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Organic Solvent-Free Reversed-Phase Ion-Pairing Liquid Chromatography Coupled to Atomic Fluorescence Spectrometry for Organoarsenic Species Determination in Several Matrices

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ABSTRACT: A novel method has been developed to determine As-containing animal feed additives including roxarsone (ROX), *p*-arsanilic acid (*p*-ASA) and nitarsone (NIT), as well as other organic As species (dimethylarsonic acid (DMAA) and monomethylarsonic acid (MMAA)) by ion-pairing high-performance liquid chromatography coupled to hydride generation atomic fluorescence spectrometry (IP-HPLC−HG-AFS). A simple isocratic reversed-phase (RP) HPLC method with a mobile phase containing citric acid and sodium hexanesulfonate (pH 2.0) was developed using a C_{18} column. The use of an organic solvent free mobile phase turns this methodology into an environmentally friendly alternative. Several ion pair forming agents, such as sodium hexanesulfonate, tetrabutylammonium bisulfate and perfluoroheptanoic acid, were studied. The limits of detection for As species were calculated in standard solution and resulted to be 0.2, 0.5, 0.6, 1.6, and 1.6 μg As L^{-1} for MMAA, DMAA, *p*-ASA, ROX and NIT, respectively. This method exhibited convenient operation, high sensitivity and good repeatability. It was applied to As speciation in different samples including arugula, dog food, dog urine and chicken liver.

KEYWORDS: Roxarsone, liquid chromatography, ion-pairing, arsenic, speciation, atomic fluorescence spectrometry

1. INTRODUCTION

The presence of organoarsenic compounds in environmental systems can be attributed to a variety of natural (methylation of inorganic As by microorganism yielding monomethylarsonic acid (MMAA), dimethylarsonic acid (DMAA), etc.) and anthropogenic sources.[1] On the other hand, a number of phenylarsonic compounds (PheAs), including Roxarsone (ROX), *p*-arsanilic acid (*p*-ASA) and nitarsone (NIT), have been used as additives to chicken and swine feed.[2−4] ROX (3-nitro-4-hydroxyphenylarsonic acid) and *p*-ASA (4-aminophenylarsonic acid) are normally used to prevent and treat coccidiosis in poultry, improve weight gain, promote growth by providing improved feed conversion, better feathering and increased egg production and pigmentation.[5−7] These PheAs are widely used in the USA and China as growth promoters in broilers and pigs.[5,8] NIT is the drug of choice for the prevention and treatment of histomoniasis, a disease caused by protozoan.[9] Although a fraction of ingested PheAs is retained in chicken or pork, they are mainly excreted.[8,10,11] Therefore, it can be stated that repeated annual poultry litter applications to agricultural soils may result in As buildup in soil, leading to plant uptake and subsequent transfer to the human food chain or leaching to groundwater.[10,12]

Inorganic As (arsenite and arsenate) undergoes enzymatic methylation in cells of various organisms (e.g., rat, fish and human) producing methylated organoarsenicals, chiefly MMAA and DMAA. These methylated species are important because methylated arsenicals are more rapidly excreted in urine than inorganic arsenicals.[13] Thus, methylation has been considered to be a detoxification mechanism. However, a more recent study has shown that some methylated As species, such as monomethylarsonous acid and dimethyarsinic acid, could be more toxic than originally thought or even than inorganic As. Therefore, methylation may not solely be a detoxification mechanism for this metalloid but could instead be considered a pathway for its activation.[14−16]

Analytical methods reported for separation and determination of PheAs and other As organic species include thin layer chromatography with a chromogenic agent, gas and liquid chromatography (GC and LC, respectively).[17] In LC, high-performance liquid chromatography (HPLC) is most used, coupled to several detectors including electrospray ionization (ESI),[2,18] inductively coupled plasma mass spectrometry (ICP-MS),[18−21] hydride-generation atomic absorption spectrometer (HG-AAS),[22] hydride-generation atomic fluorescence spectrometry (HG-AFS)[23−26] and electrospray mass spectrometry (ES-MS),[27] among others. Gas chromatography−mass spectrometry (GC−MS) with derivatization has been also used.[3] Another technique includes capillary electrophoresis (CE) with direct UV[28,29] or ICP-MS detection.[30] Although some of these methods have acceptable detection limits for determination of As species in a broad variety of samples, they involve more complex instrumental setup or have relatively high cost.

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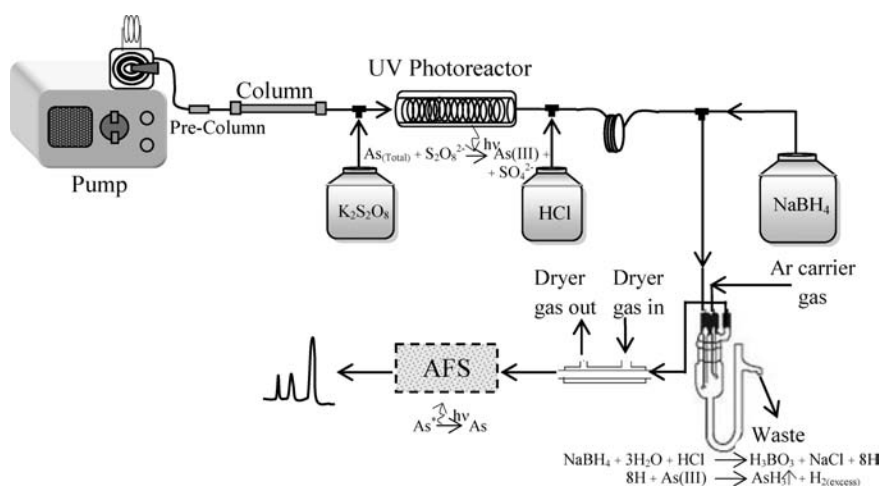

Figure 1. Schematic design of manifold and instrumental setup used for IP-HPLC−HG-AFS determination of As species. Detection and chromatographic conditions were as mentioned in Table 2.

Arsenic species have been separated by HPLC using ion exchange and ion-pairing principles.[31−34] Ion exchange is normally used in separations of charged analytes, preferably with simple matrices as it can be affected by interferences.[31] On the other hand, ion-pairing chromatography has the potential of separating both charged and uncharged analytes, while matrix effects are minimized.[34] Furthermore, hydrophobicity of ion pairs can be controlled by a careful choice of the ion-pairing agent, thus enhancing the selectivity. Typical ion-pairing agents for As species separation have involved trifluoroacetic acid, as well as alkylammonium or alkylsulfonate group containing ions.[34−36] Ion-pairing chromatography is an adsorptive process, which relies on a partitioning mechanism for achieving separation. Ion pairs formed between these agents and As species were in general highly polar, and for this reason had an important affinity for aqueous mobile phase. Therefore, separation of As species has been performed using mobile phases with some percentage of solvent, thus increasing ion-pair affinity for stationary phase to attain separation.[35]

The aim this work was the development of a novel chromatographic method for separation of several organic As species with no organic solvents in the mobile phase. Thus, sensitivity loss for As detection by hydride generation atomic fluorescence spectrometry (HG-AFS) in the presence of organic solvents was avoided. Moreover, the proposed chromatography could be suitable for coupling with more expensive detectors, such as ICP-MS. The choice of HPLC separation conditions was based on several parameters (i.e., type of ion-pairing agent, pH, and composition of mobile phase, etc.). The isocratic ion-pairing high-performance liquid chromatographic (IP-HPLC) separation coupled to AFS detection was applied for As speciation studies in arugula, dog food, dog urine and chicken liver samples.

2. EXPERIMENTAL PROCEDURES

2.1. Instrumentation. Separation of As species was developed with a chromatographic system consisting of an Alltech 301 HPLC pump (NZ. Alltech Inc., Auckland, New Zealand), a Rheodyne valve with a 100 μL loop injector (Cotati, CA, USA) and a column heater (Thermo Electron Corporation, Japan). Separation of ROX, NIT, *p*-ASA, DMAA and MMAA was performed with a reversed-phase chromatography Zorbax SB-AQ column from Agilent Technologies (Santa Clara, CA, USA). Photo-oxidation of As species was achieved with a PSA 10.570 ultraviolet oxidation system (PS Analytical, Orpington, U.K.) using a $K_2S_2O_8$ solution. The measurements were performed with a PSA 10.055 Millennium Excalibur atomic fluorescence spectrometer (PS Analytical). Analytical conditions are mentioned in Table 2, and IP-HPLC−HG-AFS instrumental setup is shown in Figure 1.

2.2. Reagents and Standards. Roxarsone (98.1%) ($C_6H_6NO_6As$), nitarsone (99.5%) ($C_6H_6NO_5As$) and *p*-arsanilic acid ($C_6H_8NO_3As$) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Disodium methylarsonate (98%) ($CH_3AsNa_2O_3 \cdot 6H_2O$) and dimethylarsonic acid (98.6%) ($C_2H_7AsO_2$) were obtained from Fluka (Buchs, Switzerland). Stock standards solutions containing all As species were prepared with ultrapure water (18 MΩ cm) (Labconco WaterPro PS water purification system, Kansas City, MO) and stored at 4 °C in amber-colored glass bottles. Working solutions were prepared by dilution of these stock standard solutions. A mobile phase solution containing citric acid (JT Baker, Xalostoc, Mexico) and sodium hexanesulfonate (NaHS, $C_6H_{13}NaO_3S$) (98%) (Sigma-Aldrich) was prepared by weighting accurate amounts of each reagent followed by dissolution in ultrapure water. Potassium peroxodisulfate (>99%) (Sigma-Aldrich) was used as oxidant for photo-oxidation process. Sodium borohydride (Fluka), dissolved in sodium hydroxide (Aldrich), and hydrochloric acid (Merck, Darmstadt, Germany) were used to form arsine. Concentrations and other specifications are mentioned in Table 2.

Tygon type pump tubing (Gilson, Villiers Le-Bell, France) was used to carry these reagents. All bottles used for storing samples and standard solutions and glassware were washed in 10% (v/v) nitric acid for 24 h and later rinsed with ultrapure water.

2.3. Sample Collection, Conditioning and Extraction Procedure. Urine samples were collected from adult domestic dogs of no specific race, fed based on a regular diet (based mainly on meat, chicken, rice and dog food). Early morning urine samples were collected from dogs in 10% HNO_3-washed glass bottles. Immediately after sampling, urine aliquots were stored at 4 °C. The samples were filtered through 0.45 μm pore membrane filters (Millipore Corporation, Bedford, MA, USA) before the analysis. Arugula, chicken liver and dog food samples were purchased in different local markets from Buenos Aires city (Argentina). Manipulation and analysis of samples were performed immediately after sampling.

For extraction of As species, arugula and chicken liver samples were cut in small pieces, freeze-dried and homogenized by grinding. Then, 0.5 g of arugula and 1 g of chicken liver were weighed and placed individually in 50 mL polypropylene tubes. The samples were extracted with 20 mL

of a mixture 1:1 methanol/water. The procedure was assisted by ultrasound for 20 min at 40 °C in an ultrasonic bath. The extraction of As species from dog food was performed first weighing 15 g of material followed by homogenization and grinding to a fine powder and final addition of 30 mL of water. The mixture was shaken for 20 min. Finally, all extracts were filtrated through 0.45 μm pore membrane filters and later injected into the HPLC column.[19−21]

All samples were spiked at two levels, 20 and 40 μg As L^{-1} of each As species in the extracts. Solids samples were spiked after complete grinding and before performing the extraction procedure. On the other hand, urine samples were directly spiked prior to filtration with equal As levels than solid samples.

2.4. Total As Determination. Total As occurring in the samples was determined by HG-AFS after mineralization in a muffle furnace for 3 h at 550 °C.[19] The accuracy of the mineralization procedure was checked by analysis of a certified reference material, BCR 402 white clover, with a certified value of 0.093 ± 0.010 mg of As kg^{-1}. Using this methodology, As concentration found in this SRM was 0.092 ± 0.010 mg of As kg^{-1} (n = 6 and 95% confidence interval).

3. RESULTS AND DISCUSSION

3.1. Optimization of HPLC Parameters. Since HPLC conditions, especially mobile phase composition, can greatly influence both separation of target compounds and AFS sensitivity,[37] the effects of mobile phase composition and the presence of additives in the mobile phase were carefully investigated.

Organic solvents have been successfully used in chromatographic separations of polar analytes. Since common As species are highly polar, the effects of different organic solvents in the mobile phase were studied to increase affinity of species for the stationary phase. However, a strong interferent effect on As signal has been observed upon the presence of organic solvents with AFS detection.[38] In fact, the interference has been demonstrated for any organic solvent, such as methanol, even at low concentration. Figure 2(a) shows As signal-to-background ratio for different methanol concentrations. Likewise, a significant increase of baseline signal was observed even since 1% (v/v) methanol in the mobile phase (Figure 2(b)). This can be attributed to the formation of a fine aerosol of organic solvent being carried toward the atomizer negatively affecting arsine atomization.[38−40] Therefore, to avoid sensitivity loss, the use of organic solvents in the mobile phase should be avoided when As species are to be detected by AFS. A feasible alternative to achieve that goal could be the use of chemically stable chromatography columns with 100% aqueous mobile phase. Thus, a Zorbax SB-Aq column, showing an alkyl reversed-phase bonded phase designed to retain hydrophilic species with up to 100% aqueous mobile phases, was selected for separation of As species in this work.

3.2. Selection of Ion-Pairing Agent and Effects on Separation Performance. Since As species in this study are very polar compounds, they have strong affinity for aqueous mobile phases. Thus, separation by reversed-phase chromatography is not feasible, since minor or no interaction with stationary phase is expected, causing elution of As species in the void volume of the column. In order to diminish the affinity of As species for the aqueous mobile phase, different ion-pairing agents were tested. Ion-pair chromatography (IPC) is a strategy that has been used in a variety of fields.[37] Thus, the main advantage of IPC is that it facilitates the separation of both ions and uncharged molecular species with minor interferences as compared to ion-exchange chromatography.[41] For separating As species, both ionic charge and hydrophobic

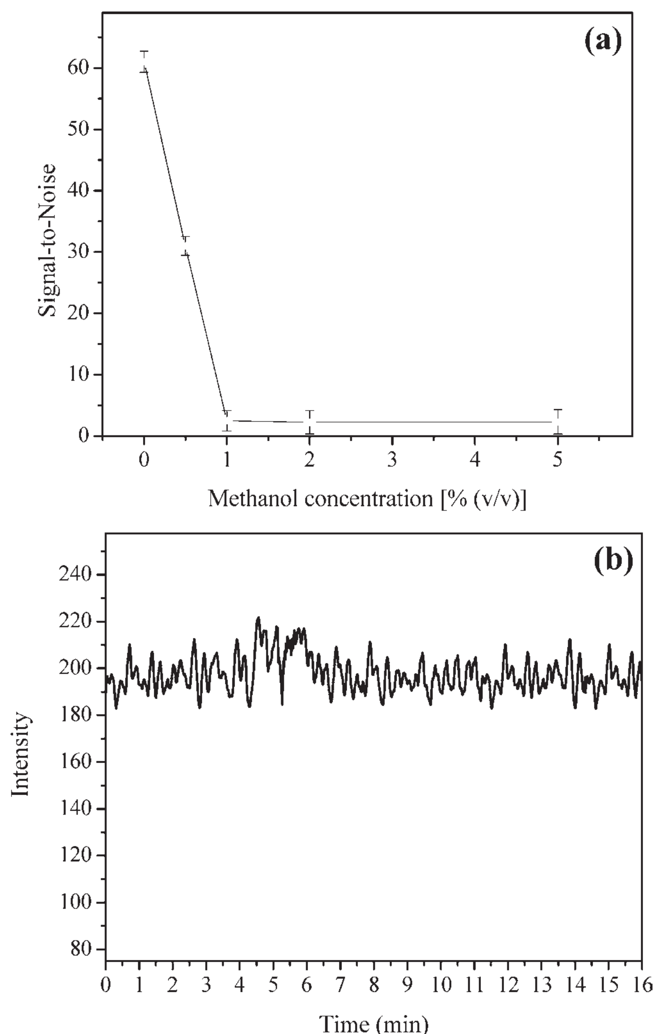

Figure 2. (a) Effect of methanol concentration in the mobile phase on As signal-to-background ratio with AFS detection. (b) Typical chromatogram obtained for As species standards (20 μg As L^{-1} of each As species) upon 1% (v/v) of methanol in the mobile phase. Instrumental conditions were as mentioned in Table 2.

character of analytes have been considered. Nevertheless, the exact mechanisms of chromatographic separation are not always clear, because the assessment of actual charge distribution in complex solutions is not a straightforward task (the dissociation equilibrium of As species is affected by parameters such as ionic strength, the presence of ion-pairing agents, etc).[37,41] Thus, several ion-pairing agents including, sodium hexanesulfonate (NaHS), tetrabutylammonium bisulfate (TBAB) and perfluoroheptanoic acid (PFHH) at different concentration levels (5, 10, and 15 mM), were studied. Different concentrations of each ion-pairing agent were assayed at different pH (1.5, 2.0, 3.0, 4.0 and 4.5). The resolution was achieved using 5 mM NaHS at pH 2.0 (Figure 3(a)). The presence of a greater number of polar groups in NaHS may be the reason to obtain better resolution as compared to other ion-pairing agents. On the other hand, when TBAB and PFHH were used, As species were not separated in the pH range under study (1.5 to 4.5) (Figure 3(b,c)). Other authors had obtained good resolution using these ion-pairing agents.[19,21,24,42] However, gradients of organic solvents and pH were required in those cases. As mentioned above, introduction of organic

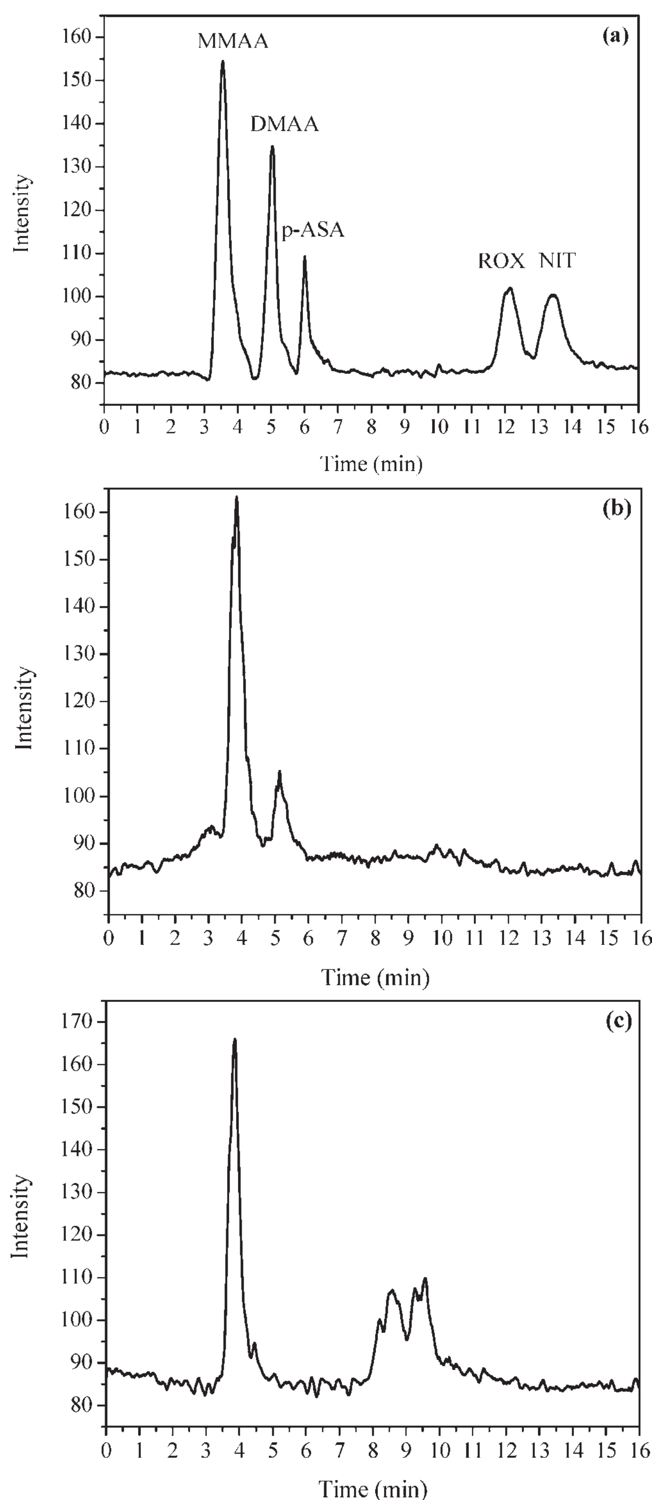

Figure 3. Chromatogram obtained for As species standards (20 μg As L^{-1} of each species) with (a) NaHS; (b) TBAB; and (c) PFHH as ion-pairing agents with a concentration of 5 mM and pH 2.0 in all cases, respectively. Other experimental parameters are illustrated in Table 2.

solvents into AFS atomizer could negatively affect analytical sensitivity, in addition to the need for higher equilibration time when gradients are employed for separation. Based on these findings, 5 mM NaHS concentration was chosen in this work.

Table 1. Chemical Names and Properties of As Species Studied in This Work[1,29,48]

Compound	Abbreviation	Elemental composition	Structure	pKa	Ref.
Roxarsone (3-nitro-4-hydroxyphenylarsonic acid)	ROX	$C_6H_6NO_6As$		3.9; 8.4; 10.1	(*1*)
Nitarsone (4-nitrophenylarsonic acid)	NIT	$C_6H_6NO_5As$		3.1; 7.8	(*48*)
p-Arsanilic acid (4-aminophenylarsonic acid)	p-ASA	$C_6H_8NO_3As$		2.0; 4.0; 8.6	(*1*)
Monomethylarsonic acid	MMAA	CH_5O_3As		4.6; 7.8	(*29*)
Dimethylarsonic acid	DMAA	$C_2H_7O_2As$		6.2	(*29*)

3.3. Effect of Buffer and pH on As Species Separation. The choice of citric acid as a buffer for the mobile phase was motivated based on the good performance obtained in previous works for separation of As species.[36,43] The pH of the mobile phase was studied in the range of 1.5 to 4.5 and between 5 and 25 mM citric acid. This range was adopted in order to achieve an ionized form of the analytes which led to ion-pair formation with NaHS. Therefore, As species under study whose pK_a values are lower than 4.5 exist in their ionic form, while NaHS is also well ionized (see Table 1). The highest resolution of the five As species under study was achieved with a 20 mM citric acid mobile phase at pH 2.0. Thus, alkylsulfonate ion-pairing agents generally requiring lower working pH values than other classes of agents were successfully applied, with the main advantage of obtaining complete separation of As species within a short time window.

It has been shown that ion-pairing agents in an aqueous mobile phase interact with hydrophobic stationary phases.[44] The ion-pairing agent is ionized at the working pH (pH 2.0), acting as an anion-exchanger toward solutes at the polar end while it interacts with the C_{18} phase at its hydrophobic end. Moreover, the interaction with the hydrophobic end of the column explains the elution order of As species (see Figure 3) as well as chromatographic parameters shown in Table 3. Interestingly, elution order is coincident with molecular size of As species. The longer retention times yielded larger capacity factors (k'), high peak broadening and lower detection limits, as compared with the first eluting analytes (see Table 3).

3.4. Mobile Phase Flow Rate and Column Temperature. With the aim of reducing separation time, the effect of mobile phase flow rate and column temperature on resolution of As species was studied between 0.3 and 1 mL min^{-1} and from 25 to 60 °C, respectively. Based on the elution time and optimum separation of each As species, 0.9 mL min^{-1} and 25 °C were selected for flow rate and column temperature, respectively (Figure 4).

Table 2. IP-HPLC−HG-AFS Instrumental Settings and Chromatographic Separation Conditions

HPLC	
column	Zorbax SB-AQ (5 μm × 4.6 mm i.d. × 150 mm)
mobile phase	20 mM citric acid−5 mM sodium hexanesulfonate (NaHS) pH 2.0
guard column	Zorbax Realiance Analytical Cartridge
flow rate	0.9 mL min^{-1}
injection volume	100 μL
column temperature	25 °C
Photo-Oxidation	
oxidant	2% (w/v) $K_2S_2O_8$ in 2% (w/v) NaOH
flow rate	0.3 mL min^{-1}
reactor coil	PTFE reactor, 11 m 0.508 mm id
Hydride Generation	
reductant and flow rate	1.1% (w/v) $NaBH_4$ in 1% (w/v) NaOH; 2 mL min^{-1}
carrier and flow rate	1.5 mol L^{-1} HCl; 2 mL min^{-1}
reactor coil	PTFE reactor, length: 1 m; 0.508 mm i.d.
Atomic Fluorescence Spectrometry	
lamp	hollow cathode As lamp; wavelength: 197.3 nm
primary current	27.5 mA
boost current	35.0 mA
carrier gas and flow rate	argon; 300 mL min^{-1}

Table 3. Chromatographic Parameters and Analytical Performance for As Species Determination[a]

	MMAA	DMAA	*p*-ASA	ROX	NIT
lineal range (μg L^{-1})	1.0−200	2.0−100	2.5−200	6.0−200	6.0−100
r^2	0.998	0.997	0.995	0.990	0.989
RSD (%)	3.1	3.9	4.5	6.3	6.8
LOD (μg L^{-1})	0.2	0.5	0.6	1.6	1.6
LOQ (μg L^{-1})	0.8	1.6	2.1	5.5	5.4
R^b		0.9	1.3	5.3	0.7
k'^c	0.9	1.4	2.0	5.3	6.0
N^d	318	353	678	1101	803
α^e		1.4	1.4	2.5	1.1

[a] Extraction conditions as described in section 2.3. [b] Resolution, $2(t_{R2} - t_{R1})/(w_2 + w_1)$. [c] Capacity factor, $k' = (t_R - t_0)/t_0$; t_R is the migration time and t_0 is the dead time. [d] Number of theoretical plates, $N = 16\,(t_R/w)^2$; w is the peak base width.[49] [e] Selectivity, $\alpha = k'_n/k'_{n-1}$.

3.5. UV Photo-Oxidation. In accordance with literature data on UV photo-oxidation alkaline peroxodisulfate has been selected owing to its high oxidation potential and relatively fast kinetics for digesting organoarsenical compounds.[45] Previous experience with online microwave-assisted digestion has come upon some problems with online microwave digestion. Several drawbacks, such as inhomogeneity of power distribution within the microwave cavity, evolution of gases and vapors that disturb the liquid flow, increased dispersion of the sample zone, aerosol formation and condensation of water vapors from heated liquid flows, have been reported.[32] To avoid those drawbacks, the method consisting of UV photo-oxidation of organoarsenic species with $K_2S_2O_8$ was chosen in this work. Different variables that could affect oxidation efficiency of the system were optimized. Poor analytical sensitivity resulting from insufficient photo-oxidation of As species was observed for low flow rates and concentration of $K_2S_2O_8$ solution. On the contrary, high flow rates caused a significant dilution effect on As peaks and hence, lack of chromatographic resolution. Therefore, a 2% (w/v) $K_2S_2O_8$ solution at a flow of 0.3 mL min^{-1} was finally selected. Lower $K_2S_2O_8$ concentrations (1 and 1.5%) were not sufficient to obtain total UV photo-oxidation of As species, while preparation of solutions with higher $K_2S_2O_8$ concentration was limited by solubility of this salt.

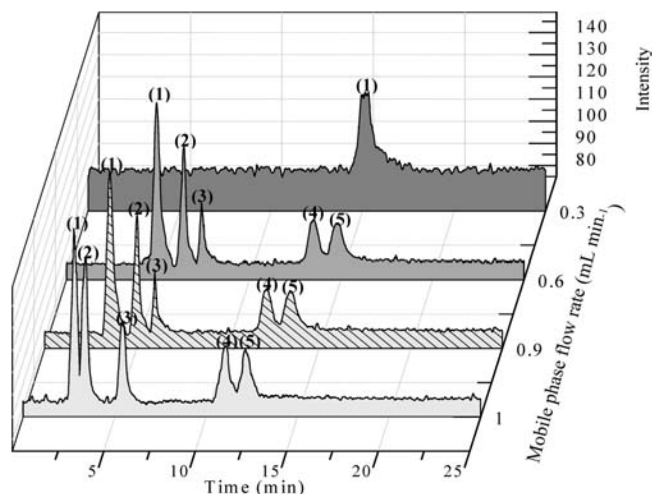

Figure 4. Effect of mobile phase flow rate on chromatography separation for As species standards (20 μg As L^{-1} each): (1) MMAA; (2) DMAA; (3) *p*-ASA; (4) ROX; (5) NIT. Other experimental parameters were as shown in Table 2.

3.6. Optimization of HG-AFS Conditions. In order to optimize the proposed HPLC-AFS method, it was necessary to study the conditions for optimal hydride generation under the influence of mobile phase composition. Thus, reductant flow and concentration were optimized, along with HCl flow and concentration. Minimal dilution and dispersion effects of the chromatographic peaks were pursued when reagent flows were optimized. Moreover, stability of hydrogen flame was also considered during the selection of different flows. The optimal conditions are shown in Table 2. On the other hand, the use of a loop (see Figure 1) after addition of HCl to the flow allowed appropriate acidification and later UV photo-oxidation of the As species separated during chromatography. Furthermore, and due to elimination of organic solvent from the mobile phase, hydride generation conditions were similar, in terms of HCl (1.5 M) and $NaBH_4$ (1.1% (w/v)) concentrations, to those normally employed in As determination by AFS.

3.7. Analytical Performance. The analytical figures of merit of the proposed methodology are summarized in Table 3. The limits of detection (LODs), calculated based on the signal at intercept and three times the standard deviation about regression of the calibration curve, obtained for As species, ranged between 0.2 and 1.6 μg As L^{-1} (Table 3). The calibration graphs showed a satisfactory linearity for different species within a concentration range of 1−200 μg As L^{-1} (see Table 3 for more details). The correlation coefficients were between 0.989 and 0.998. Table 3 also shows different parameters that characterize the chromatographic

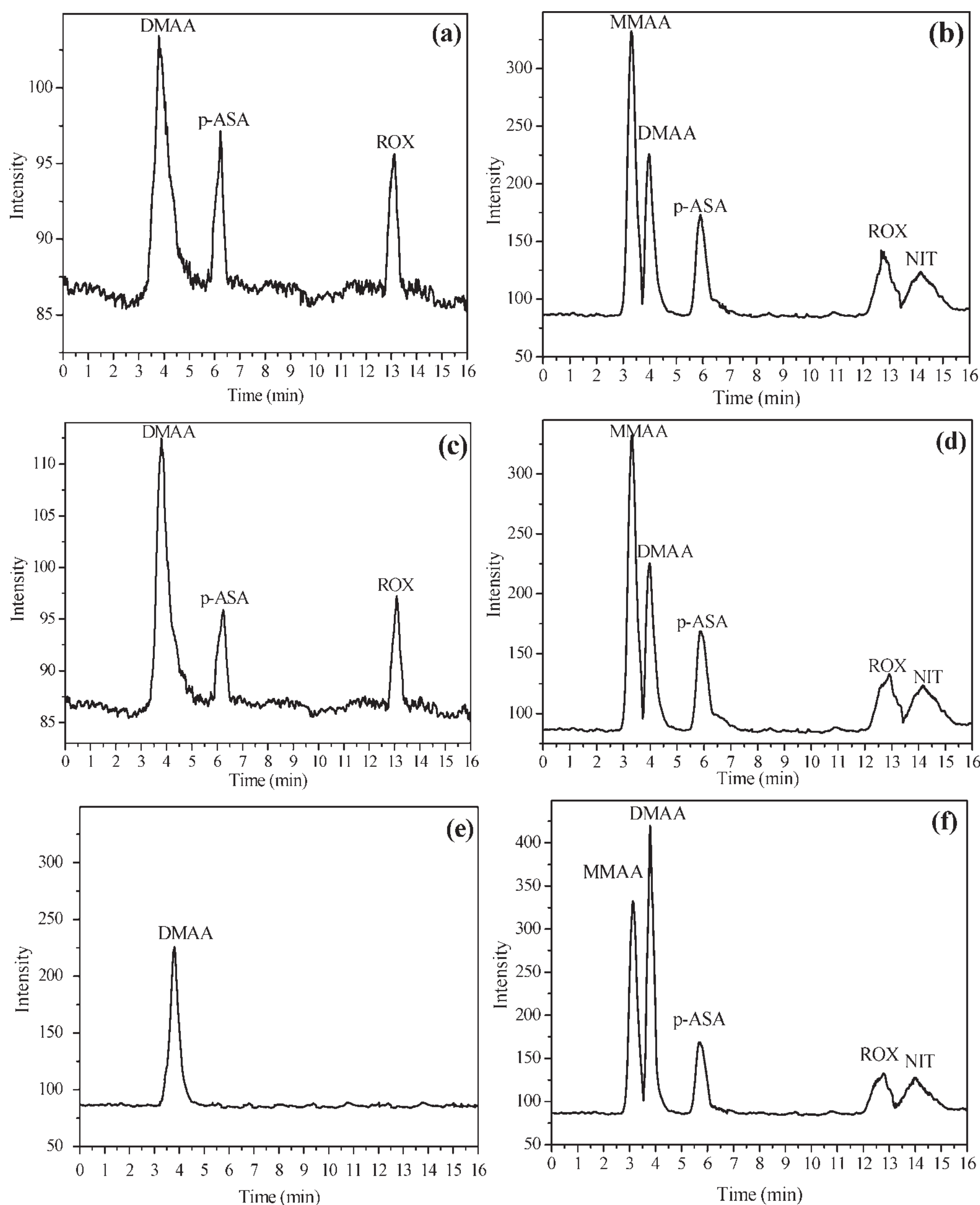

Figure 5. Speciation analysis of As in sample studied with IP-HPLC−HG-AFS: (a) chicken liver sample without As standard addition; (b) chicken liver sample spiked at 40 μg As L^{-1} of each As species; (c) dog food sample without spike; (d) dog food sample spiked at 40 μg As L^{-1} of each As species; (e) dog urine sample without spike; (f) dog urine sample spiked at 40 As μg L^{-1} of each As species. For other conditions see Table 2.

performance of the method, including capacity factor, selectivity and plate number. The parameters were calculated with respect to a previous peak, considering MMAA as peak 1. Reproducible retention times were observed throughout a regular working day (8−12 h of analysis). Relative standard deviations (RSD) were calculated for six replicate measurements of 10 μg As L^{-1} for each species varying between 3.1% and 6.8%. Furthermore, matrix effects were evaluated following a recovery study by adding each As species at different concentration levels to the samples (see Figure 5 and Table 4). Recovery values obtained at two concentration levels (20 and 40 μg As L^{-1}) of each As species varied between 94 and 100%.

Capability of IP-HPLC−HG-AFS method to quantify individual As species in the extracts was evaluated by comparing the sum of As species concentrations measured by IP-HPLC−HG-AFS with the total As concentration determined by HG-AFS in the sample after mineralization. The sum of As species concentrations widely agreed with the corresponding total As concentrations as specified in Table 4, obtaining acceptable extraction recoveries (see Table 5). Furthermore, the results of total As content in samples extracts are shown in Table 5 and were compared with the sum of As species. The *t*-test did not reveal significant difference at 95% confidence level indicating appropriate recovery of As species during chromatography.

3.8. Determination of As Species in Different Samples. In order to demonstrate the applicability of the proposed method, As speciation studies were developed in samples showing matrices of different types and complexities, such as arugula, dog food, dog

Table 4. Results of As Speciation Analysis in Different Samples (95% Confidence Interval, $n = 6$)

	concentration[a] (μg L^{-1})									
	MMAA		DMAA		*p*-ASA		ROX		NIT	
sample	added	found	added	found	added	found	added	found	added	found
arugula	0	nd[b]	0	nd	0	nd	0	nd	0	nd
	20	19.9 ± 0.6 (100%)	20	19.7 ± 0.6 (99%)	20	19.8 ± 0.7 (99%)	20	19.6 ± 1.2 (98%)	20	19.7 ± 1.9 (99%)
	40	39.8 ± 1.2 (100%)	40	39.8 ± 1.0 (99%)	40	40.1 ± 1.2 (100%)	40	39.8 ± 1.7 (100%)	40	39.9 ± 1.6 (100%)
dog food	0	nd	0	2.6 ± 0.5	0	2.3 ± 0.6	0	6.1 ± 0.6	0	nd
	20	19.7 ± 0.5 (99%)	20	22.6 ± 0.6 (100%)	20	22.2 ± 0.8 (99%)	20	26.1 ± 1.4 (100%)	20	19.4 ± 1.6 (97%)
	40	39.6 ± 0.9 (99%)	40	42.4 ± 0.9 (100%)	40	42.3 ± 1.4 (100%)	40	45.9 ± 2.3 (99%)	40	39.5 ± 2.1 (99%)
dog urine	0	nd	0	42.5 ± 1.0	0	nd	0	nd	0	nd
	20	19.8 ± 0.5 (99%)	20	62.3 ± 1.4 (99%)	20	19.8 ± 0.7 (99%)	20	18.9 ± 0.8 (94%)	20	19.5 ± 1.6 (98%)
	40	39.9 ± 0.9 (100%)	40	82.4 ± 1.7 (100%)	40	39.8 ± 1.3 (100%)	40	39.6 ± 1.3 (99%)	40	39.7 ± 2.1 (99%)
chicken liver	0	nd	0	15.7 ± 0.5	0	2.2 ± 0.6	0	5.6 ± 0.6	0	nd
	20	19.6 ± 0.6 (98%)	20	35.6 ± 1.0 (100%)	20	22.1 ± 0.9 (100%)	20	25.5 ± 1.4 (100%)	20	19.7 ± 1.6 (99%)
	40	39.5 ± 1.0 (99%)	40	55.7 ± 1.3 (100%)	40	41.9 ± 1.2 (99%)	40	45.3 ± 2.3 (99%)	40	39.7 ± 2.3 (99%)

[a] Expressed in sample extracts. Values within parentheses indicate % recovery values obtained upon different As species additions. % recovery = 100 × [(found − initial)/added]. [b] Nondetectable.

Table 5. Results of Total As in Different Samples and Recovery of As Species (95% Confidence Interval, $n = 6$)

sample	sum of As species[a] (μg L^{-1})	total As in extract[b] (μg L^{-1})	total As[c] (μg L^{-1})	extraction recovery[d] (%)
arugula	nd[e]	nd	nd	
dog food	11.0 ± 1.0	10.9 ± 0.9	12.1 ± 0.9	90
dog urine	42.5 ± 0.9	42.6 ± 1.1	43.4 ± 1.0	98
chicken liver	23.5 ± 1.0	23.4 ± 1.0	27.3 ± 0.9	86

[a] Sum of all species shown in Table 4 expressed as As concentration. [b] Total As content in samples extracts determined by HG-AFS after mineralization. [c] Total As content in the sample determined by HG-AFS after mineralization. [d] Recovery = (total As in extract/total As in sample) × 100. [e] Nondetectable.

urine and chicken liver. Figures 5(a) and 5(c) show the chromatograms for As speciation in chicken liver and dog food samples. ROX was found in these samples, which can be explained considering the low metabolization of this compound by chicken. In fact, ROX could be found in chicken organs, such as liver.[46] Animal feeds are generally manufactured with meat processing plant byproducts, such as meat and viscera.[47] Therefore, ROX drug as well as some byproduct could be still present in dog food elaborated with chicken or derivates after manufacturing. Likewise, the presence of *p*-ASA in these samples could be explained considering that no evidence of its metabolization has been found so far.

The occurrence of DMAA species in chicken liver and dog urine was also confirmed (Figure 5(a) and Figure 5(e)). This is certainly due to fact that DMAA is the major metabolite in animals and humans exposed to As.[8] Moreover, some experimental studies performed in urine have shown 75−85% As excreted as DMAA species, while a small percentage is excreted as MMAA (5−10%).[8] In experimental animals, DMAA has been found to be formed *in vivo* by methylation with distribution toward all tissues, and hence DMAA could be present in tissues such as liver.[8] Consequently, the presence of As species in dog food is feasible as it is usually made with animal tissues among other raw materials, such as liver (Figure 5(c)).

Furthermore, the absence of NIT in the samples under study could be explained considering its application in turkey rather than chicken. It has to be mentioned that information in the literature regarding consumption of PheAs compounds by animals, such as dogs and other mammals, their possible metabolites and elimination is really scarce. In fact, to the best of our knowledge, this work is one of the first revealing important data about As speciation in such samples, especially dog food and dog urine.

Studies on accumulation and metabolization of different PheAs compounds in plants growing on contaminated soils have been developed in the past. In these studies, ROX and *p*-ASA were investigated among other species, finding that all compounds were taken up by the roots and transferred to stalks and leaves, but with predominant accumulation in roots.[21] Thus, none of the As species under study were observed in arugula samples, which could be attributed to its minor accumulation in leaf. Furthermore, arsenobetaine and arsenocholine are two As species often considered in speciation analysis. Nevertheless, these species are mainly found in several sea foods and for this reason they were not expected to be present in the samples under study.

With the ever increasing levels of As in the environment due to natural and anthropogenic activities and the growing awareness of the need for elemental species determination, development of highly efficient analytical separation methods represents an important task. In this work, different organoarsenical species (i.e., MMAA, DMAA, *p*-ASA, NIT and ROX) have been studied via IP-HPLC−HG-AFS. The isocratic reversed-phase method proposed in this work allowed the separation of five As species in 16 min with low detection limits. The use of an organic solvent free mobile phase turns this methodology into a low cost and

environmentally friendly alternative, while leading to reach high sensitivity for As species when they are detected by AFS under optimal conditions. Finally, the absence of an organic solvent in the mobile phase makes this methodology compatible with state-of-the-art techniques, such as ICP-MS, for determination of As species at ultratrace levels.

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