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Speciation analysis of organoarsenic compounds in livestock feed by microwave-assisted extraction and high performance liquid chromatography coupled to atomic fluorescence spectrometry

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ABSTRACT

The development of a new method to determine the presence of the organoarsenic additives *p*-arsanilic acid (ASA), roxarsone (ROX) and nitarsone (NIT) in livestock feeds by high performance liquid chromatography coupled to ultraviolet oxidation hydride generation atomic fluorescence spectrometry (HPLC-UV/HG-AFS) after microwave assisted extraction (MAE) was proposed. Chromatographic separation was achieved on a C18 column with 2% acetic acid/methanol (96:4, v/v) as the mobile phase. The limits of detection (LODs) were 0.13, 0.09 and 0.08 mg L^{-1}, and the limits of quantification (LOQs) were 0.44, 0.30 and 0.28 mg L^{-1}. The relative standard deviations (RSDs) for ASA, ROX and NIT determined from five measurements of the mixed calibration standard were 3.3, 5.3, and 5.4%, respectively.

MAE extraction of phenylated arsenic compounds using 1.5 M H_3PO_4 at 120 °C for 45 min allowed for maximum recoveries (%) of total arsenic (As) and organoarsenic species, with no degradation of these compounds. The extraction of total As was approximately 97%, and the As species recoveries were between 95.2 and 97.0%. The results of the analysis were validated using mass balance by comparing the sum of extracted As with the total concentration of As in the corresponding samples. The method was successfully applied to determine the presence of these compounds in feed samples. ASA was the only As species detected in chicken feed samples, with a concentration between 0.72 and 12.91 mg kg^{-1}.

1. Introduction

Phenylated arsenic compounds, including 4-aminophenylarsenic acid (*p*-arsanilic acid, ASA), 4-hydroxy-3-nitrophenylarsenic acid (roxarsone, ROX), and 4-nitrophenylarsonic acid (nitarsone, NIT), are used as feed additives in animal production. These organoarsenic derivatives prevent diseases, accelerate growth, increase feed efficiency, and improve meat pigmentation. ROX and ASA are also used to control hemorrhagic enteritis in the swine industry and to improve productivity in poultry. NIT is the drug of choice for the prevention and treatment of histomoniasis in turkeys, a disease caused by a protozoan, *Histomonas meleagridis*. These organoarsenic compounds show low bioaccumulation potential and are largely excreted unchanged (Arroyo-Abad et al., 2011; Sapkota, Lefferts, McKenzie, & Walker, 2007; Sierra-Alvarez, Cortinas, & Field, 2010). Arsenic (As) contaminated poultry litter is sold and distributed as fertilizer. Therefore, contaminated poultry litter can be a source of As contamination for crops, soil or water in which phenylated As species undergo biogeochemical degradation. Thus, those compounds are transformed into stable but more toxic inorganic As compounds, such as arsenite (As(III)) and arsenate (As(V)), which may pose a potential risk to both human health and the environment (Fisher, Yonkos, & Staver, 2015; Makris, Quazi, Punamiya, Sarkar, & Datta, 2008; Sapkota et al., 2007; Yao, Huang, He, Zhou, & Li, 2013). The maximum allowable dosages in feed for ROX and ASA that have been approved in China are 50 and 100 mg kg^{-1}, respectively (L. Wang & Cheng, 2015). However, the European Food Safety Authority recommends the administration of 187.5 mg kg^{-1} NIT as an additive in feed (European Food Safety, 2004). Therefore, it is very important to develop rapid and simple analytical methods for determination of organoarsenic compounds in feed samples.

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Analytical methods that have been reported for the determination of phenylarsenic species are hyphenated techniques based on the combination of a chromatographic separation technique and element-selective/specific detector. Different separation techniques, including high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE) coupled with inductively coupled plasma mass spectrometry (ICP-MS) (Conklin, Shockey, Kubachka, Howard, & Carson, 2012; L. Liu, He, Yun, Sun, & Jiang, 2013; Mao, Chen, Huang, He, & Hu, 2011; Schmidt, Kutschera, Mattusch, & Otto, 2008; Sierra-Alvarez et al., 2010; P. Wang, Zhao, Tian, & Su, 2010) and atomic absorption or fluorescence spectrometry in combination with hydride generation (HG-AAS and HG-AFS) (Hagiwara, Inui, Koike, & Nakamura, 2013; J. Liu et al., 2008; Matoušek et al., 2008; Monasterio, Londonio, Farias, Smichowski, & Wuilloud, 2011), have been evaluated for the determination of these organoarsenic compounds in different environmental samples. Among these techniques, the use of HPLC-ICP-MS has been demonstrated to be the most effective technique for the determination of phenylarsenic compounds in complex sample matrices due to its advantages of needing no derivatization and requiring a simple interface. However, the determination of As species by HPLC coupled to HG-AFS represents a suitable alternative to the HPLC-ICP-MS technique (Cui, Xiao, Dai, Zhao, & Wang, 2012; J. Liu et al., 2008; Monasterio et al., 2011). HG-AFS has been reported to be similar to ICP-MS with regard to its sensitivity and linear calibration range. HG-AFS has some additional advantages for As speciation analysis, including simplicity, as well as lower acquisition and operating costs. Different HPLC separation modes, such as ion exchange (IE) (Conklin et al., 2012; Schmidt et al., 2008), reversed phase (RP) (Cui et al., 2012), and reversed phase ion pairing (RP-IP) (Mao et al., 2011; Monasterio et al., 2011; P. Wang et al., 2010), have been employed for As speciation in feed additives.

The goal of this work was to develop a method for quantifying three phenyl As compounds in livestock feed additives by high performance liquid chromatography coupled to ultraviolet oxidation hydride generation atomic fluorescence spectrometry (HPLC-UV/HG-AFS). Thus, the conditions for the extraction, separation, and determination of ASA, ROX and NIT in animal feeds were established. Sample preparation is a critical part of analysis because it isolates compounds of interest from the sample matrix and from potential interferences, avoiding their interconversion. The novelty of this study resides in the development of a microwave assisted extraction (MAE) protocol for organoarsenic compounds in feed samples using phosphoric acid as extracting agent. The organoarsenic species are usually extracted from environmental samples using several solutions, such as a methanol/water mixture (Cui et al., 2012; J. Liu et al., 2008; P. Wang et al., 2010), 1% (v/v) acetic acid/methanol (10:90, v/v) (Chen et al., 2011), 0.5 M phosphoric acid (X. Liu, Zhang, Hu, & Cheng, 2013), 9:1 $NH_4H_2PO_4/H_3PO_4$ mixture (Yao et al., 2013), and 25 mM sodium hydroxide (J. Wang, Nie, Fu, & Wang, 2010). Extraction procedures in the media mentioned above are usually assisted by various techniques, such as shaking with extraction times of 30 min to 16 h (X. Liu et al., 2013; J. Wang et al., 2010; P. Wang et al., 2010), sonication with a sample pre-treatment time from 2 to 10 h (Chen et al., 2011; J. Liu et al., 2008; Yao et al., 2013), or accelerated solvent extraction employing an extraction time of approximately 12 min (Cui et al., 2012). To improve the extraction efficiency and reduce both the extraction time and volume of solvent required in traditional extraction methods, such as shaking or sonication, a microwave-enhanced protocol was proposed in this study. The number of parameters needed for optimization in MAE was lower, and it was easier to set up compared with other automated procedures, such as accelerated solvent extraction (Cui et al., 2012). The parameters studied for optimization of the MAE method were temperature, extraction time, and extractant concentration. Then, As species in samples were separated by reversed-phase HPLC coupled to ultraviolet oxidation HG-AFS. The proposed method was used to analyze livestock feed samples. The analytical results were validated using mass balance by comparing the total extracted As and sum of the concentrations of the individual As species.

2. Experimental

2.1. Reagents and standards

All chemicals were of analytical grade quality. Ultrapure water from a Milli-Q system (18 MΩ cm, Millipore, Bedford, MA, USA) was used to prepare the solutions. Before use, all laboratory glassware and plastic ware were thoroughly cleaned using diluted nitric acid (<3 M) and then rinsed with Milli-Q water. Arsenic stock solutions (1000 mg L^{-1}) were prepared from As_2O_3 (99.0% purity, Sigma-Aldrich, St. Louis, MO, USA). Analytical standards of acid (4-aminophenyl) arsenic (*p*-arsanilic acid 99.0% purity, ASA), acid (4-hydroxy-3-nitro phenyl) arsenic (roxarsone 99.9% purity, ROX), and acid (4-nitrophenyl) arsenic (nitarsone 99.6% purity, NIT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard stock solutions (100 mg L^{-1}) of ASA, ROX and NIT were prepared using ultrapure water and stored at 4 °C in dark glass bottles. Hydrochloric acid (37% w/v) and nitric acid (67% w/v) were for trace metal analysis (Fisher scientific). H_2O_2 (30% v/v) and $CuSO_4$ (≥99%) were from Sigma-Aldrich (St. Louis, MO, USA). H_3PO_4 (99.99% trace metals basis) used for organoarsenic compound extraction, potassium iodide (KI, 99.5%), thiourea (CH_4N_2S, 99.0%), and L-ascorbic acid ($C_6H_8O_6$) used for reducing arsenate to arsenite during total As determination were from Sigma-Aldrich (St. Louis, MO, USA). Potassium peroxodisulfate ($K_2S_2O_8$, 99.9%, Sigma-Aldrich St. Louis, MO, USA) at 2% (w/v) prepared in sodium hydroxide (NaOH, Sigma-Aldrich St. Louis, MO, USA) at 0.5% (w/v) was used as the oxidant for the photo-oxidation processes. A solution of hydrochloric acid 10% (v/v) and a solution of sodium borohydride ($NaBH_4$, >99%, Sigma-Aldrich, St. Louis, MO, USA) at 2% (w/v) in 0.5% (w/v) NaOH were used for the hydride generation step and were prepared daily.

The certified reference materials (CRMs) DORM-2 (Dogfish Muscle CRM, National Research Council of Canada) and BCR-279 (Sea Lettuce CRM, Institute for Reference Materials and Measurements of Belgium) were used to verify the accuracy of the method during total As determination.

2.2. Instrumentation

The total content was assessed and speciation As analysis was performed by a HG-AFS (AF-640, Rayleigh Analytical Instrument Co. Beijing, China) equipped with a 193.7 nm line source with a hollow cathode lamp operating at a PMT voltage of 280 V and primary current of 100 mA.

HPLC separation was achieved using a column C8 Luna (150 × 4.6 mm i.d., 5 μm) protected by a guard column (4 × 3 mm i.d.) from Phenomenex, Inc. For separation, 2% acetic acid/methanol (96:4, v/v) at a 1 mL min^{-1} flow rate was used as the mobile phase in the isocratic elution mode with an injection volume of 300 μL. The HPLC system consisted of an SY-8100 pump (Beifen-Ruili Analytical Instrument (Group) Co., Ltd.).

The total As content and As speciation analyses in animal feed samples were based on peak areas using external standards, and measurements were performed in triplicate.

2.3. Sample collection and preparation

Seven commercial animal feed samples, including pig, horse, chicken and rooster, were obtained from different factories located within the metropolitan area of Monterrey, Mexico that produce, distribute and sell feed supplies.

The samples were pulverized using a mortar and pestle to reduce the particle size. Then, the fine powder was passed through a sieve with a pore size of 250 μm (W.S. Tyler™, USA), and stored in sealable plastic bags at 4 °C until analysis.

2.4. Total arsenic determination

The digestion of approximately 200 mg of feed sample was conducted in a microwave digestion system (Mars 6, CEM, USA) using a mixture of 3.0 mL of concentrated HNO_3, 1.0 mL of concentrated HCl and 1.0 mL of H_2O_2. The temperature was raised to 210 °C over 20 min and held at that temperature for 10 min. After cooling, the digests were centrifuged for 10 min at 8000 rpm (Sorvall ST 16 Centrifuge, Thermo Fisher Scientific) and filtered through 0.45-μm Teflon syringe filters (Phenomenex Torrance, CA, USA). Then, the samples were diluted to 50 mL with ultrapure water. The CRM samples were digested and diluted in the same way as the feed samples. For total As determination, a 1-mL aliquot of the supernatant was transferred into a 15 mL polyethylene tube; then, 1 mL of the mixture of the reductant solution (10% (w/v) thiourea/10% (w/v) KI/% (w/v) ascorbic acid) was added and diluted to 5 mL with ultrapure water. The sample was left to react for 30 min. The conditions used for arsine (AsH_3) generation were adapted from previously described studies (dos Santos, Cavalcante, Macedo, Nogueira, & da Silva, 2012; Wei & Liu, 2007).

The standards for the total As calibration curve were prepared in the range of 0.05–200 mg As L^{-1} from the 1000 mg As L^{-1} As_2O_3 stock standard solution. A matrix-matched calibration curve based on the peak area was used to quantify total As in the digested samples. The limit of detection (LOD) was determined as the concentration that gave a signal equal to three times the standard deviation of the reagent blank, and the LOD was found to be 0.02 μg L^{-1}. The limit of quantification (LOQ), defined as the concentration equivalent to ten times the standard deviation of the reagent blank, was found to be 0.06 μg L^{-1}. Each sample was analyzed in triplicate, and blank digests were measured in the same way.

2.5. Extraction of organoarsenic compounds

Phosphoric acid was used to extract organoarsenic compounds from feed samples (X. Liu et al., 2013). Approximately 500 mg of sample was accurately weighed and transferred into the microwave Teflon vessel, and then, 10 mL of 1.5 M H_3PO_4 was added to the sample. The sample was extracted for 45 min at 120 °C using the MAE system, and then, the extracts were centrifuged at 8000 rpm for 10 min. Subsequently, the supernatant was transferred to polypropylene tubes, and 2 mL of 2% (w/v) $CuSO_4$ was then added for protein precipitation (Chen et al., 2011). The resulting mixture was vortex-mixed for 2 min, followed by 10 min centrifugation at 5000 rpm. The final supernatant was transferred and diluted to 30 mL with water. All extractions were performed in triplicate. The method blank was carried throughout the entire extraction procedure. To investigate the stability of the As species, extraction of the method blank and poultry feed sample labeled as chicken 1 spiked with ROX, ASA, and NIT standard solutions at 100 μg L^{-1} each was performed. The samples were filtered through a 0.45-μm membrane syringe filter, and 300 μL of the filtered extract was injected into the chromatographic column used for As-species separation. For As speciation analysis by HG-AFS, destruction of the organic part of the phenylated arsenic molecules was required prior to the HG step and was achieved by photo-oxidation step with 2% (w/v) $K_2S_2O_8$ in 0.5% (w/v) NaOH media under UV radiation. Quantification was performed by external calibration, based on peak areas, against standard As species. Regarding calibration, the slope obtained for normal calibration was compared with the slope of the standard addition method. The results indicated no matrix effects for evaluated samples, so external calibration was used throughout this study.

3. Results

3.1. HPLC-UV/HG-AFS determination

Target As species are weak acid compounds with high polarity and water solubility that can be separated by reversed phase chromatography (Cui et al., 2012; Chen et al., 2011). The chromatographic method used was slightly modified from that previously reported by Chen et al. (Chen et al., 2011). An improvement in peak shape and separation performance was achieved using an isocratic procedure with 2% acetic acid/methanol (96:4, v/v).

Because organoarsenic compounds do not generate hydride-active As species, it was necessary to break down the C-As bonds before HG-AFS determination because of their previous conversion by UV decomposition. In this work, an on-line procedure for the photo-oxidation of organoarsenic species by the combination of potassium peroxodisulfate in an alkaline solution and UV light before hydride generation was performed. Potassium peroxodisulfate allowed relatively fast kinetics for digesting organoarsenic compounds (Monasterio et al., 2011). The concentration of potassium peroxodisulfate and the flow rate was optimized using ROX as a model compound. The results are shown in Fig. 1a. The increase in the concentration of $K_2S_2O_8$ resulted in a continuous improvement of the decomposition efficiency of ROX up to a concentration of 2.0% $K_2S_2O_8$. Further increases in the $K_2S_2O_8$ concentration resulted in a decrease in the oxidation efficiency of ROX. This effect could be attributed to the consumption of the reducing agent KBH_4 by $K_2S_2O_8$ that originated the increase in signal fluctuation and background noise. Efficient ROX UV photo-oxidation was achieved for 2% $K_2S_2O_8$ with the slower flow rate. The flow rate of peroxodisulfate was set at 1 mL min^{-1}. Therefore, 2% $K_2S_2O_8$ in 0.5% NaOH at 1 mL min^{-1} was chosen for further experiments.

It was necessary to study the conditions for optimal hydride generation under the influence of the mobile phase composition. The $NaBH_4$ concentration varied between 1.0 and 3.0% (w/v) (see Fig. 1b). Better results were obtained with 2% (w/v) $NaBH_4$ because a low concentration of KBH_4 resulted in insufficient hydride generation. However, higher concentrations resulted in more pronounced signal fluctuations that were associated with increased production of hydrogen, leading to high signal to noise ratio and detection limits. $NaBH_4$ concentrations between 1.1 and 3.0% (w/v) have been commonly described for HPLC-HG-AFS methods (He, Jiang, & Xu, 2000; Monasterio et al., 2011; Sun, Liu, Wu, & Liu, 2013; Vilanó & Rubio, 2001; Yuan, He, Gao, Lü, & Jiang, 2007). Furthermore, an acidic medium was required for the formation of hydrides. The hydride generation efficiency of the organoarsenic species strongly depends on the conditions of HG, mainly on the HCl concentration. HCl concentrations from 7.0 to 12.5% (v/v) have been used for the formation of hydrides during As speciation analysis by HPLC-HG-AFS (He et al., 2000; Monasterio et al., 2011; Sun et al., 2013; Yuan et al., 2007). Based on these previous published studies, 10% (v/v) HCl was selected for the hydride generation step.

The analytical performance of the proposed method was evaluated under the optimum conditions, and the results are listed in

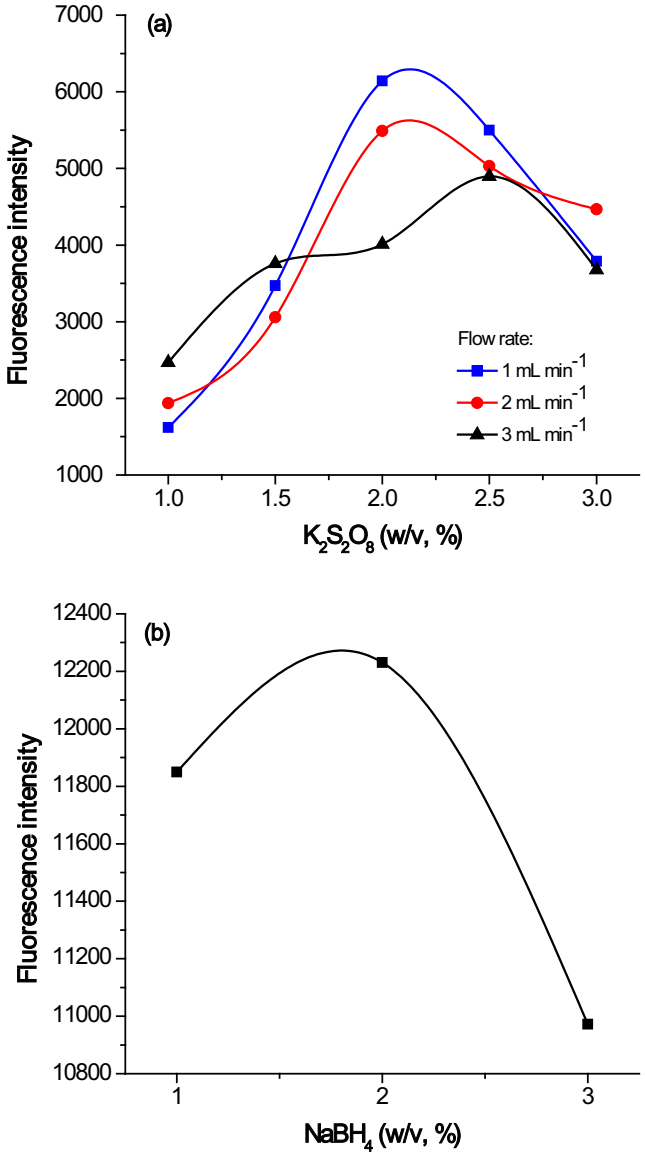

Fig. 1. Effect of: (a) the concentration and flow rate of $K_2S_2O_8$ on the oxidation efficiency and (b) the concentration of $NaBH_4$ in NaOH 0.5% (w/v) on the hydride generation efficiency of 100 μg L^{-1} of ROX.

Table 1
Analytical performance for organoarsenic compound determination by HPLC-UV/HG-AFS.

Analyte	ASA	ROX	NIT
Linear range (μg L^{-1})	5–400	5–400	5–400
Calibration curve	y = 15.68x + 35.20	x = 14.14x + 27.87	x = 12.94x − 53.94
Correlation coefficient (r)	0.9999	0.9999	0.9996
LOD (μg L^{-1})	0.13	0.09	0.08
LOQ (μg L^{-1})	0.44	0.30	0.28
RSD (%)[a]	3.26	5.30	5.35

[a] Relative Standard Deviation (RSD) was calculated for 200 μg L^{-1} standard solutions of ASA, ROX and NIT (n = 5 replicates).

Table 1. The calibration curves of the HPLC-UV/HG-AFS system were constructed for mixed standards concentrations of 5–400 μg L^{-1} with a correlation coefficient (r) in the range of 0.9996–0.9999. The three organoarsenic compounds were completely eluted within 7 min during HPLC-UV/HG-AFS analysis. A typical chromatogram is shown in Fig. 2a. The LODs were from 0.08 to 0.13 μg L^{-1}, with the relative standard deviations (RSDs) ranging from 3.26 to 5.35%, indicating good repeatability of the analysis. The detection limits for organoarsenic compounds obtained by HPLC-UV/HG-AFS were comparable with those previously reported by HPLC-UV/HG-AFS S (J. Liu et al., 2008; Monasterio et al., 2011) and HPLC/ICP-MS (X. Liu et al., 2013; P. Wang et al., 2010), while better LODs were achieved in this study than those reported by HPLC with UV detection (Chen et al., 2011). The benefits of the HG-AFS detector compared to ICP-MS are its lower operating and instrumentation costs and that it is an easier technique to perform (Gómez-Ariza, Sánchez-Rodas, Giráldez, & Morales, 2000). However, the use of a non-selective As detection technique, such as UV, could interfere with the analysis of real samples (J. Wang et al., 2010).

3.2. Extraction method development

The use of a phosphoric acid solution in the concentration range from 0.3 to 0.5 M has commonly been reported to be efficient for recovering As species from environmental and biological samples, such as soils, sediments, plants, and dietary supplements (Bohari et al., 2002; Wolle, Rahman, Kingston, & Pamuku, 2014; X. Liu et al., 2013). The better performance of phosphoric acid could be attributed to its capacity to break As-S bonds (Bohari et al., 2002) and the physicochemical similarities of As and phosphorus (Wolle et al., 2014). Phosphoric acid, as an extractant, was thus evaluated in this study using microwave irradiation. For evaluation of the extraction procedure, a feed sample labeled as chicken 1 was used due to its high total As concentration. Full factorial design with three levels is one of the most frequently applied chemometric tools in multivariate optimization for screening in order to evaluate the effect of the main factors and their interactions (Ferreira et al., 2017; Soylak, Narin, Bezerra M de, & Ferreira, 2005). The experiments were designed and analyzed using the software Statistica® version 10.0 (StatSoft, Oklahoma, USA). Preliminary experiments were conducted to select the operating conditions of the microwave oven. Thus, the extraction time (60 min), amount of sample (500 mg) and extractant volume (10 mL) were selected. A

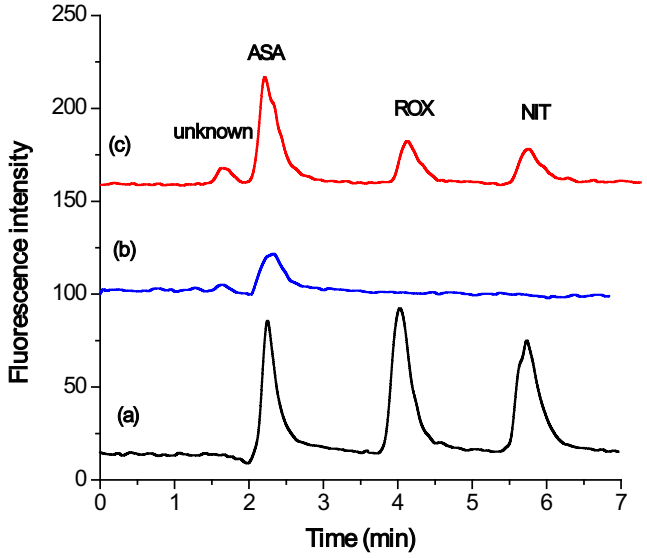

Fig. 2. HPLC-UV/HG-AFS chromatograms of: (a) the organoarsenic standards of ASA, ROX and NIT at 200 μg L^{-1} each; (b) non-spiked feed sample labeled as chicken 1 extract; and (c) chicken 1 extract spiked with standard solution of As species at 50 μg L^{-1} each.

three-level 3^2 full factorial design with 11 runs (duplicate of the central point) was used to determine the influence of the factors and their interactions on the system. In the first experimental design, the two factors studied were the extraction temperature (60, 90 and 120 °C) and H_3PO_4 concentration (0.5, 1.5 and 1.5 M). The experimental design matrix and results derived from each run (coded and real values) are indicated in Table 2. The design was evaluated using the total As extraction efficiency as the response. The values of R^2 (0.9994) and R^2-adjusted (0.9968) showed a good fit using a quadratic model. The pure error was very low (1.2321), indicating good reproducibility of the experimental data. The data of this experiment were evaluated by analysis of variance (ANOVA) by using P-value significance levels considering the coded values. The Pareto chart (Fig. 3) indicated at the 95% confidence level that the linear terms for the two parameters investigated (H_3PO_4 concentration and temperature) and the linear interaction effects were statistically significant and were positive. The quadratic terms of H_3PO_4 concentration and temperature were also significant. As can be seen in Fig. 3, the H_3PO_4 concentration was the most critical parameter for As extraction recovery. According to the results, stringent extraction conditions H_3PO_4 (1.5 M) and extraction temperature (120 °C) were required to provide the maximum As extraction efficiency. Given the above results, the optimal conditions were found by a second experimental design. Although temperature was a significant parameter, it was fixed at 120 °C because phenylated As compounds have been described as relatively stable As species at temperatures below 120 °C (Cui et al., 2012). The H_3PO_4 concentration (1.0, 1.5 and 2.0 M) and extraction time (30, 45 or 60 min) were studied by applying a second three-level full factorial design (3^2) with a duplicate of the central point. The experimental conditions (coded and real values) and the results for As extraction efficiency according to the factorial design are presented in Table 3. The factorial design model showed a good fit of experimental data using a quadratic model because the correlation coefficient R^2 and R^2-adjusted values were 0.9955 and 0.9773, respectively. The effects of each main factor as well as the respective interactions (coded values) are presented in Table 4. Regarding the statistical analysis of the experimental data, the most important effect could be attributed to the factor H_3PO_4 concentration, which provides a more efficient extraction procedure when 2 M H_3PO_4 was used. The As extraction from feed samples was also affected by the quadratic term of H_3PO_4. No significant effects were observed for the extraction time, and for H_3PO_4 concentration-extraction time linear and quadratic interactions. Thus, an extraction time of 45 min was adopted in subsequent experiments.

Additionally, it is very important in speciation analysis to investigate whether individual species are altered during the extraction procedure to confirm the reliability of the proposed analytical method. Further experiments were carried out to evaluate the stability of As species during extraction at 120 °C for 45 min using different H_3PO_4 concentrations (1.0, 1.5 and 2.0 M). The effect of the extractant concentration on the stability of the As species was evaluated using the feed sample labeled as chicken 1 spiked with a standard solution of ASA, ROX or NIT (at a 100 μg L^{-1} concentration each). The spiked sample was analyzed by HPLC-UV/HG-AFS to verify the As compound stabilities during the extraction procedure. The results are shown in Fig. 4. The total As and As species recoveries were poor using 1 M H_3PO_4. Although total As was quantitatively extracted using 2.0 M H_3PO_4, degradation of the

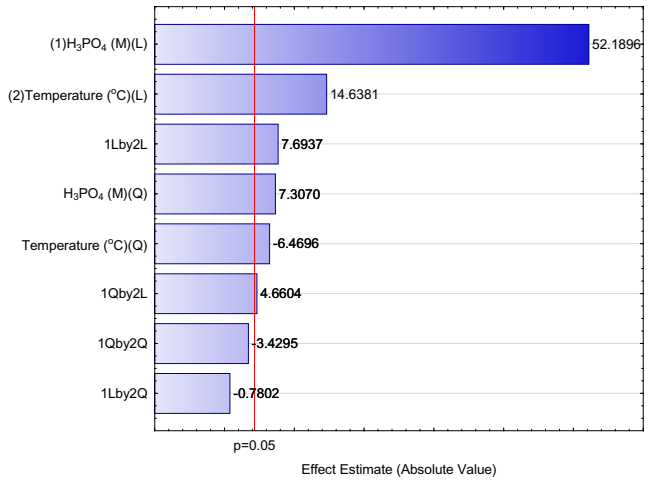

Fig. 3. Pareto chart of the effects for variables (coded values): H_3PO_4 (M) and time (min) using As extraction efficiency as response.

Table 2
Full factorial 3^2 design matrix and extraction efficiency of the As (%) response values for the first experimental design (fixed microwave extraction time at 60 min).

Experiment	H_3PO_4(M)	Temperature (°C)	Total As extraction Efficiency (%)
1	0.5 (−1)	60 (−1)	32.67
2	0.5 (−1)	90 (0)	31.20
3	0.5 (−1)	120 (+1)	34.41
4	1.0 (0)	60 (−1)	54.71
5	1.0 (0)	90 (0)	56.45
6	1.0 (0)	120 (+1)	73.95
7	1.5 (+1)	60 (−1)	71.93
8	1.5 (+1)	90 (0)	77.50
9	1.5 (+1)	120 (+1)	90.75
10	1.0 (0)	90 (0)	57.56
11	1.0 (0)	90 (0)	55.34

Table 3
Design matrix (full factorial 3^2) and response value (As extraction efficiency, %) for the second experimental design (fixed microwave extraction temperature at 120 °C).

Experiment	H_3PO_4(M)	Time (min)	Total As extraction efficiency (%)
1	1.0 (−1)	30 (−1)	68.87
2	1.0 (−1)	45 (0)	70.93
3	1.0 (−1)	60 (+1)	73.95
4	1.5 (0)	30 (−1)	95.74
5	1.5 (0)	45 (0)	91.08
6	1.5 (0)	60 (+1)	90.75
7	2.0 (+1)	30 (−1)	95.85
8	2.0 (+1)	45 (0)	99.49
9	2.0 (+1)	60 (+1)	96.94
10	1.5 (0)	45 (0)	92.75
11	1.5 (0)	45 (0)	89.41

Table 4
Statistical analysis of experimental data (coded values): effects (main and interactions factors).

Factor	Effect	Standard error(pure error)	t(2)	P
Mean/Interc.	87.0667	0.5357	162.5431	0.0000
(1)H_3PO_4 (M)(L)	26.1767	1.3635	19.1974	0.0027
H_3PO_4 (M)(Q)	8.1850	1.0899	7.5099	0.0173
(2)Time (min)(L)	0.3933	1.3635	0.2885	0.8001
Time (min)(Q)	0.1500	1.0899	0.1376	0.9031
1L by 2L	−1.9950	1.6700	−1.1946	0.3547
1L by 2Q	1.7875	1.4463	1.2359	0.3419
1Q by 2L	−4.0375	1.4463	−2.7917	0.1079
1Q by 2Q	−1.7363	1.0507	−1.6525	0.2402

*$\alpha = 0.05$.

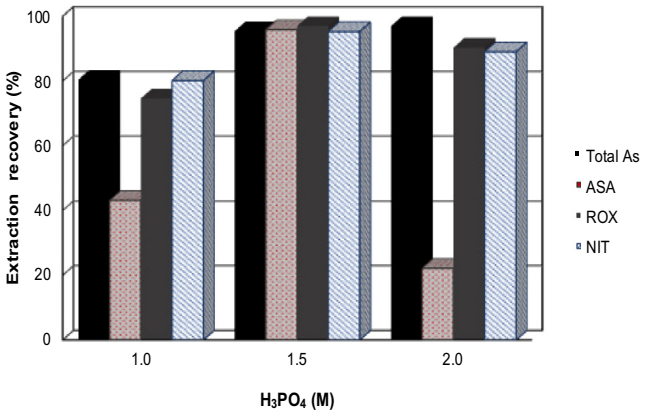

Fig. 4. Total As and organoarsenic species recoveries after the MAE procedure at 120 °C during 45 min for sample labeled as chicken 1. The concentration of As species ASA, ROX and NIT is 100 μg L^{-1} each (expressed as As).

ASA compound was achieved under these experimental conditions. Total arsenic (approximately 97%) as well as the three As species (95.2–97.0%) were quantitatively recovered when 1.5 M H_3PO_4 was used as the extracting agent. Thus, the results clearly indicated that the MAE procedure using 1.5 M H_3PO_4 at 120 °C for 45 min extracted phenylarsonic compounds quantitatively and did not lead to significant changes in these compounds in the feed samples.

3.3. Application to feed samples

The total As and As speciation in livestock feed samples were determined. The results are shown in Table 5. The accuracy of the determination procedure for total As was validated by using the certified reference materials DORM 2 (18.00 ± 1.10 mg As kg^{-1}) and BCR 279 (3.09 ± 0.21 mg As kg^{-1}). The results obtained from the triplicate analysis were 19.08 ± 1.19 and 2.94 ± 0.30 mg As kg^{-1}, respectively, and showed the absence of significant differences at the 95% confidence level between the As concentration found and certified values. Therefore, the analytical method used was suitable for total As determination in the feed samples.

The total As content in the feed samples varied from 1.47 to 19.83 mg kg^{-1}. Table 5 shows that the As concentration in the chicken feeds was higher than the As concentration in the pig feeds. The total As concentration in the chicken samples was comparable to studies reported in China (Yao et al., 2013) and the United States (Fisher et al., 2015). The concentrations found in three of the analyzed samples in this study exceeded the total As limit issued by the Chinese Hygienical Standard for Feeds (HSF) for formula feed (2.0 mg kg^{-1}) (13078-2001, 2001).

As speciation in animal feeds was carried out using the extraction and HPLC-UV/HG-AFS method developed in this study. Method detection limits (MLDs) were calculated by quantifying eight replicate extractions of feed samples spiked at low-level of phenyl As compounds. The MDLs were calculated as the product of the Student's t value of the eight replicates (at n − 1 degrees of freedom and α confidence level, 1%) and the standard deviation (SD), recommended by U.S. Environmental Protection Agency (Bloom, Preus, Katon, & Hiltner, 2003; O'Neill, Rochette, & Ramsey, 2002). The MDLs obtained were 0.06, 0.03 and 0.03 mg kg^{-1} for ASA, ROX and NIT, respectively. The mass balance between the total As concentration (digestion procedure) and total As extracted provided an estimation of the extraction yield. The recoveries achieved for the extraction of organoarsenic compounds in real samples using the MW extraction method were in the range of 76.2–99.2% and depended on the sample matrix composition that could interfere during extraction of the organoarsenic species. The protein content in the feed samples has been described as interfering with the determination of organoarsenic additives, and this interference could be avoided using $CuSO_4$ 2% (w/v) as the precipitating agent (Chen et al., 2011).

As shown in Table 5, ASA was the major species in the chicken feed samples. Fig. 2b and c show chromatograms of As species in the extract and spiked extract of chicken feed sample 1, respectively. The concentration of ASA was in the range of 0.98–

Table 5
Determination of organoarsenic compounds in commercial feed samples.[a,b]

Labeled sample	Other As species detected (mg kg^{-1})	ASA (mg kg^{-1})	ROX (mg kg^{-1})	NIT (mg kg^{-1})	Sum of species (mg kg^{-1})	Total As (mg kg^{-1})	Total As recovery (%)
Chicken 1	12.91 ± 0.50	6.44 ± 0.30	N.D.	N.D.	19.35 ± 0.58	19.83 ± 1.10	97.6
Chicken 2	0.72 ± 0.10	0.98 ± 0.10	N.D.	N.D.	1.70 ± 0.14	1.97 ± 0.01	86.3
Chicken 3	8.49 ± 0.20	4.49 ± 0.10	N.D.	N.D.	12.99 ± 0.22	13.1 ± 0.50	99.2
Pig 1	3.27 ± 0.02	N.D.	N.D.	N.D.	3.27 ± 0.02	4.29 ± 0.10	76.2
Pig 2	1.76 ± 0.07	N.D.	N.D.	N.D.	1.76 ± 0.07	1.83 ± 0.03	96.2
Rooster 1	1.32 ± 0.03	N.D.	N.D.	N.D.	1.32 ± 0.03	1.69 ± 0.01	78.1
Horse 1	1.43 ± 0.04	N.D.	N.D.	N.D.	1.43 ± 0.04	1.47 ± 0.20	97.3

[a] N.D.: not detected.
[b] Concentrations are expressed as the mean ± SD (standard deviation, n = 3).

6.44 mg kg^{-1}. Unknown As species were detected in the analyzed samples at concentrations ranging from 0.72 to 12.91 mg kg^{-1}. The most commonly detectable As impurities in feed samples were previously found to be arsenite (As(III)) and arsenate (As(V)), as well as low levels of monomethylarsonic acid (MMA) (Yao et al., 2013). The presence of these As species could be attributed to the process used for organoarsenic production.

Although the allowable limit of 100 mg of ASA kg^{-1} (namely, 34.5 mg of As kg^{-1}) has been proposed for animal feeds in China (L. Wang & Cheng, 2015), the United States Food and Drug Administration (US FDA) withdrew the use of this compound in animal feed (Fisher et al., 2015). Thus, the use of As compounds in animal production could represent an environmental and public health risk of As contamination.

4. Conclusions

In this work, a new method for the determination of phenyl As compounds in livestock feed samples based on a microwave assisted extraction procedure followed by HPLC-UV/HG-AFS detection was developed. The HPLC-UV/HG-AFS analytical method offered several advantages, including low cost, relative simplicity, high sensitivity, and good reproducibility. The MAE method for determining the organoarsenic additives was based on extraction with 1.5 M H_3PO_4 during 45 min at 120 °C. Although, the evaluation of factorial designs evidenced the importance of the parameters: phosphoric acid concentration (2 M H_3PO_4) and temperature (120 °C) on the As extraction efficiency from feed samples, it was chosen mild extraction conditions that ensures minimal species transformation or degradation. The three As species were stable under the selected extraction conditions, and the extraction efficiencies were all more than 95%. Thus, these results suggest that the developed method was suitable for the determination of phenylarsonic additives in feedstuffs. Analysis of commercial samples showed the presence of ASA compound in chicken feed samples at concentration levels between 0.72 and 12.91 mg kg^{-1}, which were below the use limit for animal feed in China.

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