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Sensitive method for the determination of roxarsone using solid-phase microextraction with multi-detector gas chromatography

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Abstract

We describe the development, optimization, and application of a novel method for the unequivocal identification and quantification of roxarsone (3-nitro-4-hydroxyphenylarsonic acid, 3-NHPAA) at low $\mu g\,L^{-1}$ levels. The method is based on capillary gas–liquid chromatography with parallel quadrupole ion-trap mass spectrometric (QIT-MS) and pulsed flame photometric detection (PFPD). The sensitive method couples the arsenic specificity of PFPD with the high selectivity of molecular MS for the determination of roxarsone, dimethylarsenic acid (DMAA), and monomethylarsonic acid (MMAA) in complex matrices. Analytes were derivatized based on the approach we previously reported [B. Szostek, J.H. Aldstadt, J. Chromatogr. A 807 (1998) 253 and D.R. Killelea, J.H. Aldstadt, J. Chromatogr. A 918 (2001) 169] for the reaction of organoarsenicals with 1,3-propanedithiol (PDT). The cyclic dithiaarsenolines formed were extracted from the sample matrix in the liquid phase by solid-phase microextraction (SPME). The optimized SPME conditions employed a 65 μm polydimethlysiloxane–divinylbenzene (PDMS–DVB) fiber, extraction temperature of 70 °C and fiber equilibration time of 15.0 min. The mass spectrum of the dithiaarsenoline of roxarsone showed a base peak that corresponded to the predicted structure at *m/z* 319 and the tell-tale peak of an arsenic compound derivatized with PDT at *m/z* 181. Further peaks at *m/z* 149 and 228 were observed and found to be unique to roxarsone, formed by an interesting internal rearrangement of the ONOH functionality. A linear calibration model was prepared for roxarsone over an environmentally relevant range (0.0–100 $\mu g\,L^{-1}$) and a detection limit of 2.69 $\mu g\,L^{-1}$ (3σ) was observed. The method was applied to several fortified environmental surface water samples (50 $\mu g\,L^{-1}$) where the average recovery for roxarsone was $103 \pm 10.9\%$.

Keywords: Gas chromatography; Pulsed flame photometric detection; Mass spectrometric detection; Solid-phase microextraction; Arsenic; 3-Nitro-4-hydroxyphenylarsonic acid (CASRN 121-91-7); Roxarsone

1. Introduction

3-Nitro-4-hydroxyphenylarsonic acid (3-NHPAA or "roxarsone") is an anthropogenic organoarsenic compound that is used widely as an additive to poultry and hog feed "... for the prevention of coccidiosis caused by *Eimeria tenella*, *E. necatrix*, *E. acervulina*, *E. mivati*, *E. maxima*, and *E. brunetti*, and for increased rate of weight gain and improved feed efficiency" [1]. The widespread usage of roxarsone in areas such as the Delaware–Maryland–Virginia ("Delmarva") Peninsula has led to recent concern over the fate of this compound. Annually, an estimated 20–50 metric tons of arsenic enters the Delmarva Peninsula when poultry manure is applied as a fertilizer [2]. Although roxarsone is apparently excreted in the animal's waste, recently detailed studies [3,4] indicate that roxarsone may ultimately decompose to water-soluble toxic arsenicals, primarily as inorganic arsenate. In these studies, a complex series of decomposition reactions was evident, with the observation of intermediate degradation product(s) of unknown structure, reactivity, and toxicity. However, because ion chromatography–inductively coupled plasma mass spectrometry (IC–ICP-MS) was the method of quantitation, the structure of the unknown arsenic compound(s) could not be determined in the absence of standards. Further investigation

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of the structure of the unknown was reported recently using electrospray ionization (ESI) MS, suggesting that it may be an azobenzene arsonic acid [5].

The ability to speciate organoarsenic compounds such as roxarsone and its degradation products in environmental samples is crucial to improve our understanding of their transport and fate. Several methods have been reported in the literature for the determination of roxarsone (Table 1). The earliest methods developed were quality control procedures that focused on the determination of roxarsone in the feed itself. These spectrophotometric methods possess neither the lower limits of detection nor the high selectivity needed for application to complex environmental sample matrices (e.g., farm run-off, soils) [6–8]. In particular, the reported limits of detection ($\sim$5 $\mu g\,g^{-1}$) are insufficient for measuring environmentally relevant concentrations at the low $\mu g\,L^{-1}$ level. Electrochemical methods have also been reported for determining roxarsone; a differential pulse voltammetry method using a chemically modified carbon paste electrode was described recently [9] in which the aromatic nitro group was reduced. Because of the large number and high concentration of aromatic nitro compounds that are often present in agricultural areas, this method would be likely to suffer from poor selectivity in these matrices. Additionally, the limit of detection was too high (260 $\mu g\,L^{-1}$) for effective application to most environmental samples.

For the measurement of trace levels of roxarsone and the elucidation of the structures of its degradation products in complex matrices, molecular MS is the obvious technique of choice. A large body of literature exists for the determination of various organoarsenic compounds by high-performance liquid chromatography (HPLC) with molecular (ESI [10–13]) and atomic (IC-ICP-MS [14–16]) detection. While methods based on reversed-phase HPLC have been published, ion-exchange HPLC is the predominant mechanism of separation for organoarsenic compounds because of problems in the observation of co-eluting compounds using reversed-phases. These methods are indeed powerful. However, in ESI the fragmentation is too "soft" in single-dimensional MS, thereby requiring extensive method development to create tandem MS methods, while in ICP-MS standards are needed to identify peaks observed in the chromatogram.

GC–MS with electron impact ionization (EI) is preferred over other molecular MS methods because it can provide the information needed to deduce the structures of unknowns. Because of the non-volatile nature of roxarsone, a derivatizing agent must be used before it can be introduced to the gas chromatograph. Given arsenic's affinity for forming strong bonds with sulfur, many methods for GC analysis have been developed using various mercaptans, such as thioglycol methylate (TGM) and 1,3-propanedithiol (PDT) [17–26]. Even though the arsenicals are chemically changed in these methods, the arsenic derivatives retain enough functionality for them to be clearly identified by molecular MS. For example, in our previous work on the improved derivatization of organoarsenicals with PDT [27,28], the products were chromatographically resolved and their individual mass spectra were distinct.

Given the widespread use of GC–MS for environmental analysis, as well as our previous experience in developing methods based on GC–MS for organoarsenicals, we describe herein our development of a new method for roxarsone based on SPME–GC–MS–PFPD.

2. Experimental

2.1. Reagents

Reagent water (18 MΩ cm) was prepared using a NanoPure Infinity filtration system equipped with an ultraviolet (254 nm) lamp (Barnstead-Thermolyne, Dubuque, IA, USA). Analyte standards were prepared from sodium arsenite (Mallinckrodt Chemical, Paris, KY, USA), sodium arsenate (Arsenic Reference Solution, 1000 mg L^{-1}, Fisher Scientific, Pittsburgh, PA, USA), monomethylarsonic acid (MMAA) (synthesized in our laboratory [18]), dimethylarsinic acid (DMAA) (98%, Sigma, St. Louis, MO, USA), and 3-amino-4-hydroxyphenylarsonic acid (3-NHPAA or roxarsone) (98%, Aldrich, Milwaukee, WI, USA). The derivatizing reagent for MMAA, DMAA, and roxarsone was 1,3-propanedithiol (PDT) (99%, Aldrich). All glassware and plasticware were washed with deionized water and then soaked for at least 36 h in 5% (v/v) nitric acid (analytical-reagent grade, Fisher), followed by copious rinsing with reagent water before use. Stock standards (1000 mg L^{-1})

Table 1
A comparison of key figures of merit for analytical methods that have been published for determining roxarsone

Major analyte(s)	Technique	Matrix	LOD ($\mu g\,L^{-1}$)	Reference
Roxarsone	Flame atomic absorption spectroscopy (AAS)	Animal feed	$\sim$10000	[35]
Roxarsone	Graphite furnace AAS	Animal feed	620	[36]
Roxarsone	Differential pulse voltammetry	roxarsone tablet	260	[9]
Roxarsone	HPLC–UV	Hog tissue	250	[37]
Roxarsone	HPLC–ICP-MS	Chicken tissue	25	[14]
Roxarsone, M, D, P	HPLC–ESI-MS–MS	Urine	0.150	[38]
Roxarsone, M, D, P	IC–ICP-MS	Soil	0.05	[16]

The reported limits of detection (LODs) for roxarsone are listed. M, MMAA; D, DMAA; P, *p*-arsanilic acid (a minor feed additive).

were stored in opaque high-density polyethylene (HDPE) bottles. Arsenic-containing standards with concentrations less than 1 mg L^{-1} were prepared on the day of use. All aqueous standards were stored at 4 °C.

2.2. Sample preparation

For the determination of organoarsencials, the sample was acidified to pH 2 with 6.0 M HCl (TraceMetal grade, Fisher) if necessary. Next, a 2.5 mL portion of the sample was pipetted into a 4.0 mL vial (clear vial, screw top, white silicone–PTFE septa; Supelco) along with a magnetic stir bar (PTFE, 10 mm length, 3 mm diameter; Fisher). The sample was then heated to 70 °C and reacted with 0.5 μL (Hamilton 10 μL syringe, Fisher) of neat PDT for 5.0 min. The organoarsenicals were extracted from the sample matrix by solid-phase microextraction (SPME). A 65 μm polydimethylsiloxane–divinylbenzene (PDMS–DVB) SPME fiber (Supelco, Bellefonte, PA, USA) was allowed to equilibrate with the sample for 15.0 min.

2.3. Gas–liquid chromatography

Organoarsenicals were determined by gas–liquid chromatography using a Varian GC–MS system (Saturn III, Walnut Creek, CA, USA) with two columns in parallel. The system consisted of the following components: Model 3800 capillary gas–liquid chromatograph (CP-Sil 8 CB Low Bleed MS column, 30 m × 0.25 mm with 0.25 μm film, Varian) with Model 1079 split/splitless; SPME apparatus (Supelco); electron impact ion source (70 eV); pulsed flame photometric detection (PFPD), performed in the arsenic mode using a high-pass optical filter (Schott RG695 nm, BES Optics, Warwick, RI, USA) based on our previously optimized conditions [27]. A Model R5070 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA) was set to 610 V with 200 mV trigger level; the Saturn 2000 quadrupole ion-trap mass spectrometer had a mass range from 10 to 650 *m*/*z* range with unit resolution. The automatic gain control (AGC) of the MS system was used throughout this study. The mobile phase was ultra-high purity (99.999%) helium (Praxair, Milwaukee, WI, USA) at a constant linear velocity of 42 cm s^{-1} through electronic flow control.

GC instrument control and data acquisition were performed on a Pentium II personal computer (Optiplex GX1, Dell, Dallas, TX, USA) using Saturn Software version 5.4 and Varian PFPD analysis software version 1.0. The response was reported as peak area (counts) for MS detection and peak height (volts) for PFPD.

For the SPME studies, the injection port was held at 250 °C for 1.0 min for the desorption step (splitless). The initial column temperature was held at 70 °C for 1 min, then programmed at 20 °C min^{-1} to 165 °C, followed by 8 °C min^{-1} to 213 °C and finally 50 °C min^{-1} to 303 °C, where it was held for 2.0 min (total run-time was 15.75 min). The transfer line between the GC and MSD instruments was maintained at 275 °C for all experiments. Mass spectra were obtained by scanning from *m*/*z* 35 to 400 with a 0.5 s scan time.

3. Results and discussion

3.1. Development of the gas chromatography method

Except for the organoarsines, most environmental organoarsenicals (e.g., DMAA, MMAA, and roxarsone) have low volatilities. Therefore, derivatization to a more volatile form is required prior to GC. By using a reducing thiol, such as PDT, arsenic compounds can be easily converted (Fig. 1) to their cyclic dithiaarsenolines [17–26]. These volatile compounds can be directly injected or they can be extracted using SPME prior to GC. The PDT-derived moieties of the cyclic dithiaarsenoline structures are straightforward to identify in molecular MS. For complex mixtures, a crowded GC–MS chromatogram is much easier to sort through when using an As-selective detector in parallel.

We have previously reported methods using PFPD in parallel to quadrupole ion-trap mass spectrometry (QIT-MS) as a means to successfully identify and quantify organoarsenic compounds at trace levels in complex samples [27,28], based on the pioneering work of Amirav at Tel Aviv University [29]. The PFPD uses the added dimension of emission time to avoid interferences in the detection of specific elements [30,31]. The sensitivity of PFPD for arsenic is ∼10-fold better than observed with atomic emission detection, and the instrument is more economical both in capital and operating costs. Combustion interferences, such as sulfur and carbon species, are greatly minimized by using a specific gate delay (the time after the pulse at which the measurement is taken), gate width (the time period during which the emission intensity is measured), and wavelength range (using cut-off filters, e.g., 695 nm for As), thereby allowing for a selective response to arsenic-containing compounds. Once the retention times of the cyclic dithiaarsinolines have been identified by use of appropriate standards (Fig. 2), one can then focus on the corresponding mass spectrum to attempt structure elucidation.

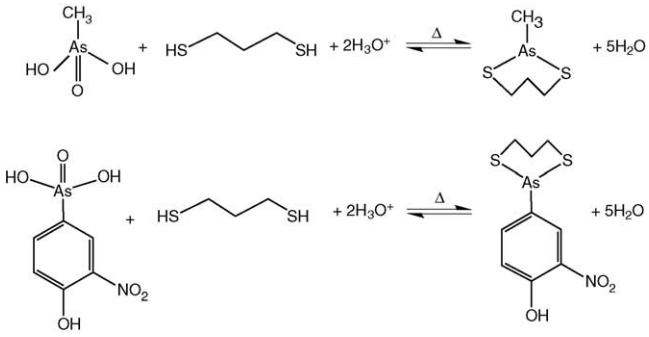

Fig. 1. Net reaction for the derivatization of organoarsenicals with 1,3-propanedithiol to form cyclic dithiaarsenolines. The reaction of MMAA is shown for comparison (above) to the reaction for roxarsone (below).

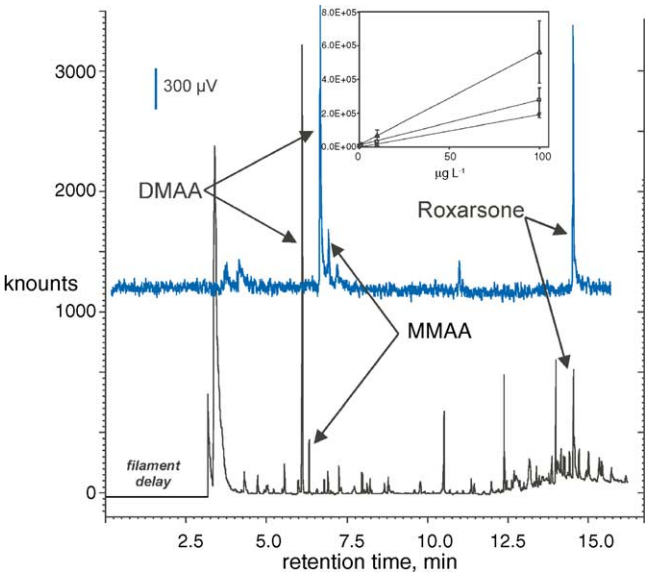

Fig. 2. Chromatograms for a standard mixture of MMAA, DMAA, and roxarsone (100 μg L^{-1} each) after derivatization with 1,3-propanedithiol and SPME. The PFPD response (blue) is off-set above and ~0.25 min later relative to the MS chromatogram for clarity. MS calibration models are shown (inset). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Derivatization by PDT of solutions containing several organoarsenicals (MMAA, DMAA, *p*-arsanilic acid, and roxarsone) was initially investigated. The responses observed for a standard solution containing MMAA, DMAA, and roxarsone (100 μg L^{-1} each) produced well-resolved peaks in the chromatograms observed for both the PFPD and MS detectors (Fig. 2). For *p*-arsanilic acid (*p*-ASA), *p*-ASA gave a substantially lower response in both detection modes. Apparently because of the amine group located on the aromatic ring, *p*-ASA retains an overall positive charge before and after the dithiol derivatization (optimally performed at pH 2). Of course, the presence of the positive charge will not permit efficient gas–liquid chromatography [32]. A lower response was observed for MMAA compared to DMAA, which we attribute to a more complex derivatization reaction and electron impact ionization chemistry, as evidenced in our previous studies of these compounds [27]. In the mass spectrum of derivatized roxarsone (Fig. 3), the base peak (*m*/*z* 319) was the predicted structure for the dithiaarsenoline molecular ion (M^+). Other major fragments, characteristic of the PDT method for organoarsenicals, were observed at *m*/*z* 181 and 106. The tell-tale fragment at *m*/*z* 181 is present in *all* mass spectra that we have collected for dithiaarsenolines, corresponding to the PDT moiety (*m*/*z* 106) bound to the monoisotopic arsenic cation (*m*/*z* 75).

While the three major peaks in the mass spectrum (Fig. 3) were straightforward to interpret, the next largest peak (at *m*/*z* 228) was less obvious. We speculate that re-arrangement of M^+ occurred, in which the As–C and C–N bonds were broken, with recombination of ONOH to form an As–N bond. The isotopic distribution for the peak at *m*/*z* 228 indicates that this ion also contains sulfur (i.e., the ^{34}S isotope with 4.5% natural abundance), thereby supporting our proposed structures. Finally, it is important to note that while As(V) may be present as a result of the degradation of roxarsone or other sources, the very weak signals observed for PDT-derivatized arsenate (or arsenite) are simple to resolve in the chromatogram (results not shown; see also [28]).

3.2. Optimization of SPME conditions

Solid-phase microextraction (SPME) was used to isolate the PDT-derivatized organoarsenicals from the sample matrix. SPME is advantageous in that it combines sampling, extraction, matrix removal, analyte enrichment, and instrument injection into a single process [33]. A set of four SPME fibers was investigated based on their polymeric functionality and practical durability: 100 μm PDMS, 65 μm PDMS–DVB, 85 μm polyacrylate (PA), and 75 μm Carboxen–polydimethylsiloxane (CAR–PDMS). Initially, the extraction efficiency of each fiber for MMAA, DMAA, and roxarsone was studied. A 2.5 mL volume of the acidified sample (pH 2.0) was heated to 70 °C in a closed vial and reacted with 0.5 μL of neat PDT. After 5 min of reaction, the SPME fiber was exposed to the sample for 30 min. Next, the SPME fiber was inserted into the (splitless) injector of the GC system and the analytes were thermally desorbed. We found that we could employ a relatively short desorption period (1 min) to prevent any "carry-over" between samples. In comparing the four phases (*results not shown*), the PDMS fiber gave the lowest response for MMAA and DMAA, and the second lowest response for roxarsone. The CAR–PDMS fiber gave the best response for DMAA and MMAA; however, CAR–PDMS gave the lowest response for roxarsone. The PA fiber gave better responses for all arsenicals than the PDMS fiber, but it did not have the best overall response. Finally, PDMS–DVB gave the best response for both DMAA and roxarsone. Because of the high sensitivity that the PDMS–DVB fiber has for the dithiaarsinoline

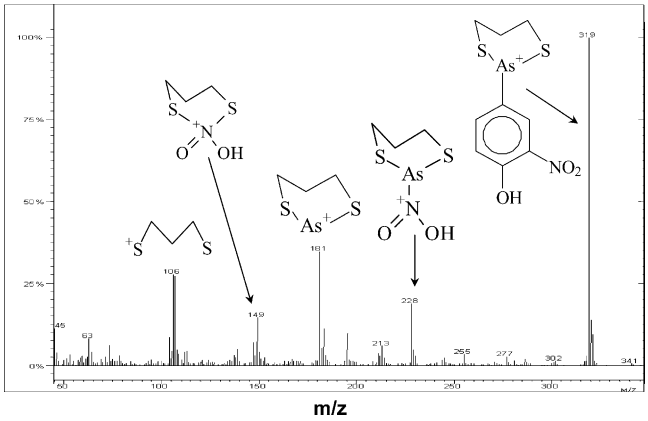

Fig. 3. Mass spectrum of the 1,3-propanedithiol derivative of roxarsone after electron impact ionization. Structures are shown for the four major peaks that were observed.

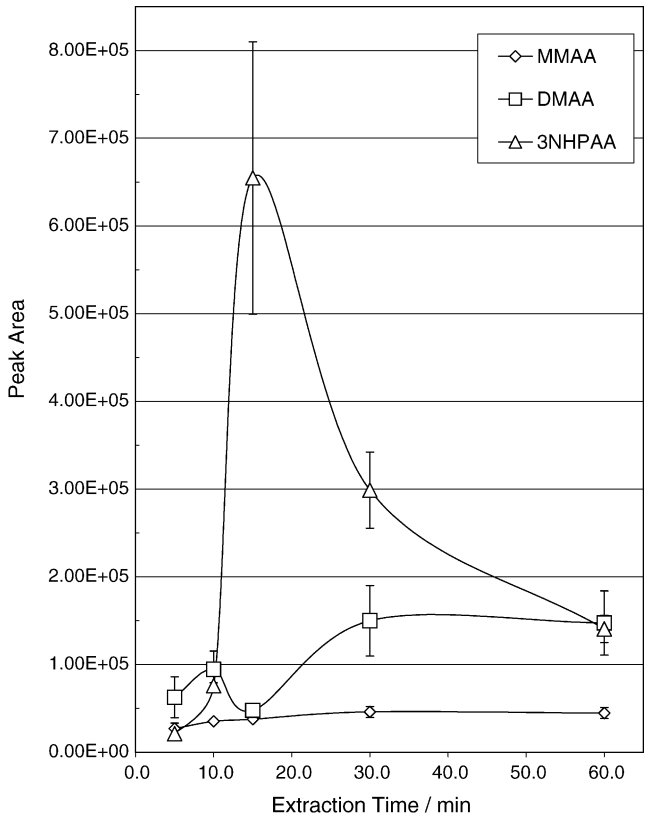

Fig. 4. Optimization of the SPME method for extraction time. PDMS–DVB fiber (65 μm film) was held at 70 °C (with stirring) in 100 μg L^{-1} solutions of the analytes. Further details are described in the text.

derivative of roxarsone, this fiber was chosen for further method optimization studies.

With the selection of the PDMS–DVB fiber, the extraction conditions were then optimized. First, the temperature at which the PDT reaction and SPME extraction was examined: 22 °C (i.e., temperature of the laboratory), 50 °C, and 70 °C (*results not shown*). The SPME fiber was exposed to the solution containing the derivatized arsenicals for 30 min. At 50 °C, the response for MMAA and DMAA was only slightly lower than the response observed at 70 °C, and within the 95% confidence limits. On the other hand, the response for roxarsone was nearly three-fold higher at 70 °C than at 50 °C. Based on these results, the derivatization of organoarsenicals and their extraction with SPME was carried out at 70 °C for the remaining experiments.

The final factor examined in the optimization of the SPME was sampling time (Fig. 4). A series of exposure times ranging from 5 to 60 min was investigated. For the extraction of MMAA and DMAA, the optimal exposure time was 15 min; after 30 min, little improvement in response for either arsenical was observed. The response for roxarsone peaked sharply at 15 min, then declined rapidly at longer extraction times.

The reason for the unusual roxarsone response may be inferred from a consideration of kinetic versus thermodynamic control of the analyte interaction with the SPME phase. That is, SPME fiber phases can be generally classified into two groups on the basis of their interaction mechanism [33], absorptive or adsorptive (or a combination thereof). In the absorptive mechanism, analytes partition into the fiber coating, i.e., a phase distribution process. On the other hand, adsorptive fibers have a finite number of active sites for binding the analyte. Analytes will compete for binding to these sites; those with faster adsorption kinetics may occupy more sites initially, but be displaced over time by other analytes that have a greater equilibrium distribution but slower uptake kinetics. With the PDMS–DVB fiber, for which adsorption is the dominant mechanism, the rate of adsorption of the *dithiaarsenoline* formed from roxarsone is greater than the rate for adsorption of the *dithiaarsenolines* that are formed from MMAA and DMAA. Undoubtedly, the aromatic ring present in roxarsone interacts more favorably with the divinylbenzene functionality in the fiber phase. This competition would thereby explain the rapid rise and subsequent decrease in the roxarsone signal. While thicker coatings for PDMS–DVB would be interesting to examine in light of this putative mechanism, the 65 μm PDMS–DVB that was studied is the thickest coating that is commercially available. Therefore, because mixtures of organoarsenicals will often be present, an extraction time of 15 min using the 65 μm PDMS–DVB fiber was chosen as optimal.

3.3. Quantitation

With the optimized conditions for the derivatization chemistry and SPME conditions for roxarsone, calibration models

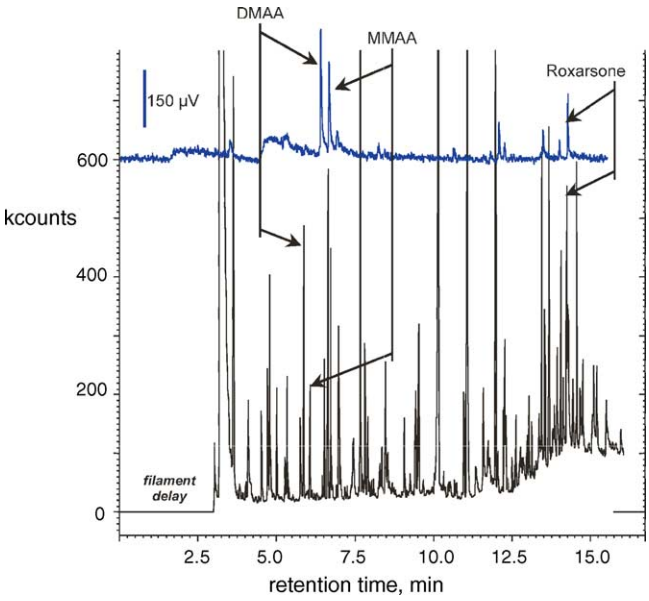

Fig. 5. Chromatograms for hog farm run-off sample fortified with roxarsone, MMAA, and DMAA (50 μg L^{-1} each). The PFPD chromatogram (blue) is off-set above and ~0.25 min later relative to the MS chromatogram (kcounts) for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 2
Determination of monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), and roxarsone (3-NHPAA) in fortified (50.0 μg L^{-1} each) environmental samples by SPME–GC–PFPD

	Hog farm		Turkey farm		Milwaukee River		Average	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
MMAA	81.6	1.70	126	13.6	99.7	15.6	103	10.3
DMAA	93.8	12.4	101	26.6	120	7.04	105	15.4
3-NHPAA	113	9.40	64.8	21.8	131	1.48	103	10.9

Phenylarsonic acid (PAA) was used as the internal standard (50.0 μg L^{-1}). Accuracy measured as percent recovery and precision measured as percent relative standard deviation (R.S.D., $n = 2$) are shown.

for MMAA, DMAA, and roxarsone were constructed using an environmentally relevant range of standard concentrations (0.0, 1.0, 10, and 100 ppb, $n = 3$ at each level), as shown in Fig. 2 (inset). Direct calibration of the SPME–GC–MS method for roxarsone produced a regression model that was linear over three orders of magnitude: y (A, peak area) = $y = 1.9 \times 10^3 \times (\mu g\,L^{-1}) - 4.71 \times 10^3$ ($R^2 = 0.9954$) with a detection limit (3σ) of 2.69 μg L^{-1}. Additionally, application of the optimized method significantly improved upon the calibration models we previously reported for MMAA and DMAA [28]. For MMAA, the regression model was: y (A, peak area) = $2.70 \times 10^3 \times (\mu g\,L^{-1}) + 6.32 \times 10^3$ ($R^2 = 0.9993$) with a detection limit (3σ) of 0.60 μg L^{-1}. For DMAA, the regression model was: y (A, peak area) = $5.55 \times 10^3 \times (\mu g\,L^{-1}) + 7.66 \times 10^3$ ($R^2 = 0.9998$) with a detection limit (3σ) calculated to be 0.22 μg L^{-1}. Finally, we used the PFPD response for qualitative identification of arsenic-containing peaks. Although the detection limit observed was ~10-fold lower, the precision was inferior compared to the MS quantitative data.

3.4. Application

Surface water was collected at a poultry (turkey) farm in Southeastern Wisconsin, at a hog farm in Western Iowa, and in the Milwaukee River near Shorewood, WI, USA. However, because we found that the samples did not contain arsenicals at the time they were taken, the samples were fortified with, MMAA, DMAA, and roxarsone at 50.0 μg L^{-1} each. In Fig. 5, the MS and PFPD chromatograms for the hog farm run-off sample matrix are shown. The PFPD measurement makes identification of the pertinent MS signals straightforward to locate and interpret. Note that the broad peaks observed in the PFPD response at a retention time of 4–5 min are routinely observed when using PDT as a derivatizing reagent. The method we optimized for the PFPD has a high selectivity in rejecting the sulfur emission, but fails for samples that have such a high amount of sulfur (i.e., remaining from the derivatization reaction). As reported by Fowler et al. [23], unreacted dithiol can be removed using Ag ion as precipitating agent; however, we chose not to include this additional step for two reasons: (a) we find that addition of $AgNO_3$ causes premature aging of the SPME fiber and (b) the analytes under study were already well-resolved from interfering peaks in the chromatogram.

Phenylarsonic acid (PAA) was used as an internal standard (50.0 μg L^{-1}) in each sample matrix. The recoveries for MMAA, DMAA, and roxarsone in hog farm run-off and the Milwaukee River were reasonable (Table 2) and the precision was typical for SPME methods at ~10% R.S.D. The variation in recovery for the three sample types is characteristic of the matrix-dependency of SPME. The recoveries of the organoarsenicals in poultry farm run-off were more variable and the variability in the data was two-fold greater than observed for the other matrices. Although PAA was not present in any of the matrices that we studied, it could be present as a decomposition product of roxarsone. We are, therefore, studying its replacement as internal standard by deuterated PAA, which could be easily differentiated in the mass spectrum from PAA. We are also investigating the use of solid-phase extraction based on modifications to the method reported by Le et al. [34] to address the matrix effects that were observed in the poultry farm samples.

Acknowledgments

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