds.

EXPERIMENTAL

Instruments and Materials

(a) *HPLC system.*—Alliance 2690 Separation Module, 2487 Dual λ Absorbance Detector, and MassLynx^{TM} 4.0 software (Waters Corporation, Milford, MA).
(b) *HPLC column.*—Anion exchange column: AS11-HC 9 μm, 250 × 4 mm with a AG11-HC 13 μm 50 × 4 mm guard column (Dionex Corporation, Sunnyvale, CA).
(c) *Shaker.*—Minishaker (IKA Works GuangZhou, China).
(d) *Centrifuge.*—TDL-5-A centrifuge (Shanghai An-ting Scientific Instrument Works, Shanghai, China).
(e) *Membrane Syringe Filter.*—Glass Microfiber GF/C, 1.2 μm; Whatman Inc. (Clifton, NJ).
(f) *Methanol.*—Optima grade (Fisher Scientific, Fair Lawn, New Jersey, US).

(g) *Sodium hydroxide.*—Analytical reagent grade (Shanghai Chemical Works, Shanghai China).
(h) *UV-Visible Spectrophotometer.*—TU-1901 Double-beam UV-VIS Spectrophotometer (Purkinje General Instrument, Beiijing, China).
(i) *Sodium dihydrogen phosphate, dihydrate.*—Analytical reagent grade (Beijing Chemical Works. Beijing China).
(j) *Copper (II) sulfate pentabydrate.*—Analytical reagent grade (Beijing Chemical Works. Beijing China).
(k) *Water.*—HPLC reagent grade, prepared by Milli-Q Reagent Water System (Millipore, Bedford, USA).
(l) *Mobile phase A.*—Optima grade methanol.
(m) *Mobile phase B.*—40 mmol/L sodium dihydrogen phosphate solution.
(n) *Extracted solution.*—25 mmol/L sodium hydroxide.
(o) *p-Arsanilic acid.*—Sigma-Aldrich Chemicals (St. Louis, Mo).
(p) *p-Arsanilic acid analytical standards.*—A stock standard solution (0.5 mg/mL)

was prepared by weighing 50 mg arsanilic acid into a 100 mL volumetric flask. Then, 5 mL of 25 mmol/L sodium hydroxide was added to the flask to dissolve the arsanilic acid, and then diluted to volume with water. A working standard solution of 0.05 mg/mL in water was prepared from the stock standard solution. The stock solution was stored at 4°C and the working standard solution was prepared fresh daily.

Sample Preparation

Commercially prepared feed samples, typical of those used for feeding pigs and poultry, were used in these experiments. The samples included complete swine and poultry feeds, swine and poultry concentrates (high in protein), and a swine vitamin-mineral premix. All feeds were made at the Pilot Workshop of the Ministry of Agricultural Feed Industry Center (MAFIC) in Beijing, China. The feeds, as prepared, did not contain any drugs or growth promoters. The feed samples were placed in plastic bags and refrigerated at 4°C until needed for analysis.

A 1% arsanilic acid premix was prepared and added to provide 10 and 50 mg/kg of arsanilic acid in the pig feeds, 150 mg/kg in the pig concentrate, and 1250 mg/kg in the pig premix. The broiler feed was spiked to contain 100 mg/kg while the broiler concentrate was spiked to contain 300 mg/kg of arsanilic acid. After mixing, all feed samples were ground through a 0.5 mm screen. Samples were kept below 45°C during grinding to prevent loss of activity.

Extraction

Feed samples (1 to 5 g) were weighed into 50 mL screw capped centrifuge tubes and 40 mL of 25 mmol/L sodium hydroxide was added. The complete feeds and concentrates were extracted at approximately 50°C in a water bath for 30 min with occasional swirling. For the swine premix, extraction was conducted at ambient temperature since contamination by organic matter (i.e., proteins, fats, and carbohydrates) would be lower for these types of samples. The extracts were centrifuged for 10 min at 3500 rpm. The supernatant was poured through a filter paper into a 100 mL amber colored volumetric flask, and then the sediment was re-extracted two times with 20 mL of 25 mmol/L sodium hydroxide. The extracts were diluted to volume with water before 5 mL of copper sulfate solution (10 g/L) was added to precipitate the protein in the extracted solutions. It was then filtered through a 0.22 μm nylon membrane with a glass filter, and a suitable aliquot was injected into the HPLC system.

RESULTS AND DISCUSSION

A stock standard solution (50 mg arsanilic acid, 5 mL of 25 mmol/L sodium hydroxide and 95 mL water) was scanned over the range of the UV spectrum (200 to 400 nm) and the maximum absorbance was obtained at 244 nm (Figure 1). Therefore, 244 nm was chosen as detection wavelength for use in this study.

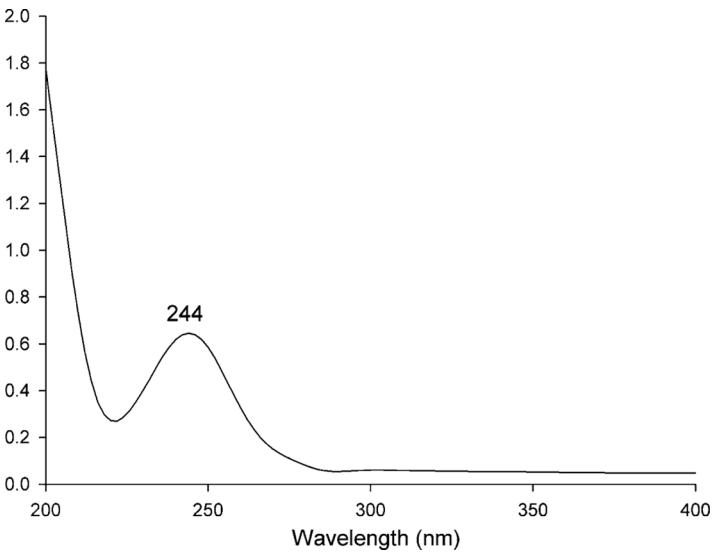

FIGURE 1 The scan spectrogram of arsanilic acid from 200 to 400 nm wavelength.

For HPLC separation of arsanilic acid, a preliminary separation was conducted on a conventional reverse phase column C_{18} with an isocratic method. A methanol and sodium dihydrogen phosphate solution was applied as the mobile phase. The system was run with the column at ambient temperature, a flow rate of 1.0 mL/min, and an injection volume of 10 μL. However, the results obtained showed that a reverse phase column was not appropriate to separate arsanilic acid because of its short elution time (2 min or less). Most contaminating compounds in animal feeds would be eluted within 4 min on a reverse phase column, and the chromatograms of these compounds would, therefore, cause interference during the range of the retention time of arsanilic acid. This problem was solved by using an anion exchange column.

To evaluate the anion exchange column and determine the optimum mobile phase, different ratios of methanol and sodium dihydrogen phosphate solution were tested with respect to optimal peak sharpness, separation efficiency, and appropriate elution time. The optimum composition of the mobile phase was 4 parts methanol and 96 parts 40 mmol/L sodium dihydrogen phosphate solution (v/v), which allowed optimum separation of arsanilic acid from other feed components on the anion exchange column (Figure 2). The total running time of the HPLC system was 12 min.

It is necessary to ensure that arsanilic acid was completely extracted from feed samples. In preliminary investigations, a sodium dihydrogen phosphate solution, a disodium hydrogen phosphate solution, a potassium

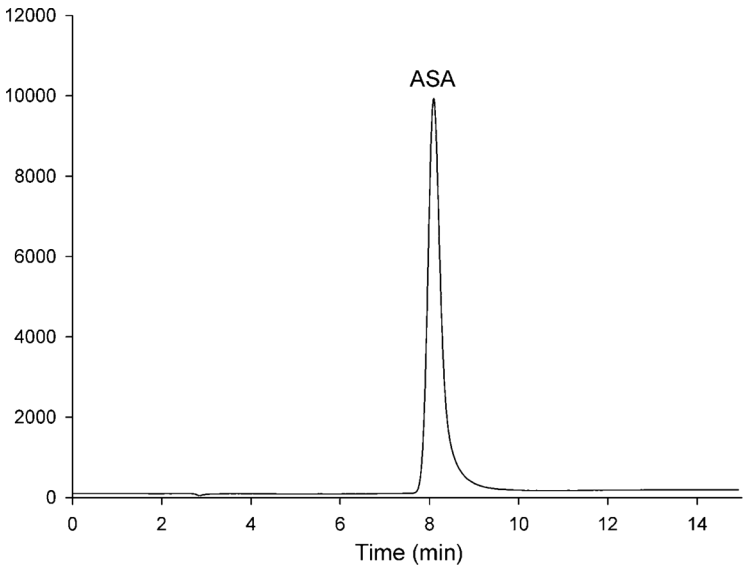

FIGURE 2 A typical chromatograph of arsanilic acid standard solution.

phosphate buffer, and a sodium hydroxide solution were tested as extraction solvents. In extraction studies, 25 mmol/L sodium hydroxide solutions obtained the highest recoveries (80 to 100%) of arsanilic acid from feed samples.

Other concentrations of sodium hydroxide solution were also tested, including 5, 10, 15, 20, 30, and 50 mmol/L sodium hydroxide solution. These concentrations were not as effective as 25 mmol/L sodium hydroxide solutions in extracting arsanilic acid from feed samples. At low concentrations of sodium hydroxide solution (i.e., 5 and 10 mmol/L), arsanilic acid was not extracted completely and recoveries were lower than 80%. When high concentrations of sodium hydroxide solutions were used as extracted solvents (i.e., 50 mmol/L), the arsanilic acid peak was not found in the analysis chromatogram. We hypothesized that the arsanilic acid was eluted more rapidly on the anion exchange column when it was dissolved in the strong alkaline solution.

Under the chromatographic conditions described above, good linearity and correlation coefficients were achieved for arsanilic acid. Replicates (n = 7) of the standard solutions of different concentrations levels were analyzed, and the detector response (peak area) was plotted against the concentrations levels. The correlations were found to be linear in the range of 2 to 10 μg/mL. The correlation of determination was >0.9999 (Table 1).

Method precision (repeatability) was calculated using a 4 μg/mL standard solution of arsanilic acid. Ten replicate injections were made within a day. The results obtained showed that the arsanilic acid peak area variability for the standard solution was within 1.2% relative standard deviations (RSD), and the retention time was 0.29% RSD. Method precision was also calculated by analyzing 7 individual replicates of a feed sample (100 mg arsanilic acid per kilogram feed). The method precision during validation was calculated to be 2.1% RSD.

Method trueness was evaluated with each recovery analysis being repeated seven times. The results presented in Table 2 showed the average spike recoveries for samples prepared at spike levels of 10, 50, 100, 150, 300,

TABLE 1 Calibration of the Proposed HPLC Method

Concentration of Arsanilic Acid, μg mL^{-1}	Mean Peak Area ($n = 7$)	RSD (%)
2	1274	1.98
4	2488	1.361
6	3679	0.85
8	4970	0.59
10	6221	0.76

Regression equation (from 2 to 10 μg mL^{-1}): $y = -0.021 + 1.62 \times 10^{-3}\, x$ ($r^2 = 0.9999$); y represents the quantity of arsanilic acid injected in μg; x represent peak areas.

TABLE 2 Trueness Data for Determination of Arsanilic Acid in Feeds

		Recoveries % ($n=7$)			
	Added Level, mg/kg	Minimum	Maximum	Mean	RSD (%)
Pig feed	10	90.2	95.3	92.8	3.6
Pig feed	50	89.6	94.8	92.4	3.9
Pig concentrate	150	93.8	96.9	94.7	1.1
Pig premix	1250	97.6	101.6	99.6	1.3
Broiler feed	100	94.3	99.2	96.1	2.7
Broiler concentrate	300	97.2	100.8	98.3	1.4

and 1250 mg/kg were 92.8, 92.4, 96.1, 94.7, 98.3, and 99.6%, respectively. It was observed that the low sample concentrations (i.e., 10 and 50 mg/kg), had larger RSD than any of the other levels(Table 2).

The limit of detection (LOD) was 0.05 mg/kg [based on a detector signal to noise (S/N) ratio of 3:1], which was calculated by considering the sensitivity of the method and the standard deviation values obtained from a standard solution. The limit of quantitation (LOQ) was determined as the lowest concentration of arsanilic acid that gives a response that could be quantified from recovery experiments with the lowest fortification level and the blank analysis. The LOQ value was 0.4 mg/kg in this study.

A feed sample chromatograph based on the UV detection may exhibit peaks that interfere with analysis. Specificity is the ability to distinguish the test compound from interferences in co-extracted materials. Animal feeds may contain other feed additives, such as antibiotics, antioxidants, vitamins,

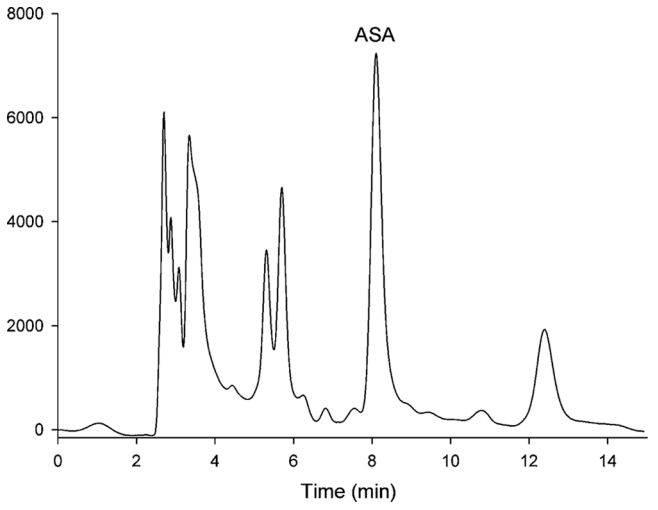

FIGURE 3 Chromatograph of pig concentrate formulated at 50 mg/kg arsanilic acid.

and mold inhibitors along with arsanilic acid. Therefore, tetracycline, bacitracin, polymycin E, flavomycin, chloramphenicol, salinomycin, monensin, chlortetracycline, and olaquindox, as well as the typical fat and water soluble vitamins normally contained in animal feeds were studied as potential interfering compounds. A series sample of pig feeds were analyzed by spiking 100 mg/kg of each of these ingredients, and no interference was found with the arsanilic acid peak (Figure 3).

CONCLUSIONS

We have established a precise and rapid method for determining arsanilic acid in animal feeds. The method could be used to test animal feeds for a label guarantee and to minimize the potential for arsanilic acid containing feeds to be inappropriately fed to animals destined to be marketed within the prescribed withdrawal time for the drug.

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