Development of a Fast Chromatographic Method for the Detection of Organoarsenic Antimicrobials

by

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ABSTRACT

New protocols were developed for the routine analysis of the organoarsenic drugs roxarsone (4-hydroxy-3-nitrophenylarsonic acid), p-arsanilic acid, and acetarsone (*N*-acetyl-4-hydroxy-*m*-arsanilic acid) based on ion-pairing high performance liquid chromatography (IP-HPLC) with rapid-resolution technology and diode array detection. The drugs under study have been used for veterinary applications as antimicrobial agents and growth promoters for poultry. The high water-solubility exhibited by these drugs represents a serious environmental problem since poultry litter is mainly used as fertilizer. Once applied to crops, these agents can leach into bodies of water and readily be absorbed by plants. The use of these drugs at high concentration levels by poultry farms have been targeted by the FDA due to QA/QC deficiencies related to feed preparations. The developed procedure is linear with a correlation coefficient (R^2) $\geq$ 0.9990 and *p*-values < 0.05; reproducible in area and t_R (%RSD < 1.0 %); and demonstrated a limit-of-quantitation (LOQ) $\leq$ 0.03 ppm (for roxarsone and p-arsanilic acid) and $\leq$ 0.24 ppm (for acetarsone). This method provides a simple, reliable and cost effective alternative for the routine analysis of organoarsenic compounds present in water due to their rapid elution times (< 1.5 minutes). The samples did not require complex preparation techniques prior to the analysis. The robustness was demonstrated by application of the method to complex matrixes such as poultry litter leachates. The application of this method for the analysis of real samples for QA/QC processes and remediation studies is discussed.

RESUMEN

Un método nuevo se desarrolló para el análisis rutinario de las drogas organoarsénicas roxarsona (acido 4-hidroxi-3-nitrofenil-arsónico), acido p-arsanílico y acetarsona (acido *N*-acetil-4-hidroxi-*m*-arsanílico) basado en la técnica de cromatografía líquida con tecnología de rápida resolución y detección con arreglo de diodo. Las drogas estudiadas se utilizan como agentes antimicrobianos y para promover el crecimiento en gallinas. La alta solubilidad que los caracteriza representa un peligro inminente para el medio ambiente debido a que la gallinaza es utilizada como agente fertilizante. Cuando se aplica a los cultivos, estos agentes organoarsénicas se desplazan a cuerpos de aguas y se absorben por las plantas de cultivos. La Administración de Drogas y Alimentos (*FDA* por sus siglas en inglés) ha efectuado auditorias exhaustivas a criaderos de gallina debido a deficiencias en los procesos de control de calidad relacionadas a la preparación de alimentos con estas drogas. El procedimiento demostró ser lineal con un coeficiente de correlación (R^2) $\geq$ 0.9990 y *p*-valor < 0.05; reproducible en área y t_R (%RSD <1.0%); y demostró limites de cuantificación (LOQ) $\leq$ 0.03 ppm (para roxarsona y acido p-arsanílico) y $\leq$ 0.24 ppm (para acetarsona). Este método provee una alternativa simple, confiable y efectivo en términos de costo para el análisis de organoarsénicos presente en agua debido a su elución rápida (< 1.5 minutos). Las muestras no requirieron una técnica de preparación compleja. La capacidad de este método fue demostrado cuando se analizaron muestras acuosas con presencia de gallinaza. La aplicación de este método para procesos de control de calidad y de remediación se discutirá.

Dedicated to my Lord and Savior Jesus Christ, my father Raymond Vélez Irizarry, Jr.

(September 1935 – June 2005), and my loving wife Marisely.

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1 Organoarsenic antimicrobials

Organoarsenic compounds have been employed in numerous applications despite the degradation byproducts that these can generate which are known to be potentially harmful to the public health.[1-3] For instance, thenarsazine oxide (10, 10'-oxydiphenoxarsine) is a registered antimicrobial in plastics (e.g. shower curtains) and as a preservative for adhesives. Cacodylic acid (dimethylarsenic acid) and monosodium methyl arsenate are well known herbicides used mainly in cotton farms and grass-growth facilitation processes for golf courses. Medications were developed during the early 19th century such as the anticancer agent *Atoxyl* (sodium-*p*-aminophenylarsonate) and *Salvarsan* (also known as asphenamine), a well-known treatment for syphilis.[4] Since the mid-1900's particular interest has been given to the use of organoarsenics, like roxarsone (3-nitro-4-hydroxyphenylarsonic acid), p-arsanilic acid, and acetarsone (*N*-acetyl-4-hydroxy-m-arsanilic acid), as feed additives for poultry and other broilers across the nation.[5, 6]

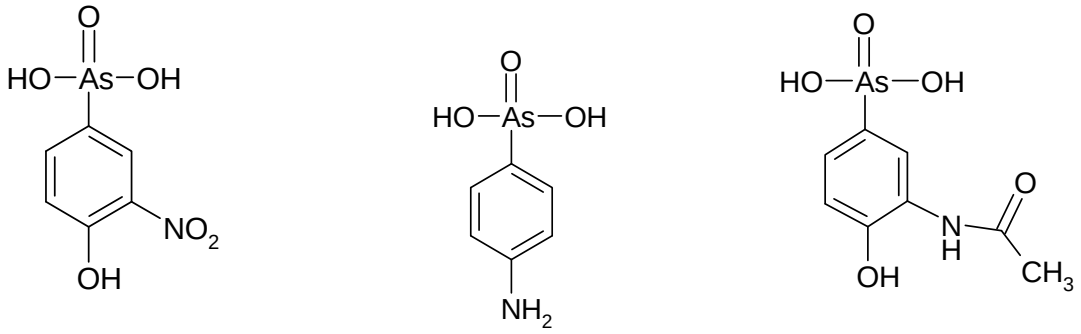

Figure 1: Representative organoarsenic drugs used in the poultry industry: (a) roxarsone, 3NHPAA; (b) p-arsanilic acid, p-ASA; (c) acetarsone, NAHAA.

The organoarsenic drug roxarsone, 3-nitro-4-hydroxyphenylarsonic acid, (Figure 1) has been approved by the Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) as a growth promoter and anti-microbial agent in poultry.[7] It was originally developed in 1946 as a therapeutic treatment of coccidiosis (intestinal parasites). However, it was not until 1949 that it was approved as a growth promoter for poultry. Toxicological effects of this organoarsenic documented for poultry, turkey, swine include tremors, hypoplastic anemia, and extreme agitation of shoulders, legs, and back.[8] A study disclosed over 80 different brands of poultry feed containing this organoarsenic drug currently used in approximately 90% of the broilers in the US.[9] The FDA and USDA approved dose ranges from 22.7 g/ton (0.03 ppm) to 45.4 g/ton (0.05 ppm). The USDA recently reported that broiler production reached over 50 billion pounds in 2007.[10] It is estimated that about 70% of this production was fed a minimum 1.7 million pounds of roxarsone.[9] According to the National Institute of Health (NIH), roxarsone has an LD_{50} value of 81 mg/kg.[11]

The organoarsenic drug p-arsanilic acid has also been approved by the FDA and USDA as an anti-microbial agent in poultry.[12] It is mainly combined with other antibiotics (e.g. penicillin, erythromycin), although its use is not as extensive as roxarsone.[9] The regulated feed dosage ranges from 45 g/ton (0.05 ppm) to 90 g/ton (0.10 ppm).[12] The organoarsenic drug p-arsanilic acid has also been employed in swine as a growth promoter and therapeutic treatment for dysentery.

Acetarsone was initially introduced as *Stovarsol*, a medical treatment of congenital herpes.[13] It also demonstrated favorable results for amebic dysentery. Currently, it is used as an anti-protozoal in turkey and geese.[14] Prolonged use of these

organoarsenics not only resulted in high levels of arsenic accumulation within the liver, but in negative physiological effects such as discoordination of the limbs and the loss of standing ability.[15]

Consequently the use of organoarsenic compounds as animal feed additives requires a precise dosage to reduce retention and accumulation in vital organs and tissue that can be consumed by humans. The FDA has established limits for total residues of combined arsenic in tissue and by-products that are edible ranging from 0.5 mg/L to 2 mg/L.[16] The Environmental Protection Agency maximum allowable concentration of arsenic in drinking water is 0.01 mg/L.[17]

The reactivity and speciation of organoarsenic compounds used as animal feed additives such as roxarsone constitutes an even greater concern due to the toxicity of their degradation products. Roxarsone is excreted virtually unchanged into poultry litter.[1] The degradation of roxarsone in this media has been the subject of various investigations directed towards biological and photolytic processes.

Stolz et.al investigated the biotransformation of roxarsone in a microbial-mediated process with *Clostridium* and *Lactobacillus* (known microorganisms in poultry litter) under anaerobic conditions.[18] They reported 4-hydroxy-3-aminophenylarsonic acid (4-HAPA) and p-arsanilic acid (p-ASA) as the main degradation products of roxarsone in fresh litter. However, As^{V} was the only species present after extended microbial action (t>200 hours) with the aforementioned microbials. Additional research concluded that the conditions under which the biotransformation process occurs favor the reduction of the nitro-group (in position 3 of the roxarsone moiety) prior to reducing

the arsenic group (in position 1). However the release of As^{V} by the cleavage of the As-C bond was not explained.

The degradation of roxarsone, 4-hydroxy-3-aminophenylarsonic acid (4-HAPA) and p-arsanilic acid was studied by Cortinas et.al using anaerobic methanogenic sludge from local waste water treatment plants.[19] According to the investigators, these organoarsenic compounds were slowly biologically eliminated as the concentration of As^{V} increased. The final results revealed that the As^{V} was evidently reduced to As^{III} under the methanogenic conditions. However, the mechanism by which the cleavage of the As-C bond is obtained was not discussed with the biotransformation process.

Bednar et.al performed photolytic degradation studies of roxarsone.[20] The main degradation product observed for roxarsone dissolved in deionized water (DIW) was As^{III} and As^{V}. The slight presence of a phenol suggested a possible photolytic reduction of the nitro group of roxarsone although at very small concentrations. The major degradation product identified when roxarsone was diluted in nitrate-rich aqueous solutions and exposed to UV light was 2,4-dinitrophenol. The mechanism for the photolytic cleavage of the As-C bond for roxarsone proposed by the investigators is shown in Figure 2.

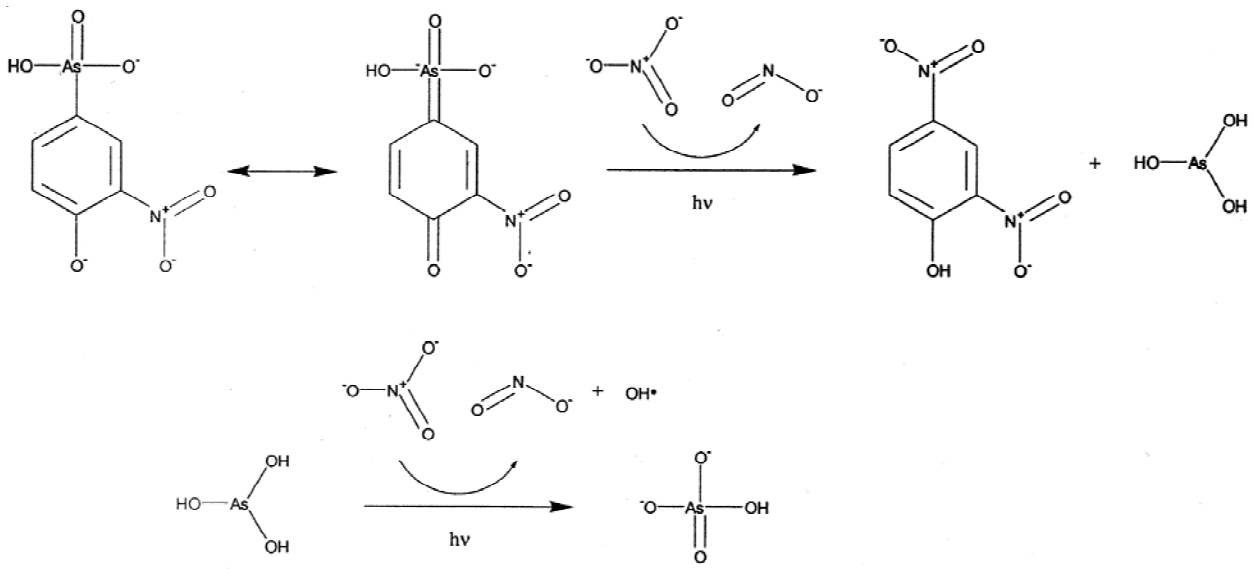

Figure 2: Proposed photolytic degradation mechanism of roxarsone by Bednar et.al[20]

About 95% of the roxarsone is released intact when excreted in poultry litter.[1, 21] Furthermore, the drug is 95% water soluble and 70% to 90% of the arsenic released in poultry litter is also water soluble.[1, 21] Poultry litter has been traditionally used as fertilizer. It is poured directly onto agricultural fields along with any material excreted by the poultry such as organoarsenics. This represents an important environmental problem since these agents can be readily absorbed by plants or leached into water bodies thus becoming a potential risk to public safety.[22] Furthermore, studies demonstrated that roxarsone's main degradation products through biotransformation processes and photolytic reactions occurring within the poultry litter are the highly toxic species of arsenic, As^{III} and As^{V}.[18-20]

2 Arsenic overview

Arsenic is a metalloid belonging to the group VA elements. It is the thirty-third (33^{rd}) element in the periodic table with a molecular weight of 74.9216 g/mol. Arsenic ranks twentieth (20^{th}) among the elements in abundance in the earth's crust. Elemental arsenic is seldom encountered free in the environment, although it is a major constituent of about 245 mineral species.[6, 23] Arsenic can exist in a variety of forms both organic and inorganic. Its main mineral sources include sulfur, copper, gold, and lead ores.[6, 24] According to the 2008 U.S. Geological Survey, domestic arsenic production has not occurred since 1985. Current reports indicate that approximately 58,354 tons of arsenic was imported between 2003 and 2007, from which 8% corresponds to its metalloid form while the remaining 92% correspond to arsenic trioxide. China is the main importer of metal arsenic (86%) and arsenic trioxide (63%).[24]

Once arsenic is ingested by a person, part of it is excreted very slowly as a methylated species in urine.[23] This makes arsenic poisoning persistent and inherently cumulative in the human body were it is mainly accrued in the hair and nails. Arsenic toxicity depends on factors such as its physical state, route of intake, chemical substituent of the compound, and bioavailability. The following is a general summary of the toxicities for different arsenic species as described by Mandal:[23]

- As^{III} is considered more toxic than As^{V} due to the absence of a direct reaction of the latter with active sites on enzymes. As^{III} is capable of forming strong bonds to sulfur groups (-SH) and hydroxyl groups (-OH). As^{III} binds to the sulfur groups of dihydrolipoic acid, thus preventing further enzymatic activity. In addition, the formation of adenosine-5-triphosphate (ATP) is prevented due

to the selectivity of As^{III} over phosphate during the reaction with glyceraldehyde 3-phosphate. It is widely known that ATP is responsible for cellular energy transport.

- Ionic and soluble species of arsenic are considered more toxic than insoluble arsenic. This behavior was attributed to the greater absorption of soluble arsenic when compare to its insoluble counterpart. However, there are differences in toxicity levels between soluble forms of inorganic arsenic and organoarsenic. The organoarsenic were considered less toxic based on the fact that this species can be excreted by the human body faster than inorganic arsenic.

The main routes of human intake may be through the respiratory tract (e.g. dusts, particulate matter) and oral (e.g. food, water). According to Mandal, the fate of arsenic after consumption will depend on (1) REDOX reactions between As^{III} and As^{V} which is most likely mediated with glutathione S-transferase and (2) oxidative methylation reactions within the liver with S-adenosyl-methionine as the methyl donor. As^{III} and As^{V} is metabolized into toxic and suspect-carcinogenic methylated organoarsenic compounds such as monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}), dimethylarsenous acid (DMA^{III}), trimethyl arsine oxide ($TMAO^{V}$), and trimethyl arsine (TMA^{III}).[2] Arsenic toxicity studies have attributed DNA damage in human peripheral lymphocytes due to the presence of MMA^{III} and DMA^{III}.[2] Figure 3 is a schematic representation (adapted from Sams) for the *In Vivo* metabolism of inorganic arsenic.[2]

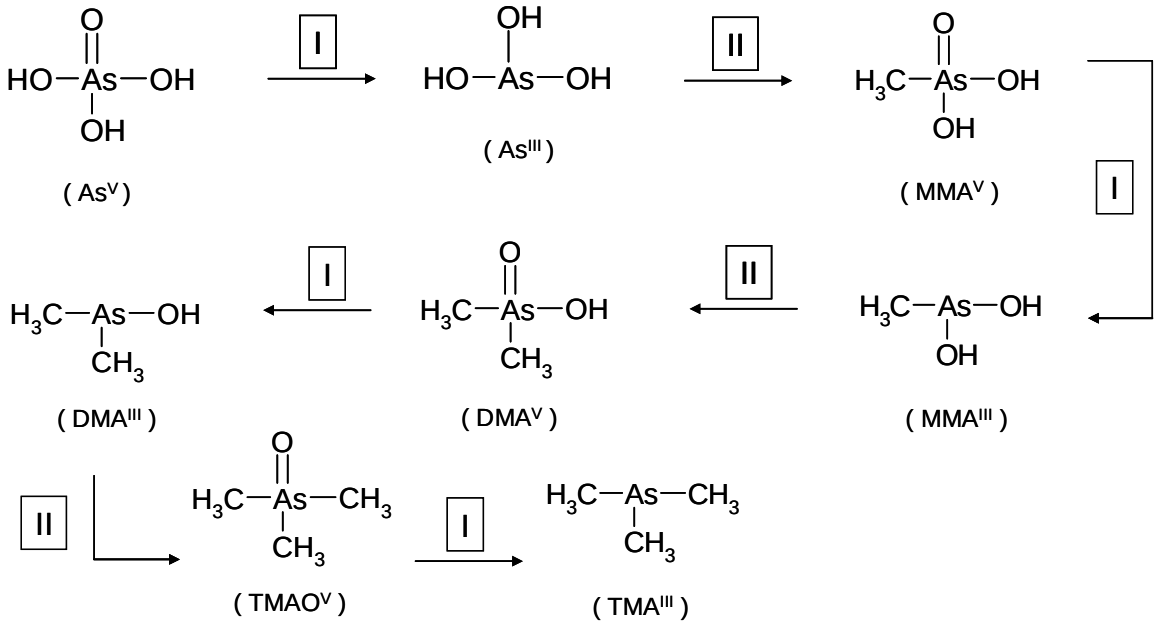

Figure 3: *In Vivo* metabolism of inorganic arsenic, where (I) is a 2e$^-$ reduction with glutathione S-transferase and (II) is an oxidative methylation mediated with S-adenosyl-methionine.[2]

History has been witness to the extensive use mankind has given to arsenic from the manufacture of cosmetics, insecticides, food, medication, wood preservatives, metallurgy, and electronics.[4] In 1809 Fowler's solution (potassium arsenite) was considered a "cure-all" therapeutic treatment, although it was specifically used as an anti-tumor drug and for dermatology applications. Despite the toxicity of arsenic-based compounds, the FDA approved Trisenox®, (arsenic trioxide for intravenous administration) during the year of 2000. Trisenox® is used for the treatment of acute promyelocytic leukemia (a disease corresponding to recurring white-blood cell cancer) or in cases were standard cancer therapeutic treatments are ineffective.[25]

3 Previous studies

3.1 Introduction

Previous studies have been directed toward the development of detection methods for organic and inorganic arsenic-containing compounds due to their potential toxicities to living organisms once introduced to the environment. The utilization of a specific method requires that factors, such as the moiety of the compound, sample origin, matrix complexity, and method efficiency, be taken into consideration. In addition, the cost of the analysis itself can become a decisive factor in the design and selection of an analytical assay for the detection of these agents in real samples.

Attempts toward the development of analytical procedures for the fast and effective identification of organoarsenics include the use of hyphenated techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and inductively coupled plasma-mass spectrometry (ICP-MS).

3.2 Atomic absorption with graphite furnace (ASS-GF) studies

Atomic absorption with a graphite furnace (AAS-GF) has been used for arsenic determinations.[26, 27] The main problem arising from such methods is the lack of selectivity. The AAS-GF technique cannot differ between arsenite (As^{III}), and arsenate (As^{V}). This means that the detector response will provide the total arsenic content in a sample. Research in which arsenic speciation is relevant requires more sophisticated techniques such as LC-ICP or electroanalytical methods. Furthermore, it is necessary at times to use chemical derivatization or modifiers thus to increase the thermal stability

of the species of interest in order to displace it from a complex matrix prior to the atomization step. Developments of specialized methods have pursued to enhance the specificity of organoarsenic analyses. Latva utilized metal-loaded activated carbon thus to pre-concentrate different arsenic species at different pH levels.[26]

3.3 Gas chromatography (GC) and gas chromatography with mass spectrometry (GC-MS) studies

Over the past 50 years GC-MS have been employed as a routine tool for the identification and chemical characterization of target molecules and unknown species. Nevertheless, as with all methods related to GC, the analytes must possess a volatile character. Species that are non-volatile must be derivatized in order to employ this application. Stable derivatives are a key step in GC in order to ensure the effective reaction completeness between the target analyte and the derivatization agent. Koster and Aldstadt developed GC-MS methodology, using electron ionization (EI) and pulsed flame photometric detection (PFFD) respectively, for the identification of organoarsenic compounds such as monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), and arsenious acid.[28, 29] Roxarsone and its degradation products required the use of derivatization agents due to their non-volatile nature. The analytes understudy were derivatized with either sodium borohydride ($NaBH_4$), 1,3-propanedithiol, or benzothiophene. Although structure elucidation was performed by careful study of the chromatograms for potential fragmentation patterns, several key experimental issues were not completely resolved. For instance, the concentrations of the organoarsenic compounds studied were not reported by Koster.[28] Thiol-containing derivatization agents posed problems for accurate detection due to the co-elution of sulfur with the

organoarsenic compounds during the studies performed by Aldstadt and collaborators. In addition, evident self-chemical ionization (CI) of the species in the ion-trap spectrometer complicated the interpretation of molecular ions. Aldstadt further performed studies on roxarsone using SPME-GC-MS method for the detection of roxarsone, MMAA, and DMAA.[30] Derivatization with 1,3-propanedithiol was necessary, thus transforming the analytes into cyclic dithiaarsenolines for ease of volatization. The reported limit of detection of roxarsone was 2.69 μg/L, although it is not mentioned how the self-chemical ionization ceased to be a negative factor for the unequivocal identification of the molecular ions.

3.4 HPLC (High-performance liquid chromatography) studies

Methods using HPLC-UV have been developed for the detection of various organoarsenicals such as aminophenylarsonic acids and nitrophenylarsonic acids. Roxarsone has been targeted by few investigators for HPLC-UV methods. Arsenic compound studies with ion chromatography and normal phase chromatographywere performed by Kuwamoto and Uden respectively.[31, 32] Compounds such as *o*-aminophenylarsonic acid (*o*-APA), *p*-aminophenylarsonic acid (*p*-APA), *o*-nitrophenylarsonic acid (*o*-NPA), triphenylarsine, As^{III}, and As^{V} were targeted. Eigendor and Momplaisir developed reverse phase HPLC-UV methods for the detection and quantitation of 3-nitro-4-hydroxyphenolarsonic acid (roxarsone), 4-nitro-phenylarsonic acid (4-NPAA), p-arsanilic acid (p-ASA), and other arsenic compounds.[33, 34] Conventional C_{18} columns with a mobile phase containing low amounts of organic solvent were used for the analysis. Although these investigations have postulated wide linear ranges (0.4 ppm to 50 ppm) and low detection limits (0.06 ppm), the methods lack

the ability to elute all the specific analytes they targeted in one chromatogram. In some cases, detector responses for some analytes under study were not reported. In other cases, the authors did not specify if they were undetectable or if they just co-eluted with the unretained species. In addition, the analytical runtimes were significantly high for practical applications.

3.5 High performance liquid chromatography with mass spectrometry (HPLC-MS) studies

The introduction of the LC-MS methodology can be considered as an advance in structural characterization techniques. LC-MS does not require derivatization agents for ease of volatization as in GC-MS since analytes are directly ionized by an electrospray. Moreover, the integrity of the target analytes are somewhat preserved since they are not subject to high temperatures. It provides the atomic mass of the analytes injected just as in GC-MS. In combination with other spectroscopic methods (e.g. IR, Raman, NMR), LC-MS can provide an adequate approach as to the identification and elucidation of organoarsenic structures. Research on organoarsenic compounds have been performed utilizing LC-MS. Various structures of organoarsenic compounds were elucidated by Larsen using an HPLC containing an ion trap mass spectrometer.[35] collision induced dissociation (CID) was used to fragment the target analytes which included dimethylarsinic acid (DMAA) and monomethylarsonic acid (MMAA). Wu used electrospray ionization (EI) for the LC-MS analysis of organoarsenic compounds such as monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), arsenobetaine (AsB), and arsenocholine (AsC).[36] Chromatograms of total ion current (TIC) and

selected ion monitoring (SIM) both demonstrated peaks of the aforementioned compounds.

3.6 High performance liquid chromatography with inductive coupled plasma –mass spectrometry (HPLC-ICP-MS) studies

Extensive work has been performed using HPLC with inductive coupled plasma mass spectrometry (HPLC-ICP-MS) for the analysis of organoarsenic compounds (including roxarsone) due to its capacity of analyte detection at concentrations in the ppb levels.[37-40] Ronkart developed an HPLC-ICP-MS method for the speciation of arsenite (As(III)), arsenate (As(V)), monomethylarsonic acid (MMAA), monomethylarsonic acid (DMAA), and arsenobetaine (AsB) in surface, well, and mineral water.[41] A Dionex Anion exchange column and a gradient program for an ammonium dihydrogen phosphate based mobile phase was found to provide adequate conditions for the separation of the target analytes. Limit of quantitations (LOQ) ranged between 56 ng/L to 88 ng/L. Limit of detections (LOD) ranged between 17 ng/L to 26 ng/L. HPLC-ICP-MS was used by Garbarino in various occasions for the determination of Roxarsone in contaminated soils.[1, 21] Detection limits were reported in the parts per billion (ppb) ranges.

Although these methods provided detector responses in low analyte concentration levels, there are several factors that should be considered prior to the implementation as a routine environmental lab technique. HPLC-ICP-MS methods have been found to suffer from detector interfacing problems.[39] Buffers based on non-volatile salts, such as phosphate and sodium, are widely used for HPLC-UV methods due to their UV transparency. Yet, with ICP-MS detectors, non-volatile buffer salts are

not suitable since these may accumulate in different equipment parts (e.g. lenses, skimmer cones), thus affecting the detector response. Furthermore, large volumes of organic modifiers (e.g. methanol) in the mobile phase may also compromise the stability of the plasma. HPLC-ICP-MS techniques for arsenic-containing compounds are subject to plasma-detector related interferences.[39] Argon from the plasma detector and chlorine from a sample matrix may combine to form argon chloride (ArCl). ArCl may exhibit a m/z = 75, which is similar to the arsenic m/z. The instrumentation must be capable of controlling the elution of the chloride prior or post the species of interests thus to avoid interference for a correct identification. Caruso et al suggests mathematical corrections which subtract the signal generated by ArCl may be used to reduce the impact of the interfering molecules.[39]

4 Statement of the Problem

Currently, the high-levels of organoarsenic compounds in poultry feed have been targeted by the FDA due to QA/QC deficiencies.[42-44] However, the role of the FDA is directed towards regulating the final product (edible poultry meat) and not the by-products produced in the feces. Consequently, the excess of organoarsenicals in poultry feed will contribute to an increase arsenic content in poultry litter. To make matters worse, the contaminated litter will be used as fertilizer for agricultural fields where it will be exposed to the environment at a greater extent. The reactivity and speciation of these organoarsenic compounds is of great concern due to their extensive runoff towards soil and water. Analytical methods are necessary for the rapid detection and accurate quantification of organoarsenicals in complex matrixes such as feed water, natural waters with dissolved ions, and poultry litter leachates.

Research has been conducted with the goal of developing analytical methodology for the analysis of organoarsenic compounds using the wide array of techniques described in the preceding section. However, these methods result to be complex, time-consuming, and costly. The ideal method should be capable of rapidly detecting organoarsenicals in the matrix to which these are exposed. The technique should be simple and capable of providing accurate results. The waste associated with the analysis should be minimal. Consideration should be given to the operational costs of the analysis in order to provide a method that is financially accessible to any laboratory despite their particular budget restraints. The subsequent sections describe how Ion-Pairing HPLC (IP-HPLC) with UV detection is an ideal method for the detection of organoarsenic drugs such as roxarsone, p-arsanilic acid, and acetarsone.

5 High-Performance Liquid Chromatography (HPLC)[45-47]

5.1 Introduction

HPLC is a separation method by which species within a sample matrix migrate through a narrow, packed column in a given direction at rates confined to different mobilities based on individual properties (e.g. adsorption, partition, solubility, ionic charge). Identification of the species can be obtained by reference to a standard material's retention time (t_R) after being introduced into the chromatographic system under equal operational parameters. Quantification is achieved by the use of the area under the curve of the bands depicted upon recognition with a suitable detector device.

The most common detector used for HPLC is based on a hyphenated ultra-violet (UV) detector. The main reason for the routine use of UV is based on the fact that most molecules exhibit some type of chromophore by which a change in detector response can be easily observed. Chromophores are unsaturated functional groups that promote the absorption of UV-VIS radiation.

HPLC analyses are common for pharmaceutical and veterinary products because they are mostly complex organic molecules with the appropriate structure for detection by UV. Various agencies have been established at a global level to regulate the scientific aspects of analytical laboratories focused on Quality Assurance and Quality Control (QA/QC) such as the United States Food and Drug Administration (FDA), the International Standardization Organization (ISO), and Japan's Pharmaceuticals and Medical Devices Evaluation Center. Guides have been established for the consistent execution of procedures by the many companies dedicated to manufacture of pharmaceuticals, medical devices, and food products. The

pharmacopoeias of the United States (USP), Europe (EP), and Japan (JP) are an example of the most common guides. The global demand for drugs and antimicrobials called for efforts to simplify the transition of new products to foreign countries like the International Conference of Harmonization (ICH) Q2 (R1), *Validation of Analytical Procedures, Text and Methodology*. Since Roxarsone, p-arsanilic acid, and Acetarsone can be marketed internationally, it was determined that the HPLC method validation would be based on ICH Q2 (R1). The USP was used as supplemental information for details not provided in the ICH guidelines (e.g. specific system suitability parameters).

5.2 System Suitability (SS)[48]

System suitability performance checks (SS) are required in order to appropriately determine adequacy of the HPLC system used for sample analysis. The successful execution of SS may be considered time consuming by many. However, this should not be taken as a set-back because the integrity of the sample results obtained will depend on the SS data. The SS parameters are established after successful completion of validation exercises. It is important to recognize that there are a vast number of parameters that form part of the validation exercises that are not necessarily performed in routine analysis on a daily basis. Although numerous parameters can be surveyed, not all are necessary to guarantee suitability of the HPLC system. The system suitability calculations used for the HPLC studies performed are normally based on USP Chapter <621>, *Chromatography*. Figure 4 depicts the common variables used for suitability calculations of a typical chromatogram obtained by a HPLC. A system suitability accounts for the following parameters according to USP Chapter <621> protocols: theoretical plates (N), resolution (R), tailing factor (T_f), and precision.

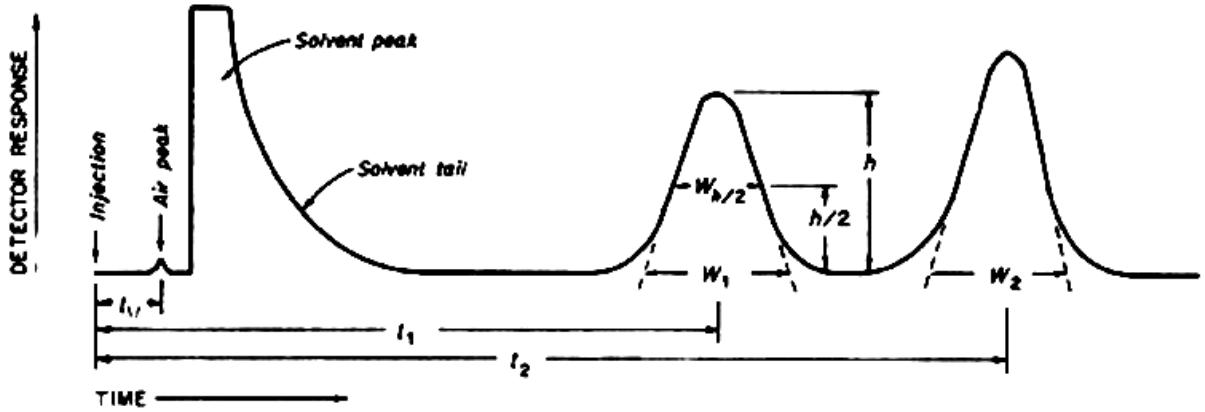

Figure 4: Generic chromatogram with common variables identified as per USP Chapter <621>.

5.2.1 Theoretical Plates (*N*)

The column efficiency can be evaluated by the theoretical plates (*N*) of the bands corresponding to the eluting species. The t_R and the bandwidth (*W*) of the unretained species are the basic chromatographic elements used for determination of *N* (1).

$$N = 16\ (t_R / W)^2 \qquad (1)$$

Values of *N* can fluctuate from a few 100 to amounts greater than 10,000. Acceptance limits have not been established since these should be method specific and are commonly determined from validation data. For example, if a column which usually depicted values for $N > 5000$ has suddenly decrease to $N = 100$, then it can be safely assumed that the column efficiency has been compromised after confirmation that all solutions have been prepared correctly. However, significant decreases in *N*

values can be corroborated with other parameters. The values of *W* and *N* are inversely proportional. *W* is surveyed with the tailing factor (see subsequent section). Therefore, a decrease in *N* can be corroborated with an increase in the tailing factor.

5.2.2 Resolution (*R*)

The resolution (*R*) is used to determine the baseline separation of a species and the peak eluting prior to it. Their individual t_R and *W* are used for this calculation (2). Values of $R \geq 1.5$ are indicators that the HPLC operational parameters are optimum for integration of the species under study. However, values less than 1.5 are acceptable if appropriate integration can be accomplished of the individual peaks.

$$R = \frac{2\,[\,(t_R)_2 - (t_R)_1\,]}{W_1 + W_2} \qquad (2)$$

5.2.3 Tailing Factor (T_f)

The tailing factor (T_f) is an indicator of the peaks symmetry (Eq. 3).

$$T_f = \frac{W_{0.05}}{2f} \qquad (3)$$

$W_{0.05}$ is the bandwidth at 5% of the peak height from the baseline. The variable *f* is the distance from the peak maximum to the point that is 5% of the peak height from the baseline (refer to Figure 5).

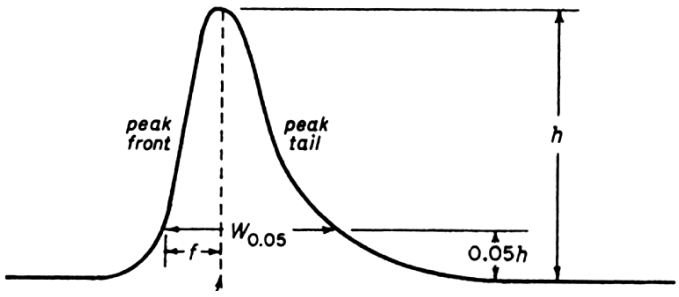

Figure 5: Peak tailing calculation variables as per USP Chapter <621> for a typical chromatogram.

Acceptance values for T_f depend on the chromatographic system used, packing material, and sample matrix. For example, it has been postulated that Ion-Pairing Reverse Phase (RP) HPLC methods will depict increased band tailing than common RP HPLC methods due to the dissociation of ion pairs in the organic phase.[45]

5.2.4 Relative Standard Deviation (%RSD)

Precision for HPLC SS is mainly assessed by the %RSD of the areas and t_R of the species understudy.

$$\%RSD = 100 \times \frac{\left[\sum (X_i - X_{Avg})^2 / \text{N-1}\right]^{0.5}}{X_{Avg}} \qquad (4)$$

The USP establishes SS precision acceptability with %RSD $\leq$ 2.0% for data of five (5) injections. Precision SS data was acquired at the beginning of the chromatographic analysis of unknown samples post-validation exercises. The acquisition of reference standard injections dispersed throughout the chromatographic

runs were performed thus to assess the suitability of the HPLC system of the whole analysis.

5.2.5 Retention Factor (*k*)

The retention factor (also known as the capacity factor, *k)* is considered the time the solute resides in the stationary phase (t_R) relative the time it resides in the mobile phase (t_M).[49] It has also been regarded as the ratio of the adjusted retention time of a compound and an unretained species.[50]

$$k = \frac{t_R - t_M}{t_M} \qquad (5)$$

USP Chapter <621> does not require the retention factor (*k*) for SS determination. However, *k* will be calculated as part of the method development studies in order to evaluate significant changes in chromatography due to variation of equipment operational parameters.

5.2.6 Selectivity factor (α)

The selectivity factor (also known as the separation factor, α) is defined as the measure of relative retention of two sample components.[50]

$$\alpha = \frac{k_2}{k_1} \qquad (6)$$

As in the case of the retention factor (*k*), USP Chapter <621> does not require the selectivity factor (α) for SS determination. However, α will be calculated as part of the method development studies in order to evaluate significant changes in chromatography due to variation of equipment operational parameters.

5.3 ICH Validation Guidelines[51]

ICH Q2 (R1), *Validation of Analytical Procedures, Text and Methodology*, was used as the guide to establish validation parameters for the development of a novel HPLC method for the analysis of organoarsenic drugs in this study (roxarsone, p-arsanilic acid, and acetarsone). The subsequent sections define the parameters surveyed during the validation process as established by the guide.

5.3.1 Specificity

Specificity is defined as the ability to assess unequivocally the analyte in the presence of components expected to be present. The developed method was subject to numerous sample matrixes (e.g. potable water, creek water, chicken litter). Roxarsone (the main species under study) was exposed to compounds of similar molecular structure which presence in unknown samples is possible (e.g. p-arsanilic acid, acetarsone). Identification of the species under study was assessed by t_R of individual preparations containing a specific reference standard material. The samples were submitted to peak purity tests using a diode array detector to further authenticate the absence of co-eluting species.

5.3.2 Precision

Precision is the degree of scatter between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. It is considered at three levels: repeatability, intermediate precision, and Reproducibility. For the purposes of this study, repeatability and intermediate precision were performed. These are defined as follows:

- Reproducibility (or repeatability) corresponds to the precision of the same operating conditions over a short interval of time. This was assessed from the %RSD of the specific species variables (areas and t_R). The precision parameters surveyed for SS exercises post-validation exercises are categorized under this definition.

- Intermediate precision assess variations within the same laboratory corresponding to different days and equipments. Intermediate precision was evaluated using varying the HPLC column serial number, mobile phase preparations, standard preparation, and analysis dates.

5.3.3 Linearity

Linearity is defined as the ability of an analytical procedure to obtain results which are directly proportional to the concentration (amount) of an analyte in the sample. Linearity was established in the developed method for the analysis of organoarsenic drugs under study using a minimum of five (5) standard solutions with different concentration levels. Data obtained from ANOVA was employed to establish a linear relationship for area response vs. concentration.

5.3.4 Accuracy

Accuracy is corresponds to the closeness of agreement between the reference value and the value found. According to ICH Q2 (R1), the accuracy of a method can be inferred once precision, linearity, and specificity has been established. In general terms, an HPLC method can be considered accurate if it can unequivocally detect a species in a reproducible manner while maintaining a linear response with its concentration.

5.3.5 Range

The range established for the developed method was derived from the linearity study. The concentration of roxarsone ranged from a high level, corresponding to the Limit-of-Linearity (LOL), to low levels corresponding to the Limit-of-Quantitation (LOQ) and Limit-of-Detection (LOD).

5.3.6 Limit-of-Quantitation (LOQ)

The Limit-of-Quantitation (LOQ) is the minimum concentration at which a quantifiable response can be produced. Despite that current ICH guidelines provide various manners for calculating LOQ values for a method, the acceptance criteria established remains the same for all. The concentration corresponding to the LOQ must exhibit a minimum signal-to-noise ratio (S/N) of 10. In this study, the standard deviation (*s*) of the noise in various blank injections and the slope (*m*) of the calibration curve were used to calculate the LOQ.

$$\mathrm{LOQ} = \frac{10s}{m} \qquad (7)$$

5.3.7 Limit-of-Detection (LOD)

The Limit-of-Detection (LOD) is the minimum concentration at which a detectable response can be produced. A detectable response is that which can be correctly distinguished from baseline noise. As in the case of LOQ, current ICH guidelines provide various manners for calculating LOD values for a method. However, the acceptance criteria established remains the same for all. The concentration corresponding to the LOD must exhibit a minimum signal-to-noise ratio (S/N) of 3. In this study, the standard deviation (*s*) of the noise in various blank injections and the slope (*m*) of the calibration curve were used to calculate the LOD.

$$\text{LOD} = \frac{3.3s}{m} \qquad (8)$$

5.4 Ion-Pair High Performance Liquid Chromatography (IP-HPLC) coupled with Rapid-Resolution HPLC Column[45-47, 49]

The successful use of HPLC for a given problem requires the right combination of operating conditions which include (but is not necessarily limited to) column dimensions, column packing type/size, mobile phase type/flow rate, separation temperature, and sample size. However, the type of sample and its matrix play the most important role in the determining the methodology that will provide the sensitivity and accuracy desired. The species under study are water-soluble organoarsenic drugs with an acidic pH nature. They may be found in organic solid matter or in run-off water

that has been in contact with contaminated soil. Roxarsone and p-arsanilic acid may be found within the same sample matrix since they are common in poultry leachates. Acetarsone, although not routinely employed in poultry feed, has a molecular structure similar to roxarsone. Therefore, the ideal method will have the capability of analyzing aqueous samples with ionizable species of similar nature.

Ion-Pair HPLC (IP-HPLC) is a chromatographic method that fits the description of the “ideal method” required for the analysis of the aforementioned organoarsenic drugs. IP-HPLC is an application fit for compounds that exhibit high polarity and/or multiple-ionization. Aqueous samples can be injected directly thus eliminating sample preparation steps requiring the use of solvents. Most importantly, typical reverse phase HPLC columns. This is highly attractive because ionizable compounds which lack specificity in a reverse phase column can be resolved by the use of an ion paring agent. Therefore it is necessary to understand the role of the counter-ion in order to apply efficiently the IP-HPLC methodology.

The mobile phase of IP-HPLC mainly consists of an aqueous solution and an organic solvent (e.g. methanol, acetonitrile). The aqueous phase contains the ion-pairing agent (IpA) and any other necessary modifiers required to obtain adequate peak shape and specificity. The counter-ion used for the IP-HPLC method must possess an opposite-charge to that exhibited by the target analyte. There are two mechanisms which describe the retention of the analytes with the ion pairing agent.

The first retention mechanism postulated is the attraction of the opposite charged ions. A neutral species produced from the attraction of sample species and the ion pairing agent migrates through the HPLC column. The migration rates of these neutral

species will be influenced by interactions with the packing material. It is highly preferable for the analyte to be completely ionized thus to maximize the ion-pair formation. If ion-pair formation is maximized, the solvent strength will depend mainly on the mobile phase composition. Figure 6 depicts the ion pair formation postulated for roxarsone with methyl-tributyl ammonium hydroxide (MTBA-OH).

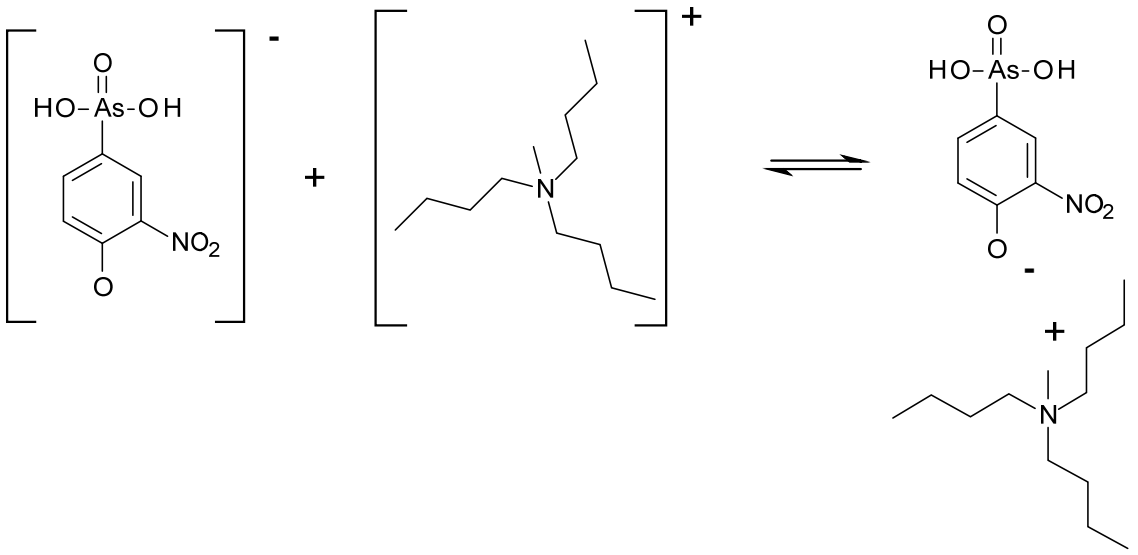

Figure 6: Ion pair formation between roxarsone and the ion-pairing agent methyl-tributyl ammonium hydroxide (MTBA-OH). The ionized forms are shown.

A second model suggested (the most widely accepted) retention mechanism for IP-HPLC analysis consists of several steps during the chromatographic run.[49] The hydrophobic end of the ion-pairing agent is adsorbed onto the HPLC column packing thus exposing the charged end to the mobile phase. Consequently, any ionizable species of opposite charge will be retained momentarily by the enhanced packing material. Species which are not ionized and those with similar charge of the ion pairing agent are expected to elute early in the chromatogram because interaction with the enhanced packing material will be minimal (Figure 7).

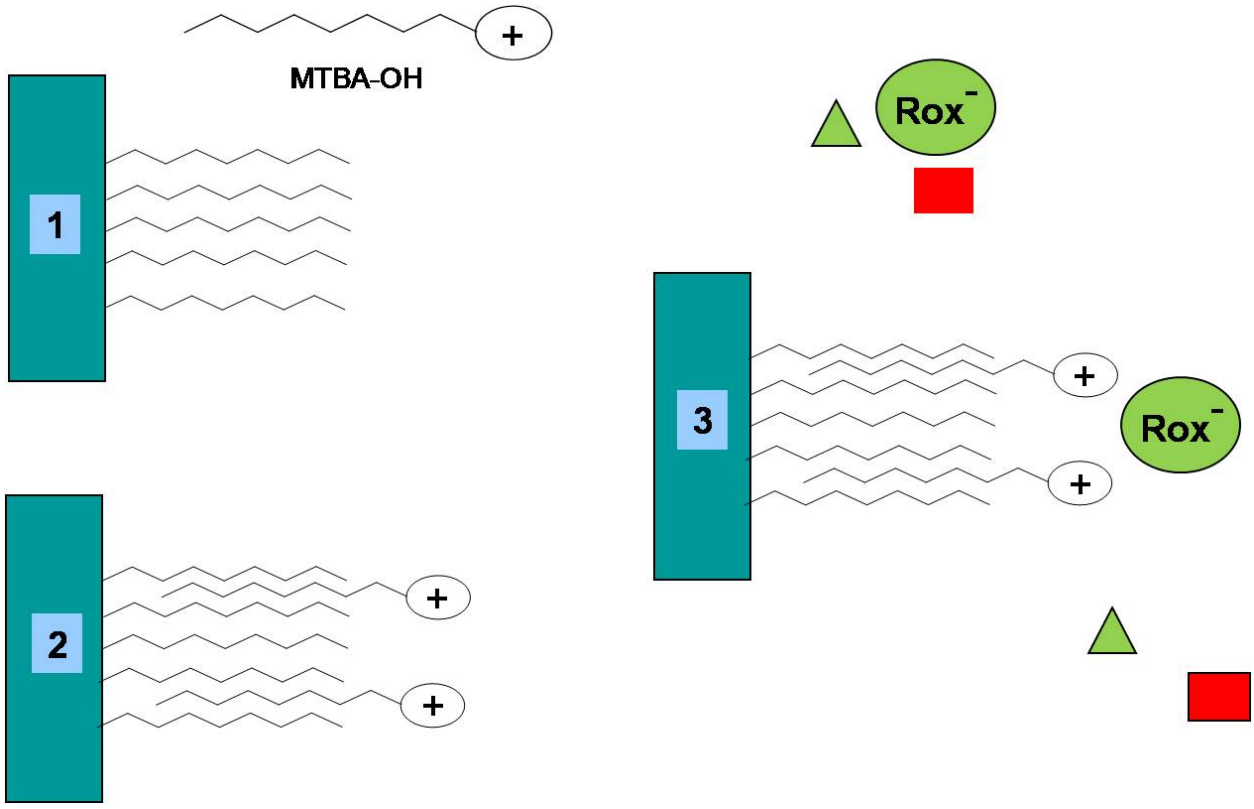

Figure 7: Second retention mechanism proposed for ion-paring HPLC (IP-HPLC). (1) An ion-pairing agent is added to the mobile phase and introduced into the HPLC column. (2) During the equilibration process, the ion-pairing agent is adsorbed onto the column packing material with the charged ionic-end exposed. (3) As the sample migrates through the column, ionizable species interact with the enhanced packing material, and the separation occurs. The figures ▲ and ■ in the diagram represent any species that could be found within the roxarsone (Rox^-) sample matrix.

Many investigations have been performed in order to explain the IP-HPLC dominant retention mechanism (neutral ion formation and column packing enhancement) and these are still under discussion today.[52, 53] However, most investigators agree that the dominant process is the second retention mechanism (enhanced column packing) due to the equilibration time required for ion pair formation prior to entering the column. The samples are exposed to the ion pairing agent when injected onto the HPLC equipment. The migration time between the injector and the column inlet is not considered suffice for appropriate equilibration between the analyte molecules and the ion pairing agent. In order to obtain appropriate ion pair formation, the analyte must be diluted in a solution containing the ion pairing agent. Furthermore, an equilibration time would be required. One main advantage of IP-HPLC is the injection of samples without complex preparatory steps. The ion pairing agent is added to the mobile phase and the baseline is allowed to equilibrate. During this equilibration process, the ion pairing agent is embedded onto the column packing material. When the sample is injected, the analyte is retained due to interactions with the ion pairing agent.

6 The composition of roxarsone as a function of pH

6.1 Introduction

The acid dissociation constants (K_a) of ionizable species play a key role when developing pH dependent analytical methodology such as IP-HPLC. The analytes understudy can be considered weak acids because (1) they do not undergo complete ionization when diluted in water and (2) the reactions are reversible. The p-functions of acid dissociation constants (pK_a) provide the starting point for the development of an IP-HPLC method.

K_a values for a known concentration of roxarsone can be determined experimentally by titration with a diluted standardized base. A plot of the pH vs. the titrant volume may produce a sigmoidal curve from which the equivalence point (EqP) and half-equivalence point ($EqP_{0.5}$) can be determined. The EqP is the point at which the stoichiometric amounts of the weak acid and the titrant are equal.[54] It is graphically described as the maximum change in slope observed in the titration curve. The $EqP_{0.5}$ is the point of the titration in which half of the weak acid has been neutralized by the titrant.[55] It is graphically located at half the volume required to reach the EqP. It is at the $EqP_{0.5}$ that a titrate and titrant exhibit a 1:1 stoichiometric concentration. From the Henderson-Hasselbalch equation (9), the $EqP_{0.5}$ is the point of the titration at which the pH is equivalent to the pKa of the weak acid (10).

$$pH = pK_a + \log [A^-] / [HA] \quad (9)$$

$$\text{if } [A^-] = [HA], \text{ then } pH = pK_a + \log 1 \quad (10)$$

$$\text{Since } \log 1 = 0, \text{ then } pH = pK_a \quad (11)$$

Considering roxarsone as a weak acid, its experimental pKa value can be determined from the pH at its $EqP_{0.5}$ (10). Consequently, the K_a value is obtained as follows:

$$K_a = 10^{pKa} \quad (12)$$

The K_a values determined for roxarsone will be the key factor for determining the composition of roxarsone as a function of pH. The molecular structure of roxarsone depicts the possibility of three consecutive deprotonations (removal of the H_3O^+ ions) as follows: H_3O^+ from the hydroxyl group in position 4 and $2H_3O^+$ from the arsonic acid group.

It can be postulated that the H_3O^+ of the hydroxyl group will correspond to the first Ka value due to its proximity to the nitro group ($-NO_2$) in position 3. The nitro group is more electronegative than the arsonic group due to the periodicities of nitrogen and arsenic. Furthermore, nitro groups have an electron withdrawing effect upon the aromatic ring, thus increasing the acidity of the hydroxyl group in its proximity. An increase in acidity is indicative of a more feasible deprotonation of the hydroxyl group in position 4 when compared to the arsonic group in position 1. This is demonstrated by the known pKa values of nitrophenol: 2.2 (ortho), 3.4 (para), and 3.5 (meta). The

second and third Ka values of roxarsone should correspond to an H_3O^+ from the arsonic group in position 1.

The Ka values obtained for roxarsone can be used to produce fractional composition graphs. The relative amounts of a species as a function of pH can be obtained by the use of these graphs. The following equations are used to plot these graphs:

$$\alpha_0 + \alpha_1 + \alpha_2 = 1 \tag{13}$$

$$\alpha_0 = \frac{[H_3O^+]^2}{[H_3O^+]^2 + K_{a1}[H_3O^+] + K_{a1}\,K_{a2}} \tag{14}$$

$$\alpha_1 = \frac{K_{a1}[H_3O^+]}{[H_3O^+]^2 + K_{a1}[H_3O^+] + K_{a1}\,K_{a2}} \tag{15}$$

$$\alpha_2 = \frac{K_{a1}\,K_{a2}}{[H_3O^+]^2 + K_{a1}[H_3O^+] + K_{a1}\,K_{a2}} \tag{16}$$

6.2 Experimental procedure

A typical set-up for a potentiometric titration was used to obtain analyte pH readings after addition of the titrant in fixed volume intervals. A solution of 1.0×10^{-3} M NaOH was used as the titrant. This solution was standardization with KHP reference standard material as per USP Method <541>. An analyte solution of 9.45×10^{-5} M roxarsone corresponded to the first titration experiment and 1.0×10^{-3} M roxarsone solution was used for the second. A glass combination electrode with a silver/silver chloride (Ag/AgCl) reference electrode was used to obtain pH readings with a pH meter, (Model Orion 710A). The electrode was submerged in the analyte solution throughout the experiments. Constant agitation at a reduced rate was obtained by the use of a magnetic stirrer. The titrant was added with an automatic-volume pipette to the roxarsone analyte solution at volumes of approximately 0.5 mL (500 μL) or 1.0 mL. The pH results documented correspond to stable readings (ΔpH = 0 for 30 seconds minimum). The temperature throughout the experiment was maintained at 25.0ºC ($\pm$ 0.2ºC). The plots of pH vs. titrant volume were prepared (Figures 8 and 9).

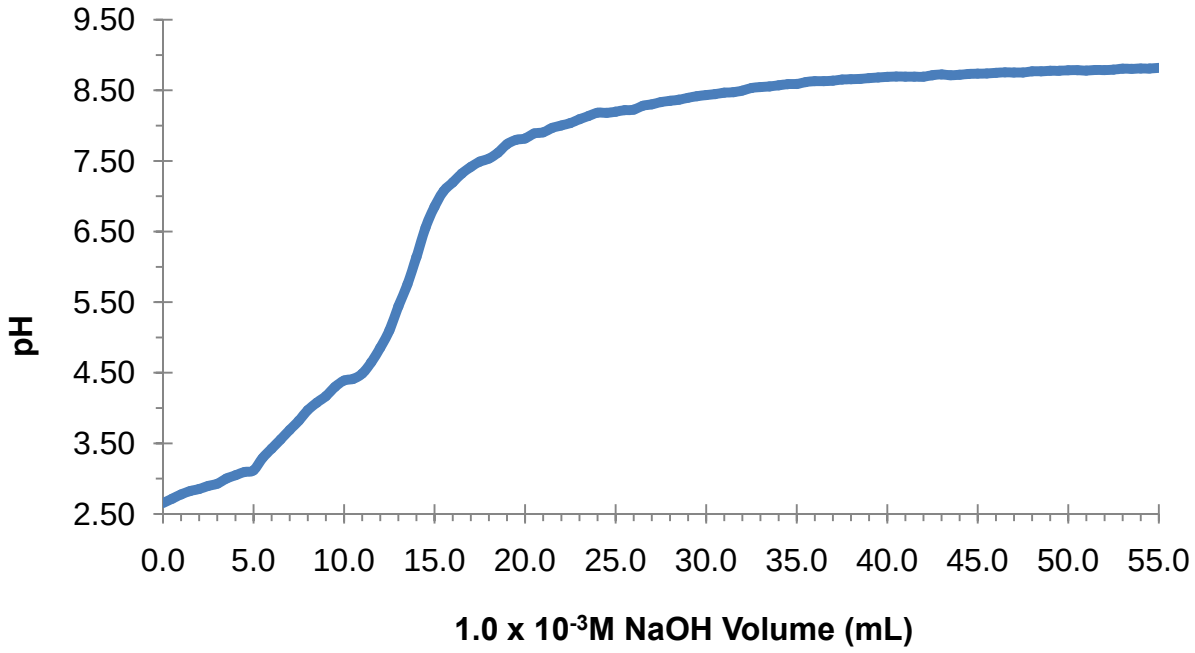

Figure 8: Roxarsone (9.45×10^{-5} M) titrated with NaOH (1.0×10^{-3} M). Refer to Appendix 1 for additional information. EqP regions and V_{eq} values could not be determined accurately due to the greater concentration magnitude of NaOH (10^{-3}M) than roxarsone (10^{-5}M). Refer to discussion in section 6.3.

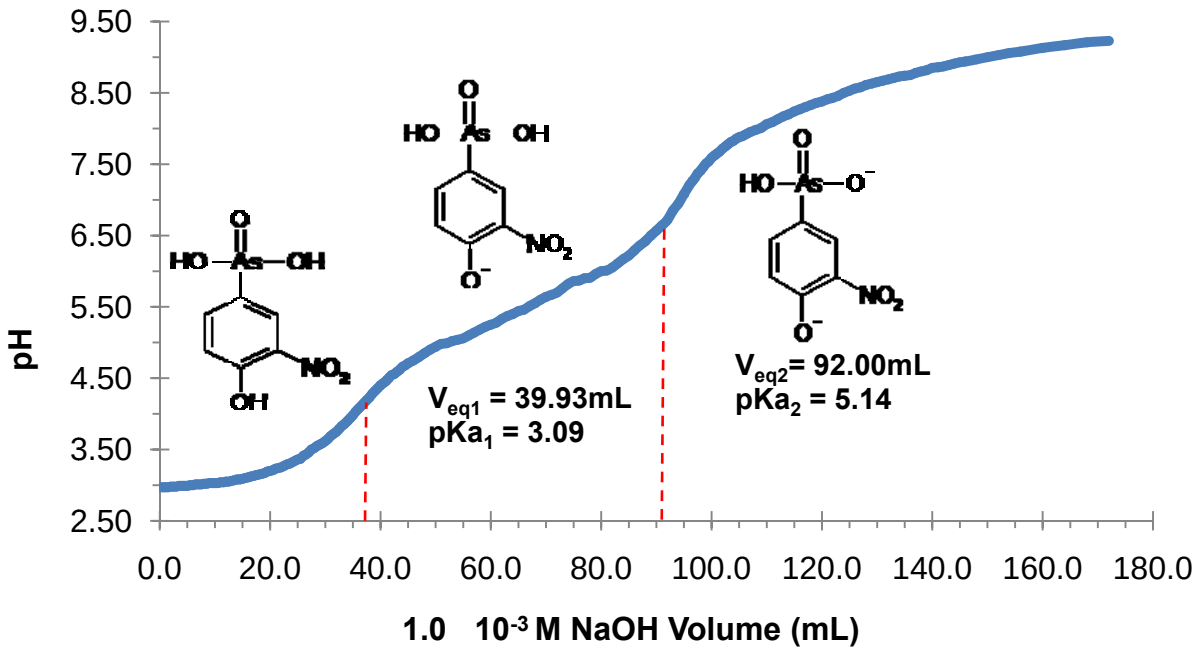

Figure 9: Roxarsone (1.0×10^{-3} M) titrated with NaOH (1.0×10^{-3} M). V_{eq} values were determined by gran plot calculations. Refer to Appendix 2 for additional information.

6.3 Results and Discussion

The plots of the pH data obtained depicted sigmoidal curves with possible regions corresponding to EqP at the following pH intervals: 3.25 to 4.50 and 5.50 to 7.50. A difference in the transition between the pH intervals is evident when figures 8 and 9 are compared. The transition between pH interval regions was scarcely observed during the first titration experiment. This is mainly due to the fact that the titrant concentration (10^{-3} M) is two orders of magnitude greater than the analyte (10^{-5} M). On the contrary, using the analyte and titrant concentrations in the same order (10^{-3} M) expanded the transition between pH interval regions thus allowing a clearer view for appropriate determination of the EqP. The sigmoidal curve depicted in figure 9 suggests the possibility of two EqP regions. The third EqP expected (as stated in

section 6.1) was not visible in either graph. The pH remained between 8.50 and 9.00 towards the end of the titration in both graphs. This suggests that the third deprotonation expected (corresponding to the second H_3O^+ in the arsonic acid group) will most likely occur at pH ranges greater than 8.50 (which are not considered suitable for the majority of reverse phase HPLC columns). The use of mobile phase at pH levels greater than 8.0 is seldom recommended. This is mainly due to the fact that most HPLC column packings are based on silanol (Si-O) groups. These types of compounds are prone to dissolution at pH levels above 8.[47]

Several methods exist for the determination of EqP from a potentiometric titration. Most introductory general chemistry courses will employ a plot similar to Figures 8 and 9. The EqP would be estimated by extrapolating a trend line from the steepest region of the graph to the x-axis (titrant volume). However, a more accurate determination of the EqP can be obtained by the use of the first and second derivatives of the titration curve.

The first derivative is determined from the change in potential per unit of titrant volume. A plot of the first-derivative data ($\Delta pH/\Delta V$) versus titrant volume will produce a curve with a maximum that corresponds to the EqP. Figure 10 depicts the first derivative plot of the curve obtained from titrating roxarsone (1.0×10^{-3} M) with NaOH (1.0×10^{-3} M). Two equivalence point regions are evident in the first derivative plot. However, the maximum cannot be precisely located due to the amount of noise depicted. A second derivative plot was not used due to the excessive amount of noise generated.

Derivative plots eliminate the use of graphical extrapolations for determinations of EqP. These are subject to error due to the nature of the calculations. Data points are evidently lost each time the results are derived and the noise increases due to the decrease in signal intensity. However, there are other mathematical methods used to determine the EqP of titration plots which can result more effective than a derivative plot.

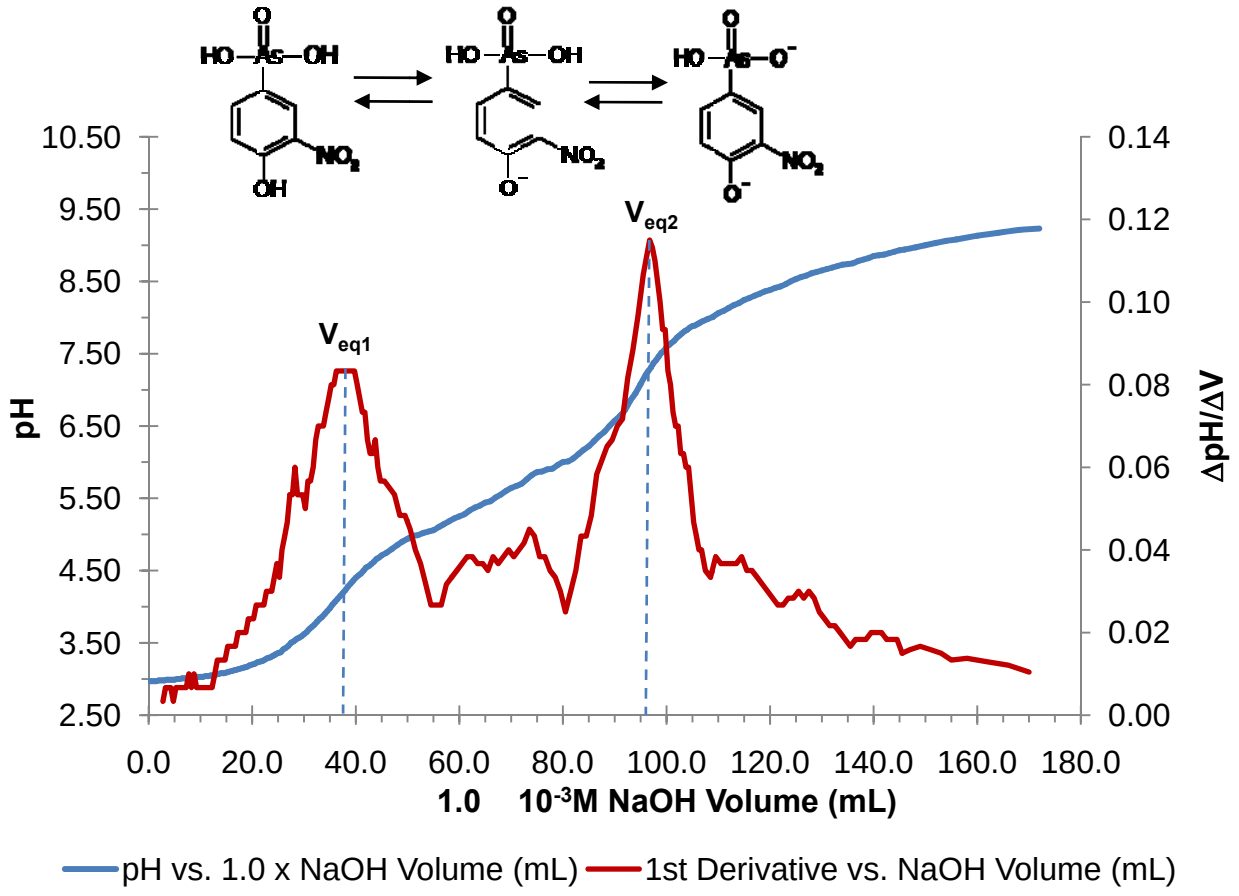

Figure 10: First derivative plot of roxarsone (1.0×10^{-3} M) titrated with NaOH (1.0×10^{-3} M). The considerable amount of noise observed in derivative plot will increase uncertainty in the V_{eq} regions. The derivative plot was not used to determine V_{eq} values for roxarsone. The gran plot calculations were used to calculate V_{eq} and pKa values. Refer to Appendix 2 for additional information.

The gran plot can be used for the determination of K_a values based on titration data obtained. This method uses the data near the EqP but it does not require the full titration profile. In this particular experiment, the full titration curve obtained facilitated the task of choosing the data set most suitable for the gran plot calculations for roxarsone.

In a weak acid titration, a linear relationship is established based on the equilibrium of the concentrations corresponding to hydronium ion (H_3O^+), the weak acid (HA), and its conjugated base (A^-) described in (17).

$$[H_3O^+] = \frac{K_a\,[HA]}{[A^-]} = \frac{K_a\,(V_{eq} - V_{NaOH})}{V_{NaOH}} \qquad (17)$$

Multiplication of both sides by V_{NaOH} resulted in the following equation for a straight line:

$$[H_3O^+]\,V_{NaOH} = -K_aV_{NaOH} + K_aV_{eq} \qquad (18)$$

where: $y = [H_3O^+]\,V_{NaOH}$ $m = -K_a$ $x = V_{NaOH}$ $b = K_aV_{eq}$

The gran plot was constructed for both proposed EqP regions identified (Figures 11 and 12). The results are tabulated in Tables 1 and 2. The K_a values acquired from the gran plot data provided the empirical information necessary for the generation of fractional composition plots (Figure 13). As a result, the dominating species of roxarsone as a function of pH is established for this study.

Table 1: Gran plot data for K_{a1} (pH region: 3.25 to 4.50)

V_{NaOH} (mL)	pH	$[H_3O^+]V_{NaOH}$	Slope	-8.06×10^{-4} ($\pm$ 2.65×10^{-5})
29.5	3.59	7.58×10^{-3}	Intercept	3.14×10^{-2} ($\pm$ 8.08×10^{-4})
30.0	3.62	7.20×10^{-3}	R^2	0.9968
30.5	3.65	6.83×10^{-3}	K_{a1}	8.06×10^{-4}
31.0	3.69	6.33×10^{-3}	V_{eq} (mL)	38.93
31.5	3.72	6.00×10^{-3}	pK_{a1}	3.09

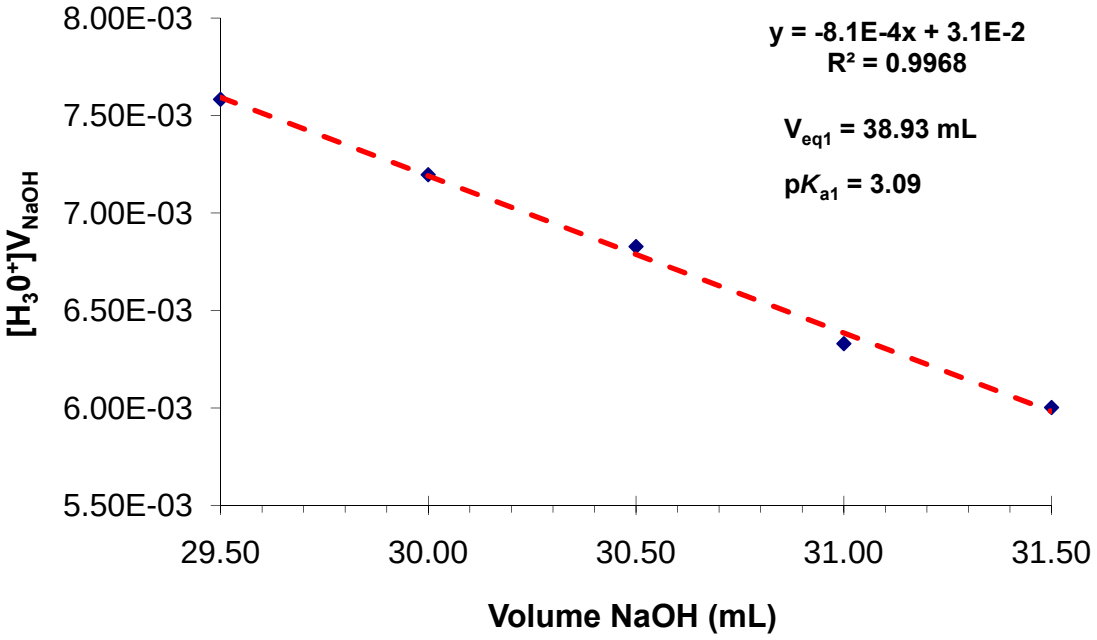

Figure 11: Gran plot for K_{a1} (pH = 3.25 to 4.50). Equation: $y = -8.06 \times 10^{-4} x + 3.14 \times 10^{-2}$; $R^2 = 0.9968$; F = 924.19; p-value = 7.82×10^{-5}; V_{eq} = 38.93 mL; pK_{a1} = 3.09.

Table 2: Gran plot data for K_{a2} (pH region: 5.50 to 7.50)

V_{NaOH} (mL)	pH	$[H_3O^+]V_{NaOH}$	Slope	-7.22×10^{-6} ($\pm$ 2.91×10^{-7})
81.0	6.01	7.92×10^{-5}	Intercept	6.64×10^{-4} ($\pm$ 2.42×10^{-5})
82.0	6.05	7.31×10^{-5}	R^2	0.9951
83.0	6.11	6.44×10^{-5}	K_{a2}	7.22×10^{-6}
84.0	6.17	5.68×10^{-5}	V_{eq} (mL)	92.00
85.0	6.22	5.12×10^{-5}	pK_{a2}	5.14

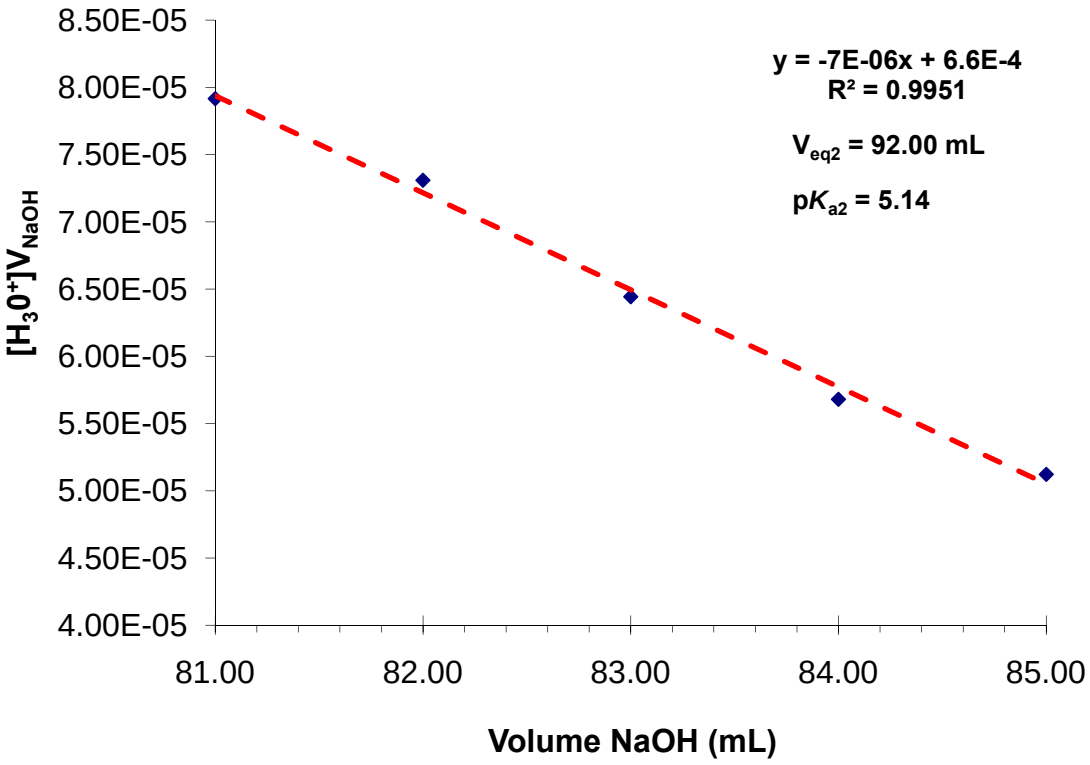

Figure 12: Gran plot for K_{a2} (pH: 5.50 to 7.50). Equation: $y = -7.22 \times 10^{-6} x + 6.64 \times 10^{-4}$; $R^2 = 0.9951$; F =615.01; p-value = 1.44×10^{-4}; $V_{eq} = 92.00$ mL; $pK_{a2} = 5.14$.

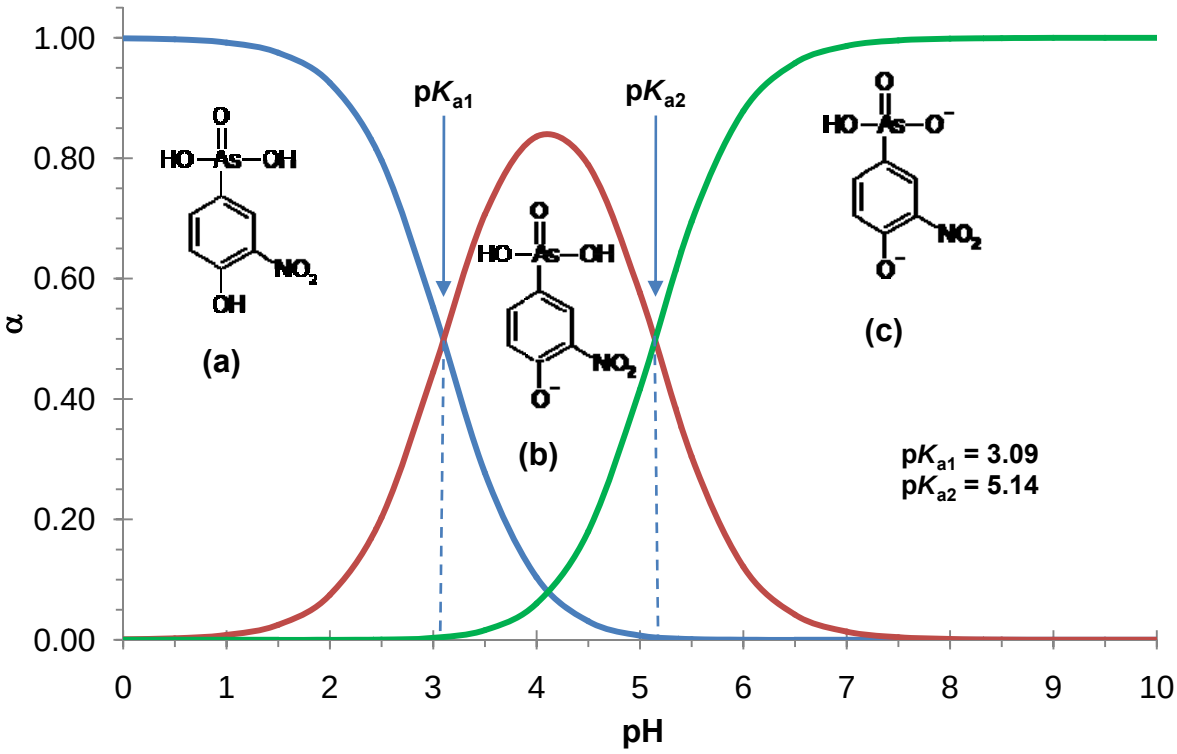

Figure 13: Fractional composition plot for roxarsone. (a) 93% of species at pH 2.0; (b) 100% of species at pH 4.0; (c) 96% of species at pH 6.5.

7 IP-HPLC method development studies

7.1 Introduction

The main objective of this stage of the study was to develop a rapid resolution IP-HPLC method capable of detecting and quantifying the organoarsenic drugs roxarsone, p-arsanilic acid, and acetarsone. It was important that adequate specificity be demonstrated with the operational parameters developed. The unequivocal identification of roxarsone is clearly important due to the presence of other organoarsenic drugs with similar molecular structures (p-arsanilic acid and acetarsone). Linearity studies were performed in order to establish appropriate values of LOQ and LOD. The methodology was subject to variations of operational conditions and sample characteristics in order to validate its robustness.

Initial studies were performed using a Zorbax Eclipse XDS, 4.6 × 150 mm, 5 μm packing HPLC column (Agilent Part Number 993967-902) for compilation of preliminary data used to further enhance the methodology related to the Rapid Resolution (RR) methodology. However, the validated method uses a Zorbax Eclipse Plus, 4.6 mm × 50 mm, 1.8 μm packing, Rapid-Resolution HPLC column (Agilent Part Number 959941-902).

A pre-column filter assembly (Agilent Part Number 5067-1553) was installed between the on the capillary tubing prior to the column. It contains a stainless steel frit, 4.6 mm diameter with a 2 μm mesh filter (Agilent Part Number 5067-1562), to prevent clogging of the column with particles. The frit's material is the same as the capillary tubing, therefore separation dynamics will not occur until the sample enters the column.

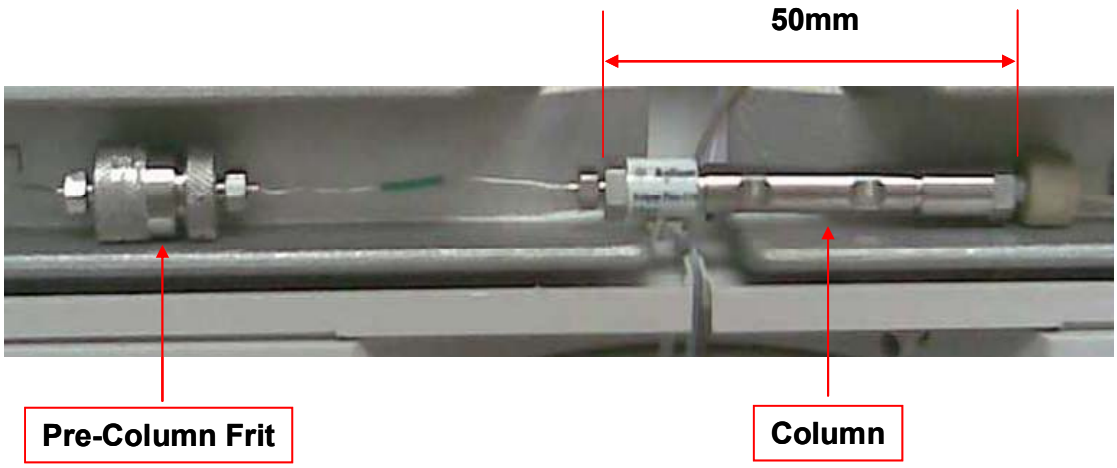

Figure 14: Eclipse Plus C18 RR HPLC Column, 4.6mm × 50 mm, 1.8 μm packing (Agilent Part Number 959941-902) with RRLC inline filter with stainless steel frit.

7.2 Equipment

An Agilent 1100 Series HPLC with a diode array detector (DAD) was used for the development studies (Figures 15-17). The eluents would first pass through a degassing unit prior to entering the mixing chamber. It was equipped with a binary pump (maximum back-pressure = 400 bar) which provided the flexibility of varying mobile phase composition without the need of manually pre-mixing the eluents prior set-up. The autosampler has 100 vial positions and a syringe capable of accurately delivering injection volumes of at least 1.0 μL. The column compartment contained a temperature control module. The DAD had the capacity of collecting and storing a 220 nm wide spectrum for each chromatographic acquisition. The equipment was set to collect the spectra from 190 nm to 400 nm. This enables the analyst to assess the purity of the eluting species in a chromatogram. Capillary tubing of 0.17 mm i.d. through which the mobile phase flowed from the binary pump to the detector output was used to reduce dead volume.

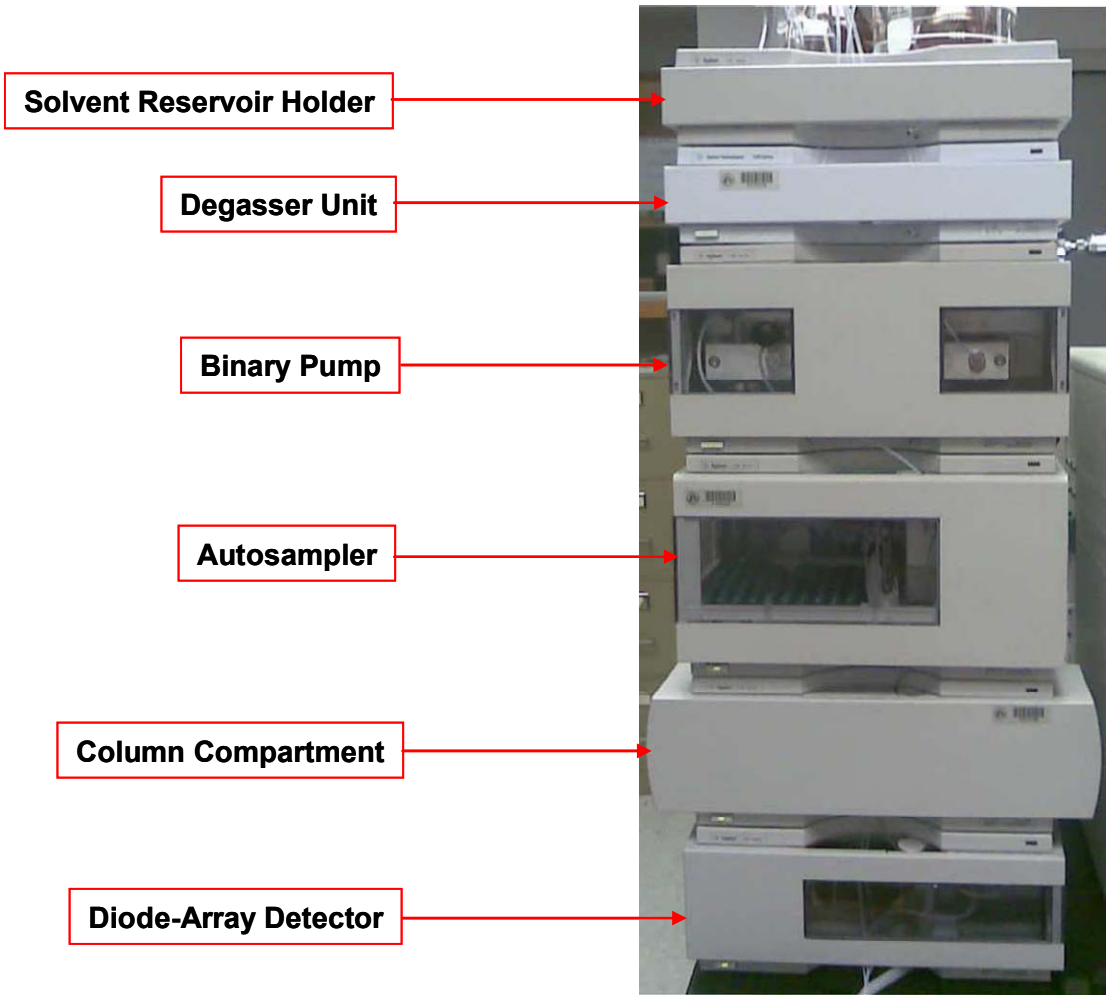

Figure 15: Agilent 1100 Series HPLC used for method development / validation.

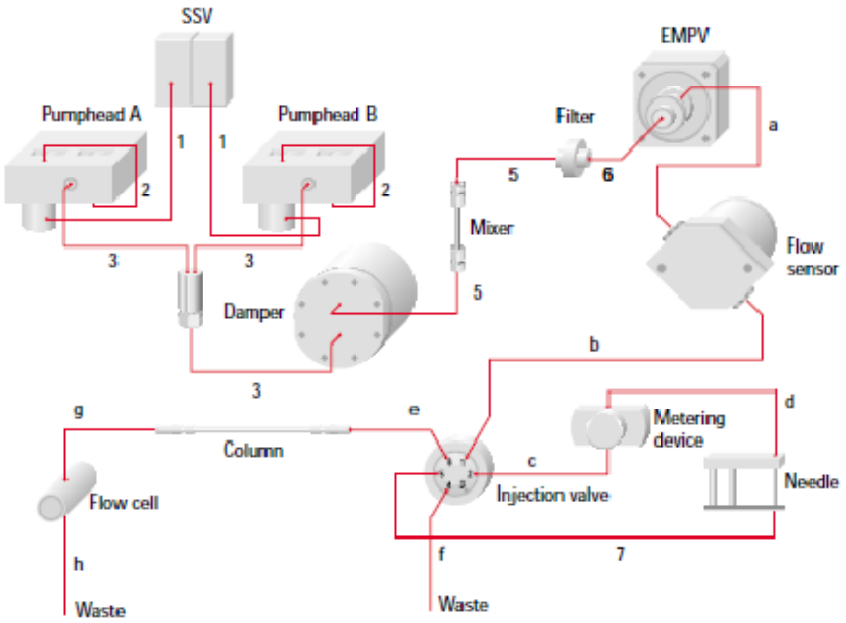

Figure 16: Schematic diagram of Agilent 1100 capillary flow path

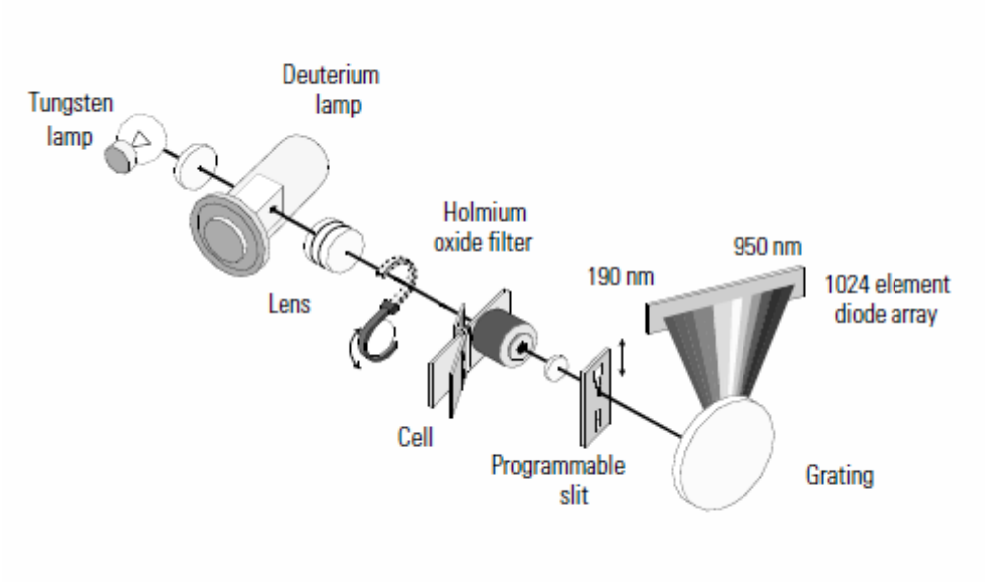

Figure 17: Diagram of an Agilent 1100 diode array detector (DAD)

7.3 Mobile phase

The empirical data (pK_a values) obtained from the pH study of roxarsone provided a foundation for the determination of mobile phase characteristics during the IP-HPLC method development. It was determined from the fractional composition plot that the suitable pH for the mobile phase would be between 6.3 and 6.5 considering the following:

- 96% of the roxarsone exists as the ionized species C (figure 13). Therefore, only 4% coexists as other ionized roxarsone species. Consequently species C favors a more efficient ion pair formation based on the fact that it depicts multiple sites with a negative charge.
- The HPLC columns used for the studies (Agilent Eclipse XDS and Eclipse Plus) have an operational pH range from 2 to 9.
- The pH of surface water (6.5 to 8.5) and ground water systems (6.0 to 8.5) was considered. The sample preparation required will most likely be a simple filtration (to remove particles that may clog the system). Consequently, the sample could be injected directly into the HPLC system, thus reducing the analysis time.

The mobile phase considered for the IP-HPLC methodology was directed towards an isocratic separation mode. All reagents used were of HPLC grade except for concentrated acids (trace metal grade). Deionized Water (DIW; 18 MΩ) was used for preparation of all aqueous solutions. The aqueous phase consisted of methyl-tributyl ammonium hydroxide (MTBA-OH) as an ion-pairing agent (IpA) diluted in DIW. Modifications were made throughout the investigation by varying the concentration of

MTBA-OH and pH of the mobile phase. However, the validated mobile phase included 1% of Triethylamine (TEA) as a modifier to further enhance the resolution between the organoarsenic drugs under study while maintaining a relatively short runtime.

In general, HPLC organic phases usually consist of either methanol (MeOH; UV cut-off = 205 nm) or acetonitrile (ACN; UV cut-off = 190 nm).[56] MeOH was chosen as the organic phase in spite of the global ACN shortage of Fall/2008. The use of methanol is compatible with the optimum wavelength (λ = 245 nm) determined as per Section 11. However, consideration should be given to the UV cut-off and eluent strength if the MeOH will be substituted with a different solvent.

7.4 HPLC Column

The HPLC column chosen for the method validation is an Eclipse Plus, 4.6 mm × 50 mm, 1.8 μm packing, Rapid-Resolution HPLC column (Agilent Part Number 959941-902). Interest in HPLC columns with Sub-2 micron (< 2 μm) packings has grown since the introduction of the first 1.7 μm porous silica column during 2004.[57] The path was drawn for the development of rapid chromatographic methods with high resolution. The species migrating through the column will have a greater interaction with the column packing material due to the greater surface area provided by the decreased particle size. Consequently, the back-pressure will increase due to the Sub-2 micron packing. However, an increment in column temperature during the chromatographic analysis will help decrease the viscosity of the mobile phase, thereby reducing the system back-pressure.

8 Study: Variation of mobile phase pH

8.1 Study Description

A study was performed to evaluate the effect of the mobile phase pH on the elution of roxarsone. The study consisted of obtaining chromatograms of a roxarsone standard solution with the mobile phase at different pH levels while maintaining a constant 4.6 mM MTBA-OH concentration. The mobile phase delivered to the column was varied from 2.79 to 3.04 by mixing two eluents as described in Table 3. The HPLC system was allowed to equilibrate (at least 15 minutes) prior to obtaining the chromatograms at the different pH levels. The equipment operational parameters are described in Table 4. These experiments correspond to initial studies performed with a Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm packing HPLC column. The acquisition time for the chromatography obtained with this column is greater than those corresponding to a rapid-resolution column of 1.8 μm packing (as demonstrated in subsequent sections).

Table 3: Eluent fractional compositions of lines (A) pH = 2.74; (B) pH = 6.00

%A	%B	$[H_3O^+]$	Final pH[(a)]
50	50	9.10×10^{-4}M	3.04
60	40	1.09×10^{-3}M	2.96
70	30	1.27×10^{-3}M	2.89
80	20	1.46×10^{-3}M	2.84
90	10	1.64×10^{-3}M	2.79

(a): $\pm$ 0.01 pH units

Table 4: Test parameters for the retention study of roxarsone as a function of mobile phase pH.

Mobile Phase (95:5 A/B)			
Eluent A		Eluent B	
4.6 mM MTBA-OH		methanol	
Equipment Operating Conditions			
Flow	1.0 mL / min	Column Temp	25ºC
Wavelength	236 nm	Injection Volume	10 μL
Column			
Description	Zorbax Eclipse XDB-C18		
Serial Number	USKH028561		
Dimensions	4.6 × 150 mm, 5 μm packing		
Sample (Diluent: DIW)			
Analyte	roxarsone	Concentration	4.99×10^{-5} M

8.2 Results and Discussion

The sample analyzed was prepared by diluting an amount of roxarsone in DIW. Therefore, the dominating retention mechanism will correspond to the adsorption of the ion paring agent (MTBA-OH) onto the column packing material followed by interactions of the ionized sample species with the enhanced packing material. Consequently, a greater amount of roxarsone anions are available for interaction with the MTBA-OH as the pH of the mobile phase increases. This leads to a more effective retention of the antimicrobial. The separation obtained during this particular study agrees with this retention behavior. Figure 18 depicts chromatography in which the t_R for roxarsone increased from 15.370 minutes to 19.177 minutes as the mobile phase pH increased from 2.79 to 3.04.

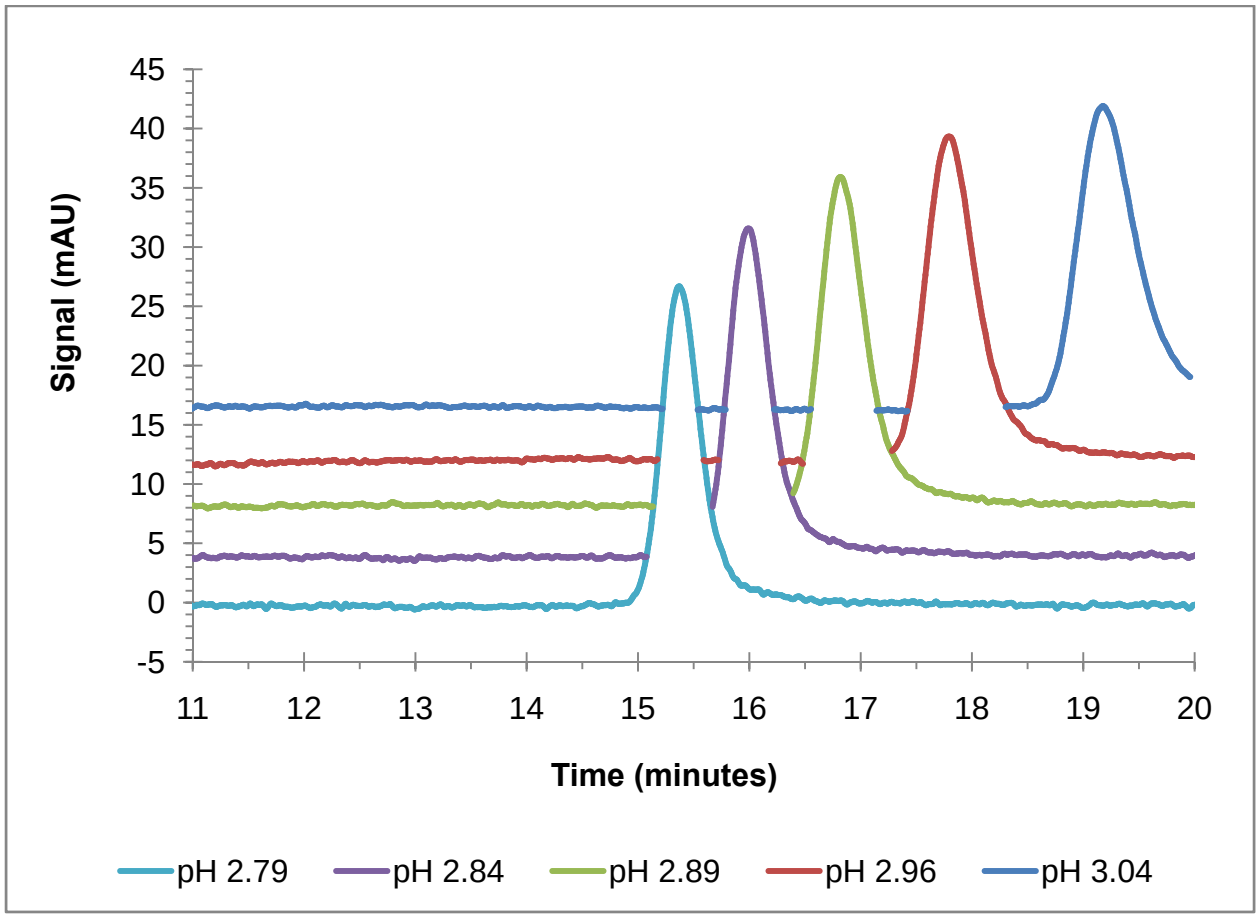

Figure 18: Chromatograms obtained for the retention study of a roxarsone (4.99×10^{-5} M) standard solution with mobile phase at various pH levels (as per Table 3). Mobile phase: (95:5) 4.6 mM MTBA-OH / methanol. Flow: 1.0 mL/min. Column temperature: 25ºC. Detector wavelength: 236 nm. Injection volume: 10 μL. Column: Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm packing.

Table 5: Performance parameters for the retention study of roxarsone as a function of mobile phase pH.

pH	t_R	Δt_R [(b)]	Area	T_f	N	W
	min	min	mAU*s			min
3.04	19.177	-	(a)	(a)	(a)	(a)
2.96	17.795	1.382	887.6410	1.4	6858	0.551
2.89	16.819	2.358	808.3850	1.4	7605	0.491
2.84	15.992	3.185	784.2721	1.4	7910	0.473
2.79	15.370	3.807	720.8112	1.4	8336	0.447

(a) Peak tailing eluted after 20 minutes of data acquisition. Appropriate integration for performance parameters was not possible. Chromatogram used for t_R reference only.

(b) Δt_R was determined referent t_R of pH = 3.04. (e.g. for sample 2.96, Δt_R = 19.177 min - 17.795 min = 1.382 min)

9 Study: Variation of roxarsone sample pH

9.1 Study Description

A study was performed to evaluate the effect of the sample pH on the elution of roxarsone. Chromatograms of roxarsone standard solutions were obtained at different pH levels while maintaining the mobile phase pH constant at 6.50. The pH of the samples was adjusted with glacial acetic acid to the levels described in table 6. The mobile phase consisted of 18.3 mM MTBA-OH with 1% TEA (pH of 6.50 with glacial acetic acid). The HPLC system was allowed to equilibrate (at least 15 minutes) prior to starting the analysis. The Eclipse Plus C18 RR HPLC, 1.8 μm HPLC column was used. The equipment operational parameters and working curve are described in Appendix 6.

Table 6: Retention study of roxarsone (6.70×10^{-6} M) as a function of sample pH. Samples were diluted in DIW.

Sample	1	2	3	4	5
pH [(a)]	3.96	4.41	6.10	7.01	7.51

(a) $\pm$ 0.01 pH units

9.2 Results and Discussion

The chromatography obtained for this particular study is depicted in Figure 19.

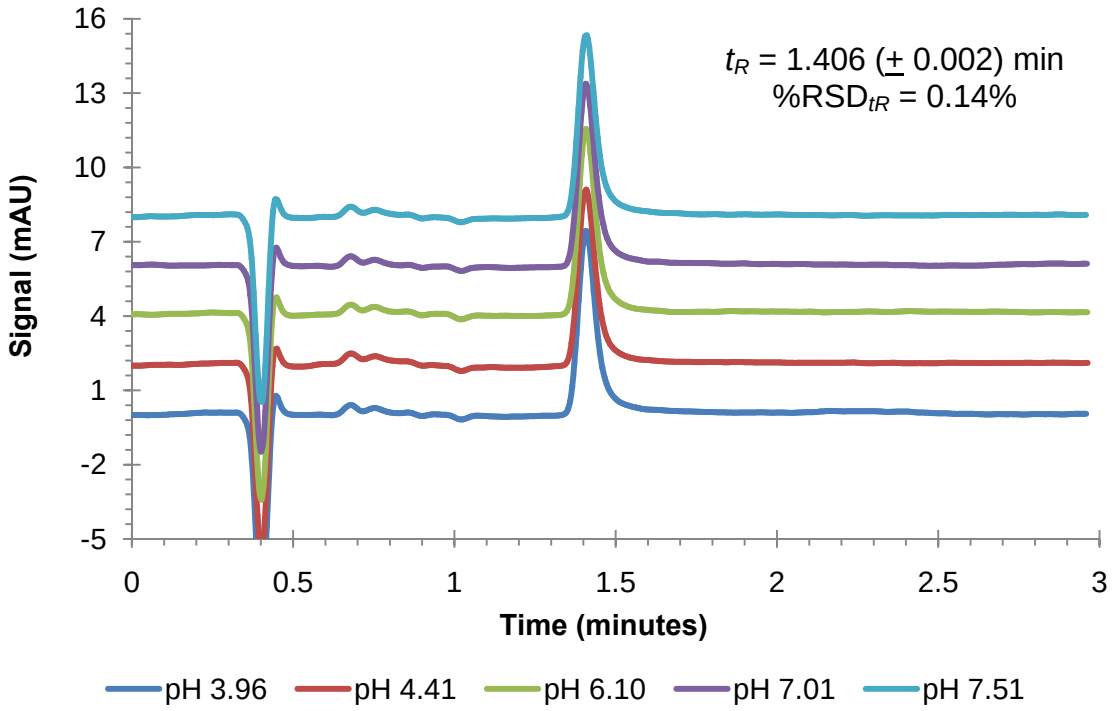

Figure 19: Chromatograms obtained for the retention study of a roxarsone standard solution (6.70×10^{-6} M) as a function of sample pH. Mobile phase: (85/15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection wavelength: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50mm, 1.8 μm packing.

The elution data reflects no apparent change in roxarsone's t_R despite the sample pH variability. The low %RSD values (< 1.3%) obtained for the performance parameters described in Table 7 indicate that the variable pH of the sample did not affect significantly the elution of roxarsone. In addition, the data suggests that the pH dominating this particular system is that of the mobile phase (pH = 6.50) and not of the sample. This confirms that the retention mechanism corresponds to the adsorption of MTBA-OH to the column followed by interaction of the ionized species with the enhanced packing material. Furthermore, this suggests that the process of deprotonation (for samples 1, 2, and 3) and protonation (for samples 4 and 5) occurs once the samples are exposed to the mobile phase.

Table 7: Performance parameters for the retention study of roxarsone solutions with variable pH.

Parameter (n=15)	t_R (min)	Area (mAU*s)	T_f	N	k
AVE	1.406	30.182	1.6	2979	2.52
s	0.002	0.37	0	17	0.01
%RSD	0.14	1.23	0	0.58	0.21
MIN	1.403	29.642	1.6	2948	2.51
MAX	1.408	30.772	1.6	3001	2.52
MODE [a]	1.408		1.6		2.52

(a) Value mostly repeated within the data set.

The %relative error (%RE) obtained for the roxarsone samples at different pH levels ranged from 3.0% to 6.2% (Table 8). This data suggests that the method quantification capability is not affected significantly by sample pH variability.

Table 8: Recovery data for roxarsone samples with variable pH.

pH	3.96	4.41	6.10	7.01	7.51
Conc. (M) [(a)]	7.12 ($\pm$0.68) $\times 10^{-6}$	6.90 ($\pm$0.68) $\times 10^{-6}$	7.09 ($\pm$0.68) $\times 10^{-6}$	6.98 ($\pm$0.68) $\times 10^{-6}$	6.99 ($\pm$0.68) $\times 10^{-6}$
%RE[(b)]	6.2	3.0	5.6	4.0	4.2

(a) n = 15; AVE = 7.02×10^{-6} M; $s_{pooled} = 8.9 \times 10^{-8}$ M; %RSD = 1.3%

(b) %RE = ($C - C_0 / C_0$) × 100, were C is roxarsone's sample concentration (M) obtained with the calibration curve and C_0 corresponds to the sample's theoretical concentration.

10 Study: Effects of column temperature variation

10.1 Description of Study

A study was performed to evaluate the effect of the column temperature on the elution of the organoarsenic drugs roxarsone, p-arsanilic acid, and acetarsone. It is well-known that temperature plays an important role in Ion-Pair chromatography that employs reverse-phase HPLC columns.[45] In general, it is expected that an increase in the column temperature will decrease the viscosity of the mobile phase. The organoarsenic drugs will migrate at a faster rate through the column due the lower viscosity. Consequently the interactions occurring between the species and the column packing material will decrease, thus resulting in a more rapid separation.

Chromatograms of reference standard solutions containing all three organoarsenic drugs were obtained at 25ºC and 40ºC. The mobile phase used was 18.3 mM MTBA-OH with 1% TEA. It was adjusted to a pH of 6.30 with glacial acetic acid. The HPLC system was allowed to equilibrate (at least 15 minutes) prior to starting the analysis. This particular study used the Eclipse Plus C18, 4.6 × 50 mm, HPLC column, with 1.8 μm packing. The equipment operational parameters and solutions used for this study are described in Table 9.

Table 9: Test parameters for the column temperature variation study for the organoarsenic drugs roxarsone, p-arsanilic acid, and acetarsone.

Mobile Phase (85:15 A/B)			
Eluent A		Eluent B	
18.3 mM MTBA-OH, 1% TEA pH = 6.30		methanol	
Equipment Operating Conditions			
Flow	1.4 mL / min	Column Temp	25ºC/40ºC
Wavelength	254 nm	Injection Volume	5 μL
Column			
Description	Zorbax Eclipse Plus C18		
Serial Number	USUXG03355		
Dimensions	4.6 × 50 mm, 1.8 μm packing		
Sample (Diluent: DIW)			
Description	High Sample	Low Sample	
Analyte	Concentration (M)		
roxarsone	4.99×10^{-5}	9.98×10^{-7}	
p-arsanilic acid	6.45×10^{-5}	1.29×10^{-6}	
acetarsone	4.50×10^{-5}	9.00×10^{-7}	

10.2 Results and Discussion

There was no significant difference between the chromatographic profiles of DIW (blank) acquired at 25ºC and 40ºC (Figure 20). The DIW data obtained at both study temperatures portrayed the Ion-Pairing Agent Background (IpA-Bkg) which is considered part of the chromatographic profile (based on all the DIW blanks evaluated throughout initial studies). The t_R of the IpA-Bkg remained constant at 0.661 minutes during both separation temperatures. In addition, the inflection at 0.400 minutes also remained constant.

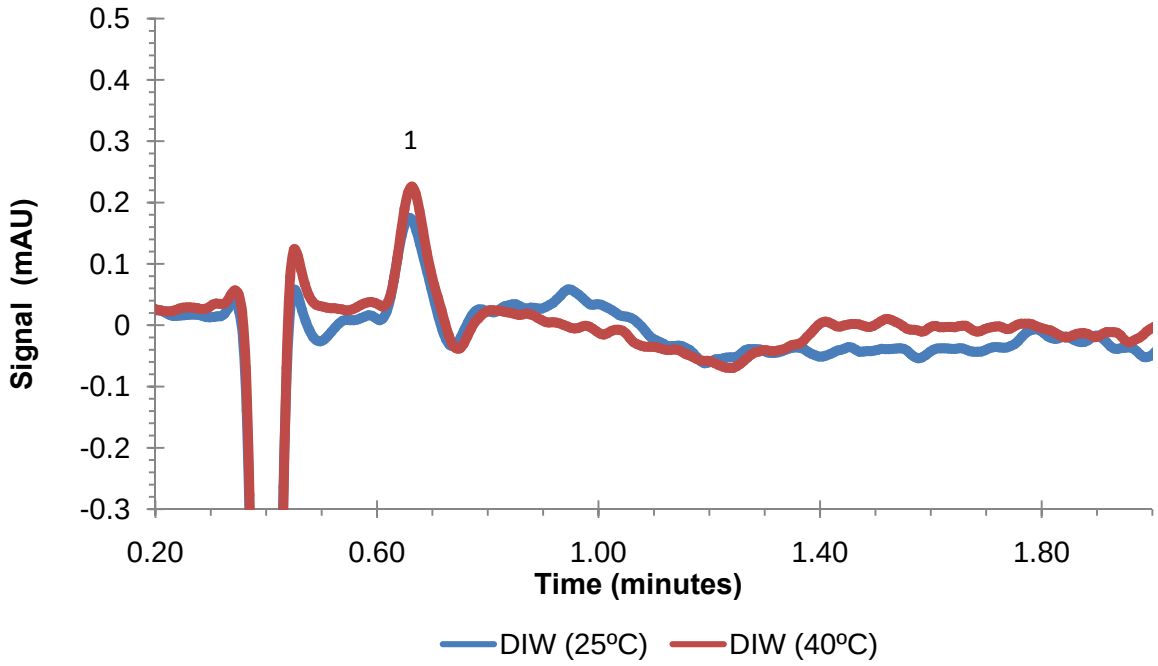

Figure 20: Chromatography of DI Water (blanks) acquired with column compartment temperature set at 25°C and 40°C. (1) IpA-Bkg. Mobile phase: (85:15) 18.3 mM MTBA-OH, pH = 6.30 / methanol. Flow: 1.4 mL/min. Detector wavelength: 254 nm. Injection volume: 5 µL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 µm packing.

The separation obtained at temperatures of 25ºC and 40ºC of the organoarsenic drugs (high concentrations) is displayed in figure 21. Visual differences were evident in the resulting chromatogram.

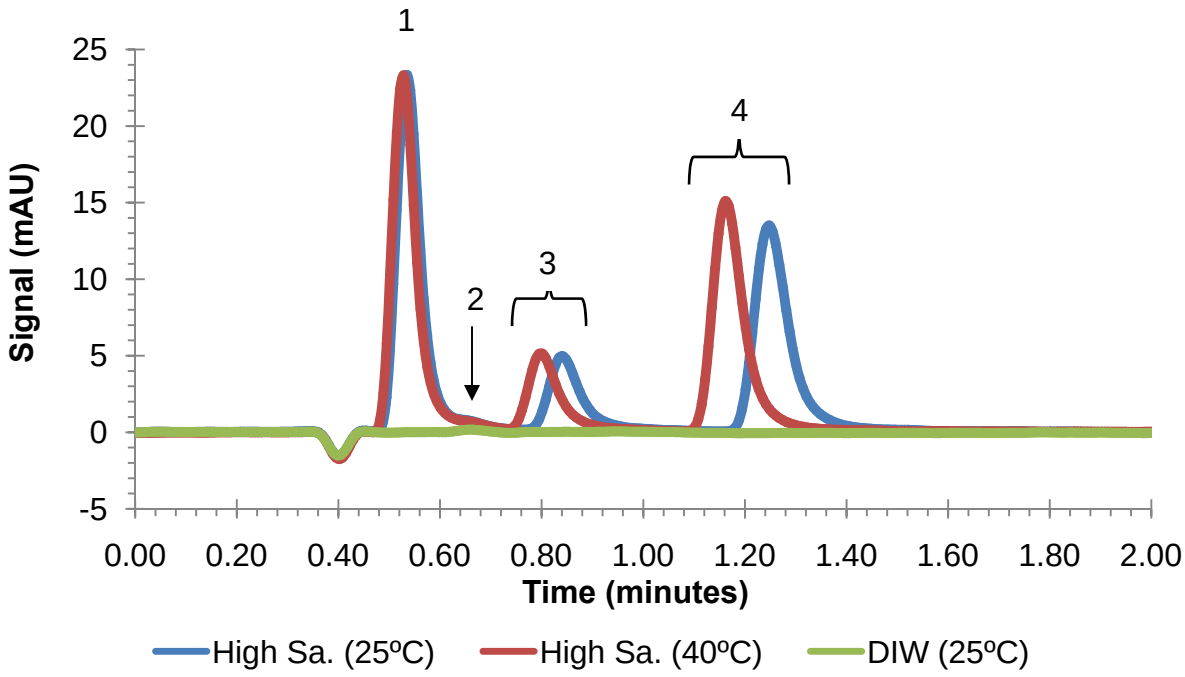

Figure 21: Chromatography of the organoarsenic drug samples (high concentration) acquired with the column compartment temperature 25ºC and 40ºC. The peak elution order for the chromatograms is (1) p-arsanilic acid, 6.45 × 10^{-5} M; (2) IpA-Bkg; (3) acetarsone, 4.50 × 10^{-5} M ; and (4) roxarsone, 4.99 × 10^{-5} M. Mobile phase: (85:15) 18.3 mM MTBA-OH, pH = 6.30 / methanol. Flow: 1.4 mL/min. Detector wavelength: 254 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 x 50 mm, 1.8 μm packing.

The inverse relationship between t_R and temperature is most noticeable when observing the elution of the organoarsenic drugs. An increase in temperature resulted in a decrease of t_R values for acetarsone (Δt_R = 0.041min) and roxarsone (Δt_R = 0.084min). There was no significant decrease in t_R for p-arsanilic acid with the temperature increase (Δt_R = 0.007min). As mentioned earlier, an increase in temperature decreases the viscosity of the mobile phase; therefore, the species migrate at a much faster rate through the column. The data obtained for the high sample agrees with this theory.

The temperature increase resulted in the chromatography of the three organoarsenicals in a shorter time while maintaining adequate separation. The decrease in t_R was observed in roxarsone (-6.7%), acetarsone (-4.8%), and p-arsanilic acid (-1.3%). The *k* and α values were not significantly affected by the decrease in t_R. A slight increase in *N* values was obtained for acetarsone and roxarsone. The most significant enhancement was observed for S/N of all three organoarsenicals. The S/N increased for p-arsanilic acid (21.1%), acetarsone (15.9%), and roxarsone (54.9%) with the separation temperature of 40ºC.

Other figures of merit used for chromatography (e.g. Area, T_f, *N*, and *R*) were calculated for the analytes at 25ºC and 40ºC (Table 10 and 11 respectively). The peak height is reported although it is not a figure of merit. Height is particularly used for determination of Signal-to-Noise Ratio (S/N), an important parameter to confirm the degree of detection and quantification for a peak. According to regulatory guidelines, the accepted Limit-of-Quantitation (LOQ) is 10 times the magnitude of the noise, whereas the Limit-of-Detection (LOD) should be 3 times the magnitude of the noise.[51]

Table 10: Figures of merit for organoarsenic drug samples (high concentration) acquired with the column compartment temperature of 25ºC.

Analyte	p-arsanilic acid	IpA-Bkg	acetarsone	roxarsone
Conc. (M)	6.45×10^{-5}	N/A	4.50×10^{-5}	4.99×10^{-5}
t_R (min)	0.535	0.661	0.840	1.247
k	0.34	0.65	1.10	2.12
α	1.93	1.69	1.93	N/A [(b)]
Area (mAU*s)	79.1347	1.9646	21.3678	65.2801
T_f	1.4	N/A [(c)]	1.5	1.5
N	585	N/A [(c)]	887	1613
R	N/A [(a)]	1.3	2.1	3.6
Height (mAU)	23.26	0.71	4.82	13.46
Baseline Noise (mAU)	1.94×10^{-2}	N/A [(d)]	1.13×10^{-2}	1.44×10^{-2}
S/N	1199	N/A [(d)]	427	935

(a) R is calculated based on the preceding integrated peak (Section 5.2.2, Equation 2).
(b) α is calculated using the k values for the present peak and the next integrated peak (Section 5.2.6, Equation 6). There are no eluting peaks after roxarsone.
(c) These figures of merit are calculated by the software for identified analytes.
(d) S/N is calculated with baseline noise of blank chromatogram. The baseline noise was not calculated for the IpA-Bkg because it is part of blank chromatogram.

Table 11: Figures of merit for organoarsenic drug samples (high concentration) acquirred with the column compartment temperature of 40ºC. The difference from the data acquired at 25ºC is displayed in parenthesis for each value.

Analyte	p-arsanilic acid	IpA-Bkg	acetarsone	roxarsone
Conc. (M)	6.45×10^{-5}	N/A	4.50×10^{-5}	4.99×10^{-5}
t_R (min)	0.528 (-0.007)	0.661 (0)	0.799 (-0.041)	1.163 (-0.084)
k	0.32 (-0.02)	0.65 (0)	1.00 (-0.10)	1.91 (-0.21)
α	2.04 (+0.11)	1.53 (-0.16)	1.91 (-0.02)	N/A [(a)]
Area (mAU*s)	79.2637 (+0.129)	1.3226 (-0.6420)	20.2592 (-1.1086)	66.0753 (+0.7952)
T_f	1.4 (0)	N/A [(a)]	1.5 (0)	1.5 (0)
N	583 (-2)	N/A [(a)]	897 (+10)	1695 (+82)
R	N/A [(a)]	1.5 (+0.2)	1.7 (-0.4)	3.4 (-0.2)
Height (mAU)	23.37 (+0.11)	0.60 (-0.11)	4.94 (+0.12)	15.06 (+1.6)
Baseline Noise (mAU)	1.61×10^{-2} (-3.30×10^{-3})	N/A [(a)]	9.98×10^{-3} (-1.32×10^{-3})	1.04×10^{-2} (-4.00×10^{-3})
S/N	1452 (+253)	N/A [(a)]	495 (+68)	1448 (+513)

(a) Refer to Table 10 for a detailed explanation.

The low sample consisted of a decreased concentration of the organoarsenic drugs (10^{-6} M and 10^{-7} M). Data was acquired at 25º and 40ºC (Figure 22).

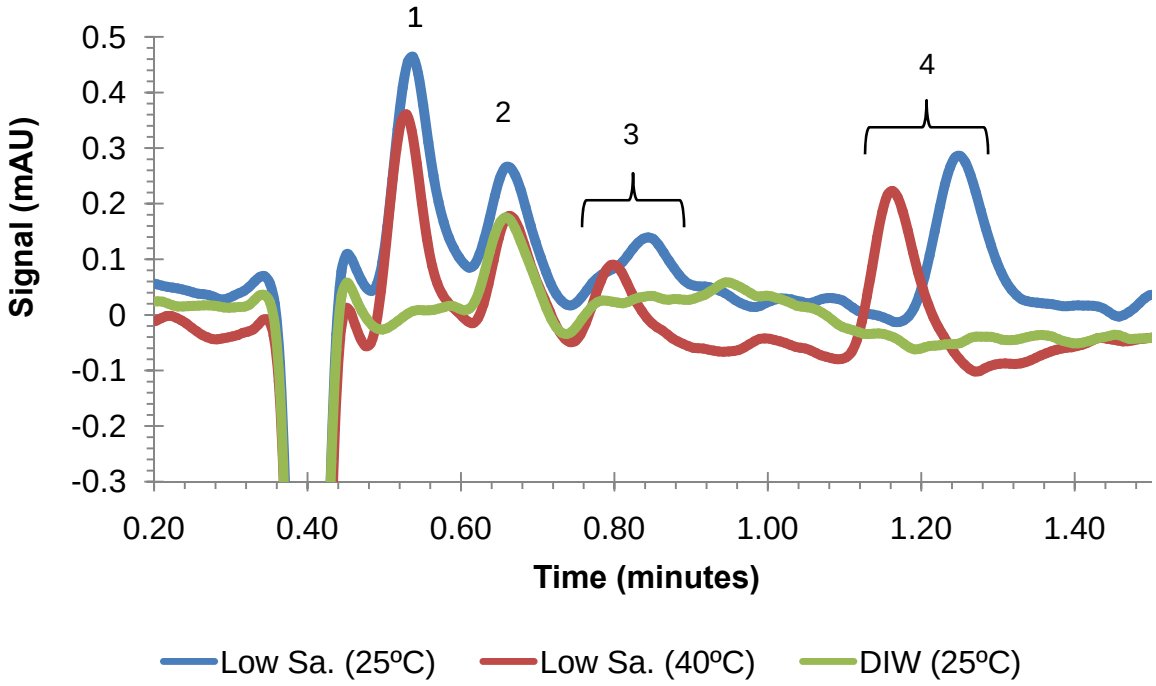

Figure 22: Chromatography of organoarsenic drug samples (low concentration) acquired with the column compartment temperature set at 25ºC and 40ºC. The peak elution order for the chromatograms is (1) p-arsanilic acid, 1.29×10^{-6} M; (2) IpA-Bkg; (3) acetarsone, 9.00×10^{-7} M; and (4) roxarsone, 9.98×10^{-7} M. Mobile phase: (85:15) 18.3 mM MTBA-OH, pH = 6.30 / methanol. Flow: 1.4 mL/min. Detector wavelength: 254 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

Visual differences were noticed in the separation of the organoarsenic drugs between acquired at 25ºC and 40ºC (Figure 21). The inverse relationship between t_R and temperature was observed. Once again, the most significant t_R differences corresponded to acetarsone (Δt_R = 0.046min) and roxarsone (Δt_R = 0.087min). There was no significant decrease in t_R for p-arsanilic acid with the temperature increase (Δt_R = 0.008min). This suggests that a stable ion pair was achieved between p-arsanilic acid and the MTBA ions, at the concentrations used for the experiment, regardless of the temperature variation. However, the t_R shifts observed for acetarsone and roxarsone is indicates ion pair formation is affected by temperature. The data demonstrates a faster rate of ion pair formation at 40ºC than at 25ºC for acetarsone and roxarsone.

The shape of the acetarsone peak is the most visually noticeable enhancement with the temperature increase. At 25ºC, acetarsone elutes as a broad peak with increased fronting. However, increasing the temperature to 40ºC provided a more gaussian shaped and better defined peak for acetarsone.

The figures of merit are displayed in tables 12 and 13 for the data acquisition at 25ºC and 40ºC respectively. The decrease in t_R observed in the low concentration sample for the peaks corresponding to roxarsone (-7.0%), acetarsone (-5.5%), and p-arsanilic acid (-1.4%) were in agreement with the more concentrated sample 1. A slight increase of 6.3% was observed in the α value between p-arsanilic acid and the IpA-Bkg with a 40ºC separation temperature. The α value between the IpA-Bkg and acetarsone decreased by 10.5%. However, this reduction in selectivity did not affect the appropriate integration of the peaks.

Table 12: Figures of merit for organoarsenic drug samples (low concentration) acquired with the column compartment temperature of 25ºC.

Analyte	p-arsanilic acid	IpA-Bkg	acetarsone	roxarsone
Conc. (M)	1.29×10^{-6}	N/A	9.00×10^{-7}	9.98×10^{-7}
t_R (min)	0.536	0.661	0.844	1.249
k	0.34	0.65	1.11	2.12
α	1.92	1.70	1.91	N/A [(a)]
Area (mAU*s)	1.5154	0.9315	0.4981	1.3198
T_f	1.3	N/A [(a)]	0.8	1.3
N	540	N/A [(a)]	1144	1766
R	N/A [(a)]	1.3	1.6	3.4
Height (mAU)	0.43	0.24	0.10	0.29
Baseline Noise (mAU)	1.94×10^{-2}	N/A [(a)]	1.13×10^{-2}	1.44×10^{-2}
S/N	22	N/A [(a)]	9	20

(a) Refer to Table 10 for a detailed explanation.

Table 13: Figures of merit for organoarsenic drug samples (low concentration) acquired with the column compartment temperature of 40ºC. The difference from the data acquired at 25ºC is displayed in parenthesis for each value.

Analyte	p-arsanilic acid	IpA-Bkg	acetarsone	roxarsone
Conc. (M)	6.45×10^{-5}	N/A	4.50×10^{-5}	4.99×10^{-5}
t_R (min)	0.528 (-0.008)	0.661 (0)	0.798 (-0.046)	1.162 (-0.087)
k	0.32 (-0.02)	0.66 (-0.01)	1.00 (-0.11)	1.91 (0.21)
α	2.04 (+0.12)	1.52 (-0.18)	1.91 (0)	N/A [(a)]
Area (mAU*s)	1.4380 (-0.0774)	0.8735 (-0.0580)	0.5559 (+0.0578)	1.3279 (+0.0081)
T_f	1.5 (+0.2)	N/A [(a)]	1.3 (+0.5)	1.2 (-0.1)
N	599 (+59)	N/A [(a)]	993 (-151)	1680 (-86)
R	N/A [(a)]	1.4 (+0.1)	1.3 (-0.3)	3.5 (+0.1)
Height (mAU)	0.42 (-0.01)	0.23 (-0.01)	0.14 (+0.04)	0.31 (+0.02)
Baseline Noise (mAU)	1.61×10^{-2} (-3.30×10^{-3})	N/A [(a)]	9.98×10^{-3} (-1.32×10^{-3})	1.04×10^{-2} (-4.00×10^{-3})
S/N	26 (+4)	N/A [(a)]	14 (+5)	30 (+10)

(a) Refer to Table 10 for a detailed explanation.

The most significant enhancement was observed for S/N of all three organoarsenicals. The S/N increased for p-arsanilic acid (18.1%), acetarsone (55.6%), and roxarsone (50.0%) with the separation temperature of 40ºC. These particular results are highly attractive since an increase in S/N represents greater opportunities for detecting low levels of the organoarsenic drugs understudy.

11 Study: Optimum wavelength determination

11.1 Description of Study

The present study evaluated the optimum wavelength for the analysis of the organoarsenic drugs targeted in this investigation. UV spectra were collected at 2 nm intervals from 190 nm to 400 nm range for the high concentration sample used for the temperature variation study (Section 10). The equipment operational parameters and standard solutions are described in table 9 (Section 10.2).

11.2 Results and Discussion

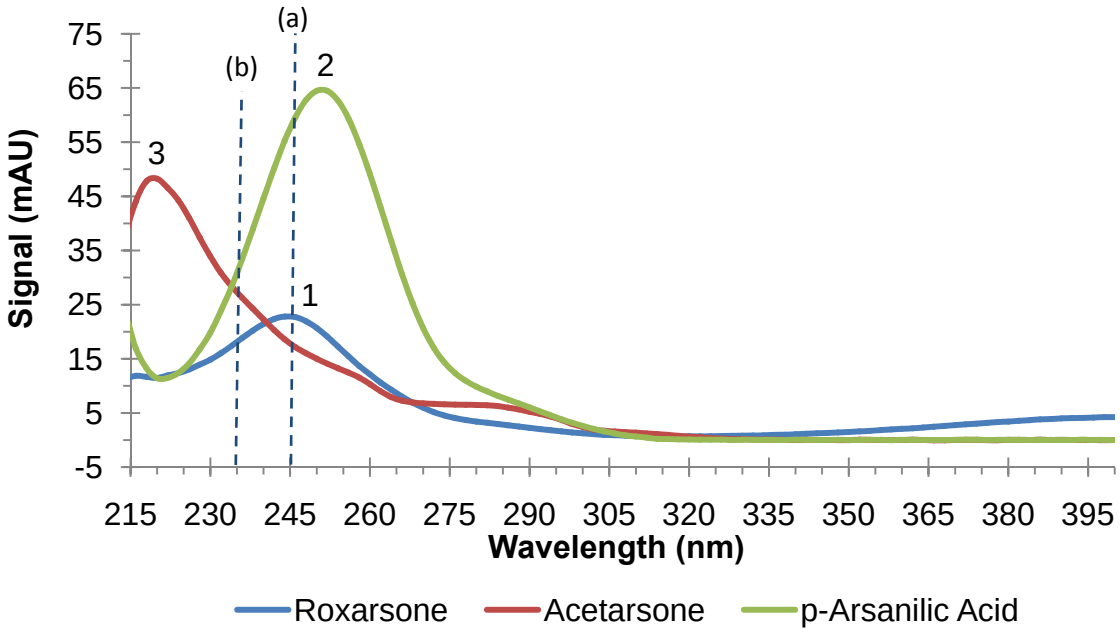

Figure 23: UV Spectra of (1) roxarsone, λ_{max} = 245 nm; (2) p-arsanilic acid, λ_{max} = 250 nm; and (3) acetarsone, λ_{max} = 220 nm. (a) Absorbance at 245 nm (roxarsone = 23 mAU; p-arsanilic acid = 57 mAU; acetarsone = 18 mAU). (b) Alternate detection wavelength for increased acetarsone sensitity at λ = 235 nm (roxarsone = 20 mAU; p-arsanilic acid = 31 mAU; acetarsone = 27 mAU).

The observed wavelength of maximum absorbance for p-arsanilic acid, acetarsone, and roxarsone were 250 nm, 220 nm, and 245 nm respectively (Figure 23). The maximum wavelength targeted corresponds to roxarsone (245 nm). The spectra suggest that all peaks will be detected at 245 nm. The response of roxarsone was 23 mAU at 245 nm. The response demonstrated at 245 nm for p-arsanilic acid was 57 mAU and 18 mAU for acetarsone. The absorbance observed for acetarsone at 245 nm is fairly small. However, a well-defined peak was observed for acetarsone in the chromatograms in Figures 21 and 22 which correspond to a at 254 nm wavelength (Section 10.3). According to the UV spectra in Figure 23, acetarsone has greater absorption at 245 nm than 254 nm. Therefore, adequate chromatographic peaks are expected for acetarsone despite the small absorbance at 245 nm. In addition, when analyzing ultra-trace levels of the analyte for a specific organoarsenic, the analyst can switch to the wavelength of maximum absorbance for enhanced sensitivity.

12 Study: Variation of the Ion-Pairing Agent Concentration

12.1 Study Description

A study was performed to evaluate the effect of varying the ion-pairing agent (IpA) concentration. A sample solution containing the three organoarsenic drugs used in this investigation was analyzed using mobile phase containing 2.3 mM and 18.3 mM of methyl-tributyl ammonium hydroxide (MTBA-OH). The equipment operational parameters and sample concentration are described in Table 14. The mobile phase consisted of the variable concentration of MTBA-OH with 1%TEA. It was adjusted to a pH of 6.30 with glacial acetic acid. The HPLC system was allowed to equilibrate (at least 15 minutes) prior to starting the analysis. This particular study used the Eclipse Plus C18 RR HPLC column.

Table 14: Test parameters for the ion pairing agent concentration variation study for the organoarsenic drugs roxarsone, p-arsanilic acid, and acetarsone.

Mobile Phase (85:15 A/B)			
Eluent A		Eluent B	
MTBA-OH (18.3 mM / 2.3 mM), pH = 6.30		methanol	
Equipment Operating Conditions			
Flow	1.4 mL / min	Column Temp	25ºC
Wavelength	254 nm	Injection Volume	5 μL
Column			
Description	Zorbax Eclipse Plus C18		
Serial Number	USUXG03355		
Dimensions	4.6 × 50mm, 1.8 μm packing		
Sample (Diluent: DIW)			
Analyte	roxarsone	Concentration	4.99×10^{-5} M
	p-arsanilic acid		6.45×10^{-5} M
	acetarsone		4.50×10^{-5} M

12.2 Results and Discussion

Figure 24 depicts the separation obtained by varying the IpA concentration. The data obtained using 2.3 mM MTBA-OH depicts two peaks, whereas there are three peaks for 18.3 mM (corresponding to the three organoarsenic species in the sample solution).

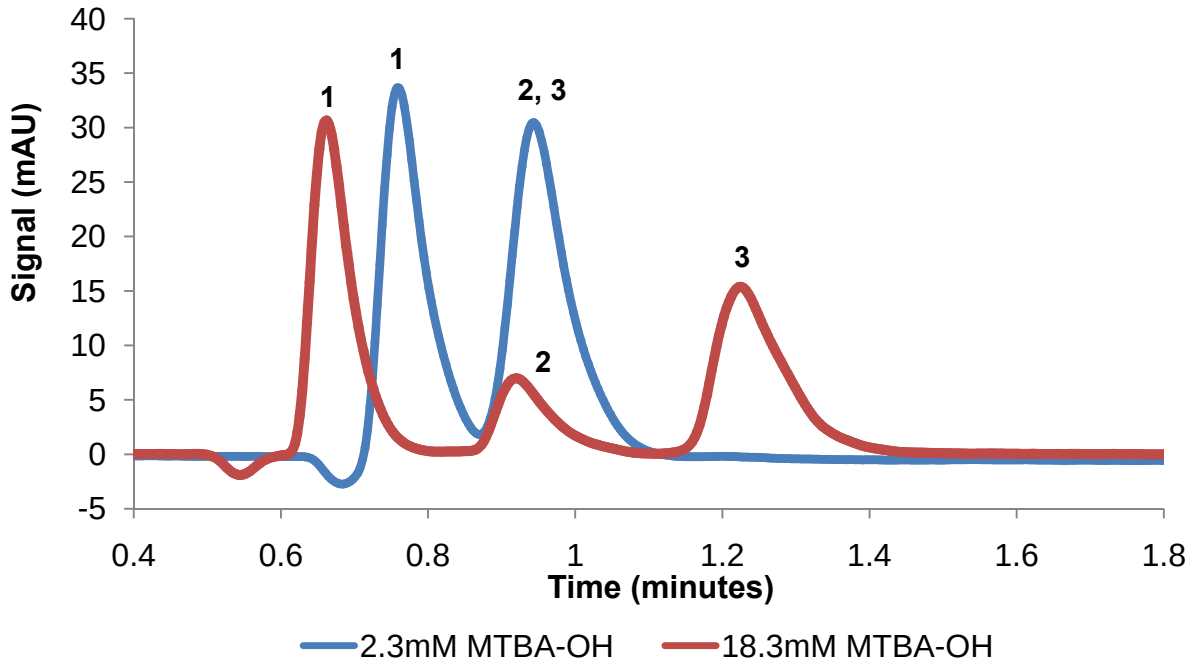

Figure 24: Chromatography rendering the effect of the MTBA-OH concentration in the mobile phase at 2.3 mM and 18.3 mM. (1) p-arsanilic acid, 6.45 × 10^{-5} M; (2) acetarsone, 4.50 × 10^{-5} M; (3) roxarsone, 4.99 × 10^{-5} M. Mobile phase: (85:15) MTBA-OH, pH = 6.30 / methanol. Flow: 1.4 mL/min. Column temperature: 25ºC. Detector wavelength: 254 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

A spectral analysis of the second peak in 2.3 mM MTBA-OH was performed using the ChemStation® software. Three segments were chosen corresponding to the beginning (0.898 min), middle (0.944 min), and end (1.014 min) of the suspect peak. The UV data demonstrates spectral profiles characteristic of acetarsone and roxarsone (Figure 25). This is indicative that roxarsone and acetarsone co-eluted under the chromatographic separation conditions. Furthermore, this suggests that although the samples are expected to be sufficiently ionized at a pH of 6.30, the concentration of 2.3 mM MTBA-OH does not provide enough MTBA ions to completely resolve the peaks. The chromatography obtained with 18.3 mM MTBA-OH resolved the three organoarsenic drugs completely. Therefore, increasing the IpA concentration was appropriate to compensate for the elution problem.

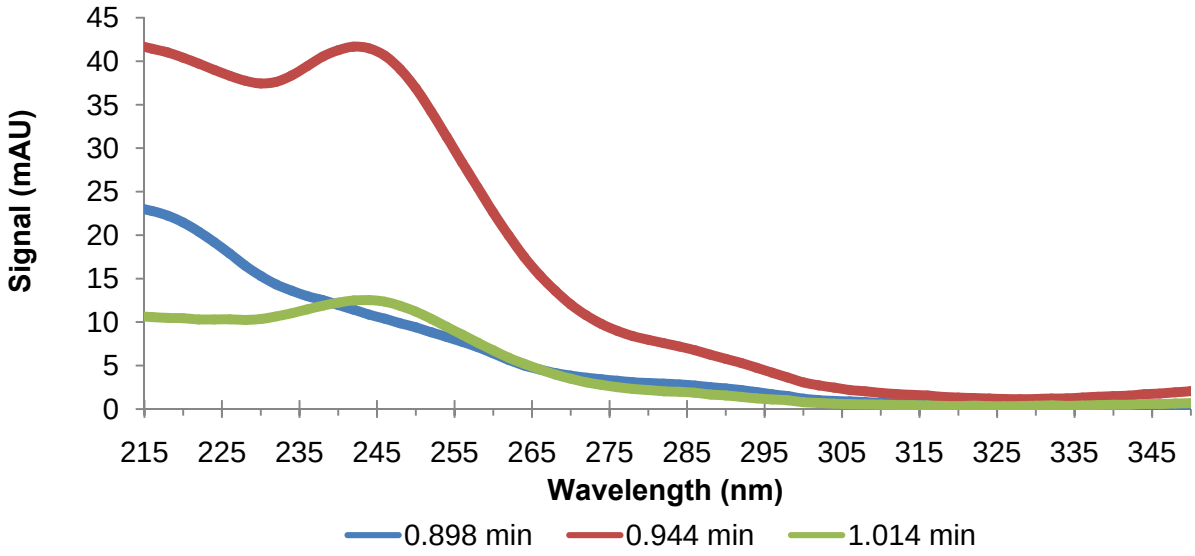

Figure 25: UV Spectra obtained for three different segments of the second peak eluting in the chromatogram of 2.3 mM MTBA-OH (see figure 24). The characteristic UV spectra for acetarsone (0.898 min) and roxarsone (0.944 min, 1.014 min) are indicators that these species co-eluted.

13 Study: Mobile phase variable composition and the addition of triethylamine (TEA) as a modifier.

13.1 Study Description

As mentioned in the previous section, solvent strength can be varied in one of two ways in IP-HPLC methods: (1) Variation of the ion-pairing agent (IPA) concentration and (2) Variation of the mobile phase composition.[45, 47] The variation of the MTBA-OH concentration was discussed and performed in the previous study (section 12). The study presented in this section will address the variations of mobile phase composition.

The main advantages of IP-HPLC over conventional reverse-phase HPLC (RP-HPLC) include the increased specificity of ionizable compounds (due to interactions with IpA), analysis of aqueous samples with a significant reduction in sample preparation steps (e.g. pH adjustments, addition of organic solvent), and the capability of analyzing in a neutral pH region (thus increasing the life of the HPLC column). However, the mobile phase used for IP-HPLC will generally behave the same way as in a conventional reverse-phase HPLC (RP-HPLC) in terms of the aqueous/organic phase composition ratio.

The organic/aqueous composition ratio should be optimized to take advantage of the IP-HPLC separation mechanism (discussed in section 5.4). An increase in the organic phase (e.g. acetonitrile, methanol) will increase the solvent strength of the mobile phase. Based on this, hydrophilic species tend to elute at a more rapid rate due to the reduced interactions with the column packaging material. The reduction of the

organic solvent will allow the hydrophilic species a more suitable environment for interactions with the packing material thus resulting in an extended migration time. The ability of the mobile phase to stabilize ion pairs dictates the mobility and retention of the species in reverse-phase IP-HPLC.

Experiments were performed in which the aqueous / organic composition was varied from 70:30 to 80:20. The aqueous phase consisted of 18.3 mM MTBA-OH with a pH of 6.50 (adjusted with glacial acetic acid). The concentration of MTBA-OH was maintained constant throughout the study. The equipment operational parameters and solutions used are described in Table 15.

Table 15: Test parameters for the mobile phase variation and addition of the TEA modifier study for roxarsone, p-arsanilic acid, and acetarsone.

Mobile Phase			
Eluent A		Eluent B	
18.3 mM MTBA-OH, pH = 6.50		methanol	
Equipment Operating Conditions			
Flow	1.4 mL / min	Column Temp	40ºC
Wavelength	245 nm	Injection Volume	5 μL
Column			
Description	Zorbax Eclipse Plus C18		
Serial Number	USUXG03355		
Dimensions	4.6 × 50 mm, 1.8 μm packing		
Sample (Diluent: DIW)			
Analyte	roxarsone	Concentration	4.99×10^{-5} M
	p-arsanilic acid		6.45×10^{-5} M
	acetarsone		4.50×10^{-5} M

13.2 Results and Discussion

The chromatography obtained for the 70:30 and 80:20 mobile phase compositions (Aq./Org.) depict a significant difference for the roxarsone t_R when compared to p-arsanilic acid and acetarsone. Based on the chromatogram displayed (Figure 26), increasing the solvent strength of the mobile phase will reduce the roxarsone t_R at the expense of decreasing resolution between p-arsanilic acid and acetarsone. In addition, the ion-pairing agent background (IpA-Bkg) was absorbed in the peak tail of p-arsanilic acid at higher concentrations of organic phase.

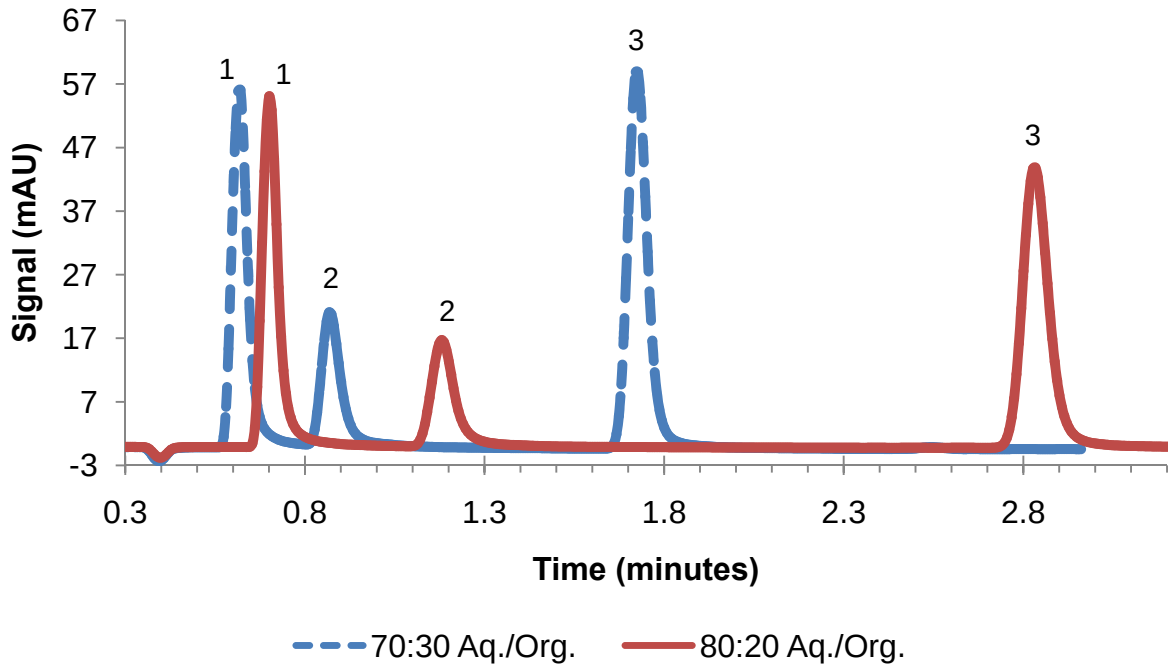

Figure 26: Chromatography rendering the difference between mobile phase aqueous/organic compositions of 70:30 (dashed line) and 80:20. (1) p-arsanilic acid, 6.45 × 10^{-5} M; (2) acetarsone, 4.50 × 10^{-5} M; (3) roxarsone, 4.99 × 10^{-5} M. Mobile phase: 18.3 mM MTBA-OH, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 µL. Column: Zorbax Eclipse Plus, 4.6 x 50 mm, 1.8 µm packing.

Triethylamine (TEA) has been used as a mobile phase modifier to improve separations. The same mobile phase compositions of 70:30 and 80:20 were used with a 1% addition of TEA.

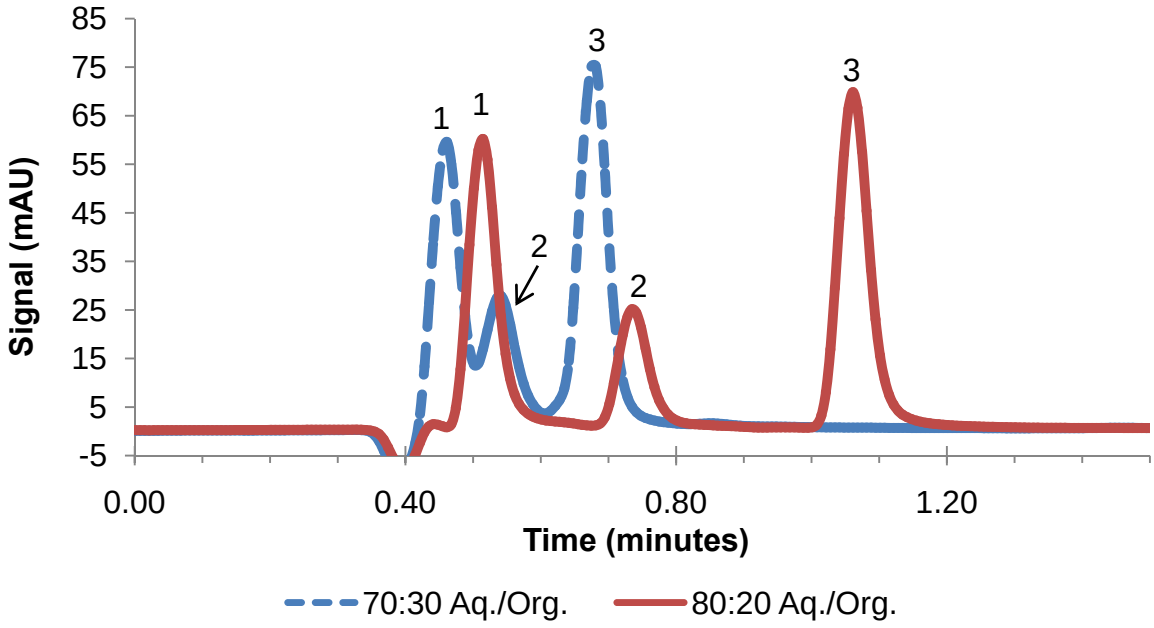

Figure 27: Chromatography rendering the effects of adding 1% TEA to the aqueous phase of the mobile phase. (1) p-arsanilic acid, 6.45 × 10^{-5} M; (2) acetarsone, 4.50 × 10^{-5} M; (3) roxarsone, 4.99 × 10^{-5} M. Mobile phase: 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

The resulting chromatography demonstrates a 60.7% decrease in acquisition time (from 2.8 minutes to 1.75 minutes) for roxarsone and a 61.1% decrease for acetarsone (from 1.1 minutes to 0.7 minutes) for the 70:30 Aq./Org. composition (Figure 27). The peaks corresponding to p-arsanilic acid and acetarsone were not fully resolved with the 70:30 Aq./Org. composition. An 80:20 Aq./Org. composition was sufficient to completely resolve these two species.

Additional chromatography was obtained using an 85:15 Aq./Org. composition which included 1% TEA (Figure 28). All peaks eluted with t_R less than 1.6 minutes. The IpA-Bkg (t_R = 0.69 minutes) slightly co-eluted with p-arsanilic acid (t_R = 0.56 minutes). However, adequate resolution was obtained in order to perform proper integration.

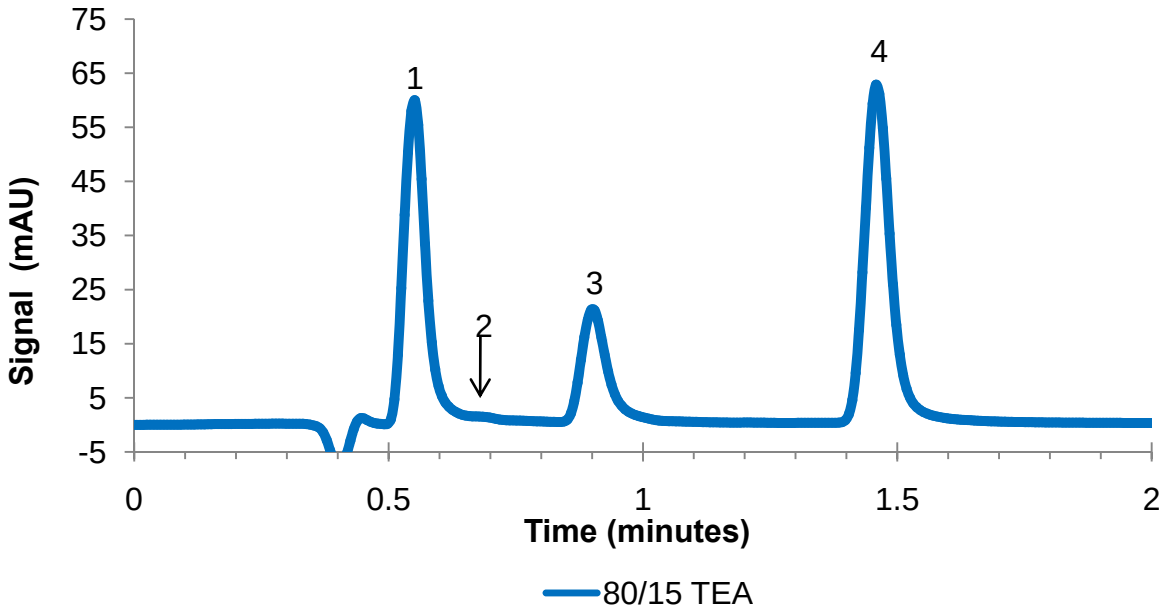

Figure 28: Chromatography obtained with additon of TEA as a mobile phase modifier. Refer to Figure 29 for an enhancement of the p-arsanlic acid region. (1) p-arsanilic acid, 6.45 × 10^{-5} M; (2) IpA-Bkg; (3) acetarsone, 4.50 × 10^{-5} M; (4) roxarsone, 4.99 × 10^{-5} M. Mobile phase: 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

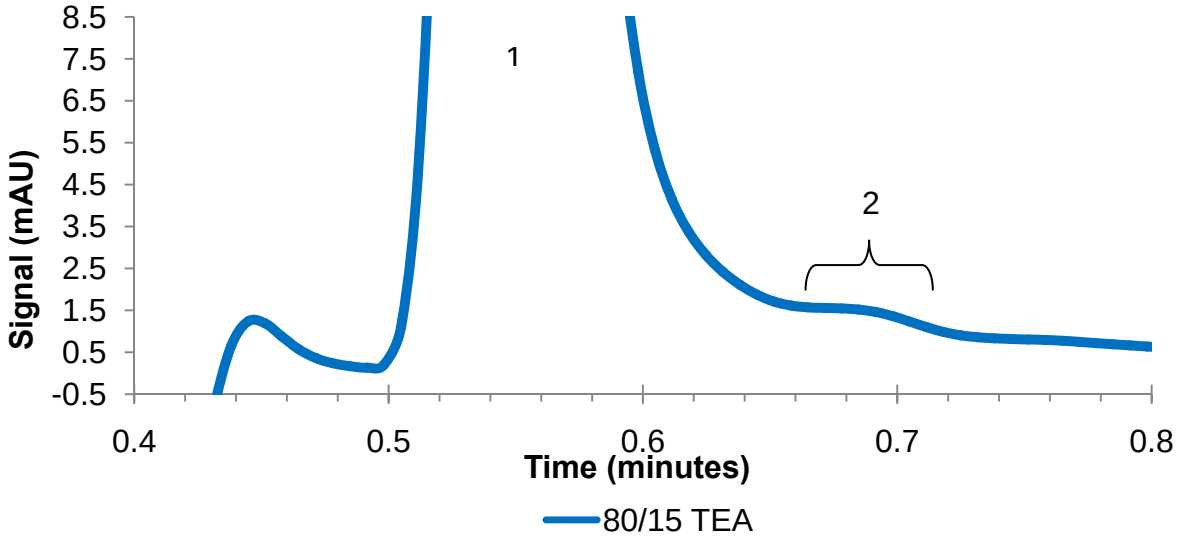

Figure 29: Enhancement of the p-arsanilic acid region for the chromatography depicted in Figure 28. (1) p-arsanilic acid, 6.45 × 10^{-5} M; (2) IpA-Bkg. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

14 IP-HPLC Validation

14.1 General Description of Studies

Throughout the investigation, standard solutions of roxarsone, p-arsanilic acid, and acetarsone were evaluated at various concentrations with different equipment operational parameters. Once the optimum conditions have been identified, it is important to demonstrate the method validity. The purpose of this particular study was to comply with validation criteria as per regulatory requirements.[48, 51]

The study described in this section was performed using standard reference material dissolved in DIW. The matrix was maintained simple in order to confirm optimal operational parameters observed throughout the pre-validation studies. Experiments involving more complex matrixes (e.g. potable water, dissolved ions, and poultry litter leachates) were performed and will be discussed in subsequent sections.

The validated methodology can be used as a routine protocol for the detection and quantitation of roxarsone, p-arsanilic acid, and acetarsone. Base on the results obtained in the previous sections, the final chromatographic conditions that were submitted for validation purposes are described in Table 16. A general summary of the validation results are provided in Table 17.

Table 16: Test parameters used for the validation of the IP-HPLC rapid-resolution method for the detection of roxarsone, p-arsanilic acid, and acetarsone.

Mobile Phase (85:15 A/B)			
Eluent A		Eluent B	
18.3 mM MTBA-OH, 1% TEA, pH = 6.30 and 6.50		methanol	
Equipment Operating Conditions			
Flow	1.4 mL / min	Column Temp	40ºC
Wavelength	245 nm	Injection Volume	5 μL
Column			
Description	Zorbax Eclipse Plus C18		
Serial Number	USUXG03355 and USUXG03364		
Dimensions	4.6 × 50mm, 1.8 μm packing		

Table 17: General summary of the validation parameters for the IP-HPLC rapid-resolution method for the detection of roxarsone, p-arsanilic acid, and acetarsone. Subsequent sections will discuss each parameter in greater detail. Additional information is found in the appendices referrenced in each section.

Validation Parameter	Result Summary
Specificity	Specificity was demonstrated by the analysis of blank chromatograms and reference standard solutions. Other possible complex matrixes (e.g. potable water, dissolved ions, poultry litter leachates) are discussed in subsequent sections. The target species eluted with peak characteristics adequate for integration. Species and mobile phase artifacts observed depicted a minimum resolution value of 1.2 from target analytes. Peak purity testing performed with diode-array detector and ChemStation software confirmed the absence of additional peaks co-eluting with the target species.
Precision (Reproducibility)	Reproducibility was demonstrated for peak area and t_R of the target species. The %RSD values obtained for the organoarsenic drugs were less than 2.0%. These results comply with USP Chapter <621> guidelines.[48]
Linearity	Linearity was demonstrated by analyzing several reference standard solutions of the target analytes with a concentration range suitable for monitoring activities. Linear responses were determined based on the following ANOVA Data Output provided by *Microsoft Excel*: • $R^2 \geq 0.9990$ • $MS_{reg} >> MS_{res}$ • Large F value • *p*-value < 0.05
Limit-of-Quantitation (LOQ)	The LOQ was calculated as per Section 5.3.6 using blank chromatograms and the slope of the calibration curve. The operational parameters were sufficient to produce an LOQ level suitable for monitoring roxarsone at the approved concentrations.[7]
Limit-of-Detection (LOD)	The LOQ was calculated as per Section 5.3.6 using blank chromatograms and the slope of the calibration curve. The operational parameters were sufficient to produce an LOD level suitable for monitoring roxarsone at the approved concentrations.[7]
Accuracy	The accuracy of the method was demonstrated for the target analytes with the compliant results of linearity, repeatability, specificity.
Range	The range of the method obtained was established for the target analytes with the data corresponding to linearity, LOQ, LOD, and precision.

14.2 Specificity

Specificity was initially assessed by carefully evaluating DI Water blank injections against the chromatographic profiles of p-arsanilic acid, acetarsone, and roxarsone. (The matrixes of a more complex nature, such as natural water and poultry litter leachates, are discussed from Section 15 through Section 17). A baseline inflection is depicted around 0.4 minutes for all DI Water chromatograms. The intensity of this negative peak fluctuates around -0.4 mAU. A peak was observed in all DI Water chromatograms around 0.7 minutes with an intensity of 1 mAU and an area response of 1.0 mAU*s. According to the chromatography obtained, the average retention times of roxarsone, p-arsanilic acid, and acetarsone are displayed in Table 18.

Table 18: Retention time (t_R) data used to determine specificity of the organoarsenic drugs p-arsanilic acid, acetarsone, and roxarsone. All compounds were diluted in DIW. Chromatography was obtained as per conditions described in Table 16. The results for t_R are given in minutes.

Description	t_M [(a)]	p-ASA	IpA-Bkg [(b)]	acetarsone	roxarsone
pH = 6.3 Col. #1 (n=18)	0.401 (± 0.001)	0.540 (± 0.001)	0.665 (± 0.001)	0.831 (± 0.001)	1.236 (± 0.001)
pH = 6.3 Col. #2 (n=18)	0.401 (± 0.001)	0.541 (± 0.001)	0.649 (± 0.001)	0.824 (± 0.001)	1.278 (± 0.001)
pH = 6.5 Col. #1 (n=18)	0.400 (± 0.000)	0.541 (± 0.001)	0.687 (± 0.001)	0.824 (± 0.001)	1.430 (± 0.001)
pH = 6.5 Col. #2 (n=18)	0.401 (± 0.000)	0.547 (± 0.001)	0.691 (± 0.001)	0.880 (0.000)	1.410 (± 0.001)

(a) The inflection eluting at 0.401 (± 0.001 min) corresponds to methanol. It is considered the marker (t_M) for the purposes of the studies.

(b) IpA-Bkg = Ion-pairing background artifact.

All components resolved free from baseline artifacts with the exception of p-arsanilic acid. The IpA-Bkg eluting around 0.7 minutes slightly co-eluted with p-arsanilic acid standard concentrations of 1.3×10^{-4} M (28 ppm) , 6.4×10^{-5} M (14 ppm), and 3.2×10^{-5} M (7 ppm). Despite the slight co-elution, adequate integration was performed for p-arsanilic acid. The IpA-Bkg was completely absorbed by the tailing end of p-arsanilic acid at a concentration of 1.9×10^{-4} M (42 ppm). However, this does not represent a problem from a regulatory and compliance point-of-view being that any concentration detected for p-arsanilic acid greater than 0.05 ppm exceeds the dosage established by law.[12] Furthermore, if necessary, appropriate dilutions could be made of the sample in order to obtain smaller peaks thereby increasing the resolution between p-arsanilic acid and the IpA-Bkg.

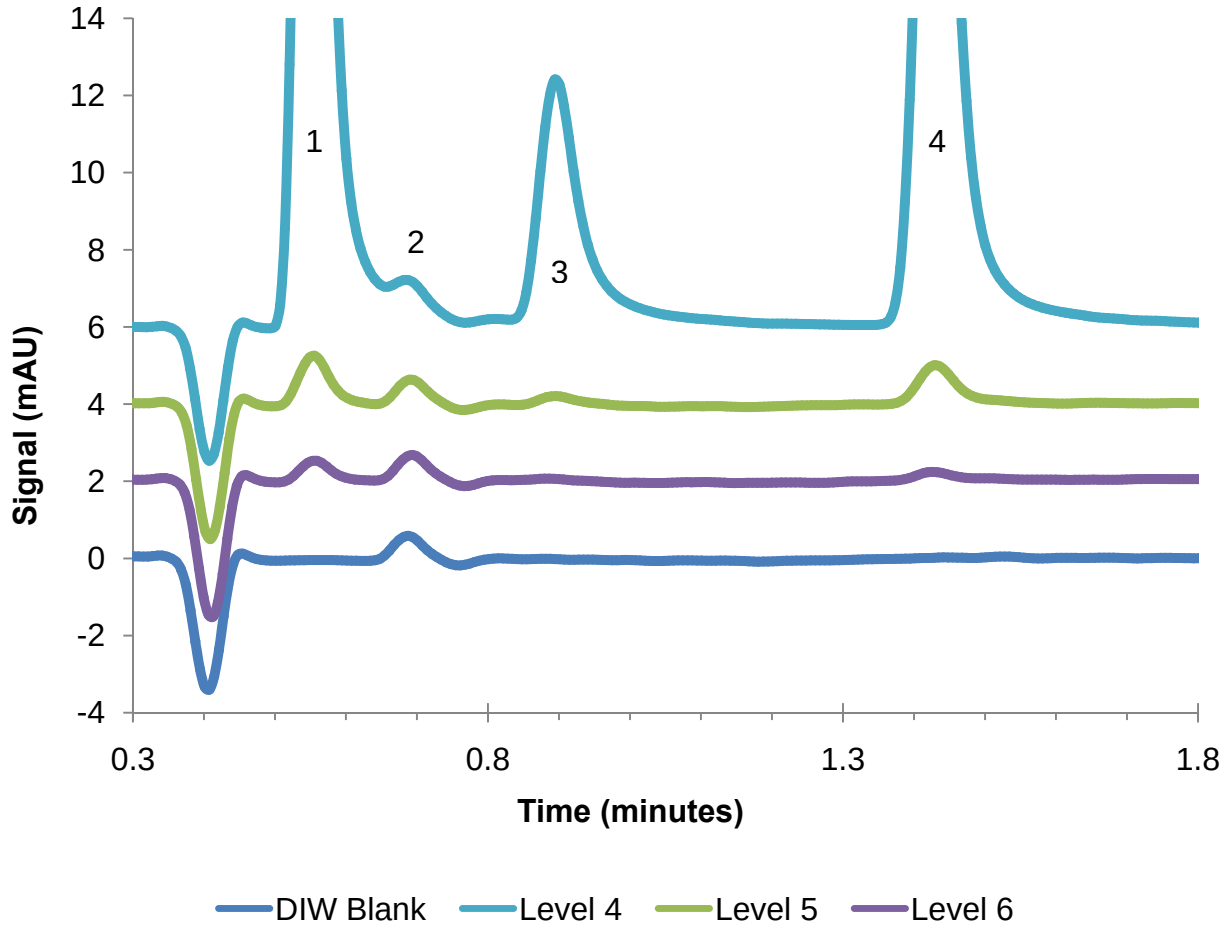

Figure 30: Chromatograms corresponding to various concentration levels of the organoarsenic drugs. (1) p-arsanilic acid; (2) IpA-Bkg; (3) Acetarsone; and (4) Roxarsone. Refer to Appendix 5 for level specific concentrations. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

Normalized spectra of a pure peak will not differ independently from which section of the peak it was taken. ChemStation® determines the peak purity using the average spectrum across the peak. A *purity factor* (also known as the *similarity factor*) is calculated based on a variety of mathematical and statistical functions. The purity factor ranges from 0 (impure peak) to 1000 (pure peak). According to the software, a purity factor $\geq$ 990 indicates that there is no significant difference in the spectral data used, therefore the peak can be considered pure.

Peak purity assessment was performed using the ChemStation® software and the spectral data obtained with the diode array detector (DAD).[58, 59] The peaks corresponding to roxarsone, p-arsanilic acid, and acetarsone for the standard level one were evaluated. The purity factors obtained were greater than 995 for roxarsone, p-arsanilic acid, and acetarsone. This suggests that the target analyte peaks obtained with the validation equipment parameters are pure.

Figures 31–33 portray the *spectral purity display* (SPD) obtained from ChemStation® for roxarsone, p-arsanilic acid, and acetarsone respectively. The green box contains the threshold area calculated by the software. In order for the peak to be pure, all spectral data used for purity calculations must remain within this area. The peak spectral data obtained for roxarsone, p-arsanilic acid, and acetarsone remained within the threshold area calculated by the ChemStation® software. Therefore, the data suggests that the peaks corresponding to these organoarsenic drugs obtained with the validation equipment operational parameters are pure.

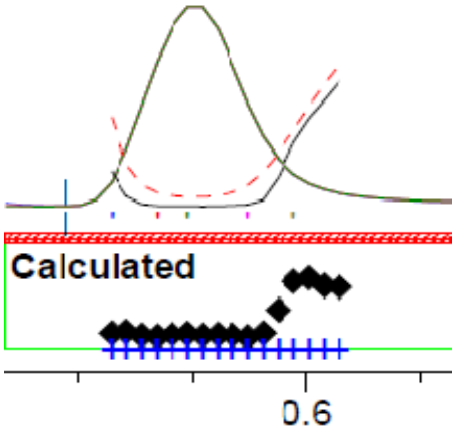

Figure 31: Spectral purity display obtained by the ChemStation® software for p-arsanilic acid (1.3×10^{-4} M).

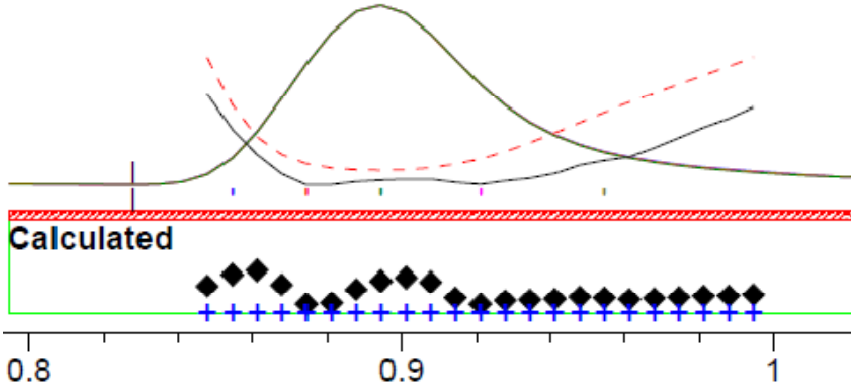

Figure 32: Spectral purity display obtained by the ChemStation® software for acetarsone (4.5×10^{-5} M).

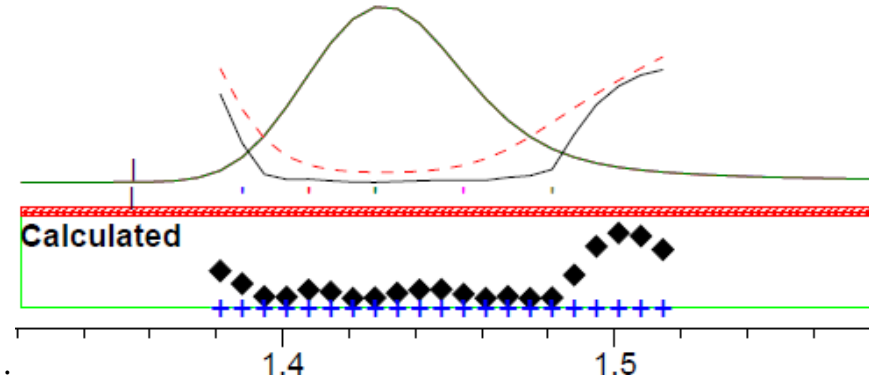

Figure 33: Spectral purity display obtained by the ChemStation® software for roxarsone (4.99×10^{-5} M).

A *similarity curve* and a *threshold* curve are calculated by ChemStation® software using all the spectra obtained for the peak. The similarity curve uses the same mathematical functions as the purity factor, yet it is displayed as a plot of purity factor vs. time. Furthermore, it is displayed directly over the peak in the peak spectral display with the y-axis values (purity factor) inversed. This particular placement provides a sense of correlation between the peak shape and the purity. An ideal similarity curve is that which depicts a flat, horizontal line (m=0) within the purity factor range of 0.990 to 1000. The signal-to-noise ratio (S/N) affects the shape of this plot. As the S/N decreases, the peak spectra will suffer baseline noise interference therefore compromising the purity of the peak. For this reason, the values for purity obtained at the beginning and the end of the peak will decrease the purity factor. However, as stated before, if the peak is pure, the spectra obtained will be similar independently from the peak section it is obtained. A plot depicting a wide, low, u-shaped curve (as seen in Figures 31-33 for the organoarsenic drugs analyzed) is expected.

The similarity curve subtracts the background noise from the spectra. However, the *threshold* curve contemplates the noise in the plot. The threshold curve is depicted as a dashed line as the “purity factor + noise” vs. time. The purity factor value decreases in the threshold curve because the noise is contemplated. Therefore, the plot of this curve is expected to be located above the similarity curve because its purity factor is less. In addition, if the background noise is consistent, the shape of the threshold curve should be similar to that of the similarity curve. The software has the capacity of detecting peak impurity in the 0.1% level.[58] If a peak were impure, the shape of the similarity curve would be different from the threshold curve. Furthermore, it would intercept the threshold curve were the spectral data differs, which would correspond to the elution time of a co-eluting impurity. The similarity curves were similar to the threshold curves for roxarsone, p-arsanilic acid, and acetarsone. The curve data suggest that the peaks corresponding to these compounds obtained with the validation equipment operational parameters are pure.

14.3 Precision

Method precision was evaluated from four (4) working curves consisting of six (6) levels of reference standard solutions preparations. Three injections were made of each working standard as per ICH validation guidelines.[51] The chromatographic data obtained during the validation exercise demonstrated a reproducible area response (mAU*s) for roxarsone (%RSD $\leq$ 0.31%), p-arsanilic acid (%RSD $\leq$ 0.31%), and acetarsone (%RSD $\leq$ 0.34%). Furthermore, the t_R between all the concentration levels were reproducible for roxarsone (%RSD $\leq$ 0.07%), p-arsanilic acid (%RSD $\leq$ 0.10%), and acetarsone (%RSD $\leq$ 0.06%). The data obtained during this stage of the validation exercises indicate that the method parameters will produce precise results. Refer to Appendix 3-6 for a detailed summary of the chromatographic data.

14.4 Linearity

Linearity was assessed by evaluation of four (4) working curves working curves consisting of six (6) levels of reference standard solutions preparations. Tables 19-21 depict the ANOVA analysis of the chromatographic data obtained indicating a linear response for roxarsone, p-arsanilic acid, and acetarsone. Refer to Appendix 3-6 for a detailed summary of the chromatographic data.

Table 19: Regression results used for linearity assessment of roxarsone. Results were calculated as per ANOVA using the chromatographic data obtained with the conditions described in Table 16.

Description	Slope	Intercept	R^2	F	*p*-value
	mAU*s / M	mAU*s			
Work. Curve 1 pH = 6.3 Col. #1	4.3×10^6	0.0414	0.9999	31095.64	6.2×10^{-9}
Work. Curve 2 pH = 6.3 Col. #2	4.2×10^6	0.2995	0.9999	31622.03	6.0×10^{-9}
Work. Curve 3 pH = 6.5 Col. #1	4.2×10^6	1.9936	0.9990	3950.89	3.8×10^{-7}
Work. Curve 4 pH = 6.5 Col. #2	4.2×10^6	1.1717	0.9996	9305.68	6.9×10^{-8}

Table 20: Regression results used for linearity assessment of p-arsanilic acid. Results were calculated as per ANOVA using the chromatographic data obtained with the conditions described in Table 16.

Description	Slope	Intercept	R^2	F	*p*-value
	mAU*s / M	mAU*s			
Work. Curve 1 pH = 6.3 Col. #1	2.7×10^6	0.4807	0.9998	21396.13	1.3×10^{-8}
Work. Curve 2 pH = 6.3 Col. #2	2.8×10^6	-0.2793	0.9998	19534.78	1.6×10^{-8}
Work. Curve 3 pH = 6.5 Col. #1	2.8×10^6	1.4805	0.9992	5223.04	2.2×10^{-7}
Work. Curve 4 pH = 6.5 Col. #2	2.8×10^6	1.2294	0.9995	8202.62	8.9×10^{-8}

Table 21: Regression results used for linearity assessment of acetarsone. Results were calculated as per ANOVA using the chromatographic data obtained with the conditions described in Table 16.

Description	Slope	Intercept	R^2	F	*p*-value
	mAU*s / M	mAU*s			
Work. Curve 1 pH = 6.3 Col. #1	1.6×10^6	-1.0405	0.9999	67410.31	1.3×10^{-9}
Work. Curve 2 pH = 6.3 Col. #2	1.5×10^6	0.0113	0.9998	16448.96	2.2×10^{-8}
Work. Curve 3 pH = 6.5 Col. #1	1.5×10^6	-0.2771	0.9998	23967.46	1.0×10^{-8}
Work. Curve 4 pH = 6.5 Col. #2	1.5×10^6	-2.2666	0.9995	8749.94	7.8×10^{-8}

The plots of area (mAU*s) vs. Concentration (M) rendered a linear graph for all three components. A representation of a working curve for each component is depicted in Figures 34- 36.

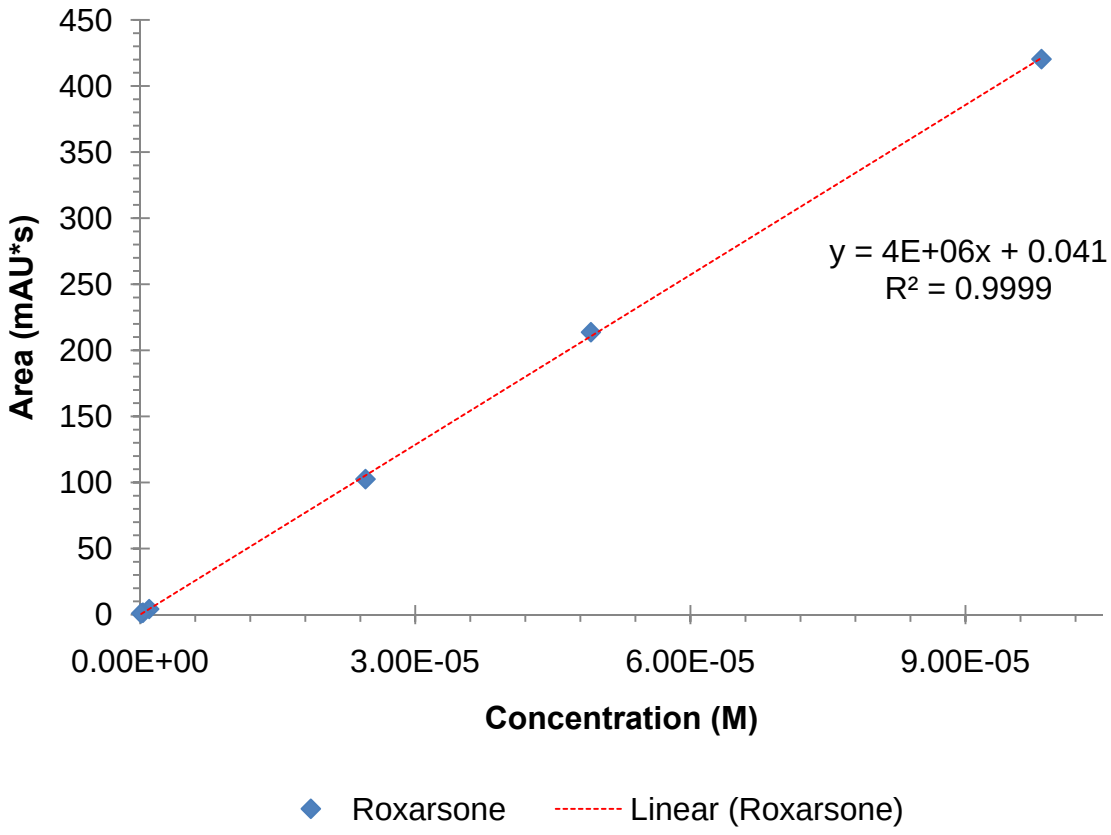

Figure 34: Working curve of roxarsone used for the linearity assessment of the IP-HPLC method; ($F = 31095.64$; p-value = 6.2×10^{-9}).

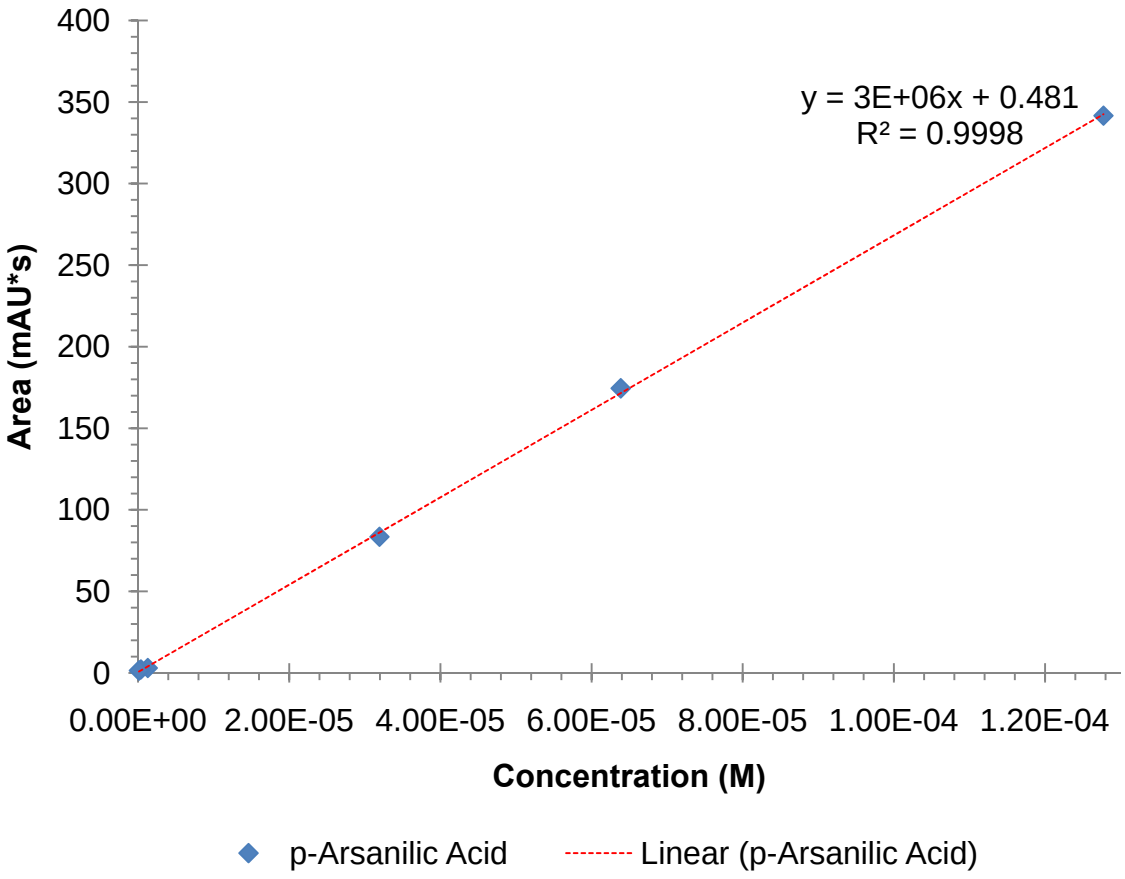

Figure 35: Working curve of p-arsanilic acid used for the linearity assessment of the IP-HPLC method; ($F = 21396.13$; p-value = 1.3×10^{-8})

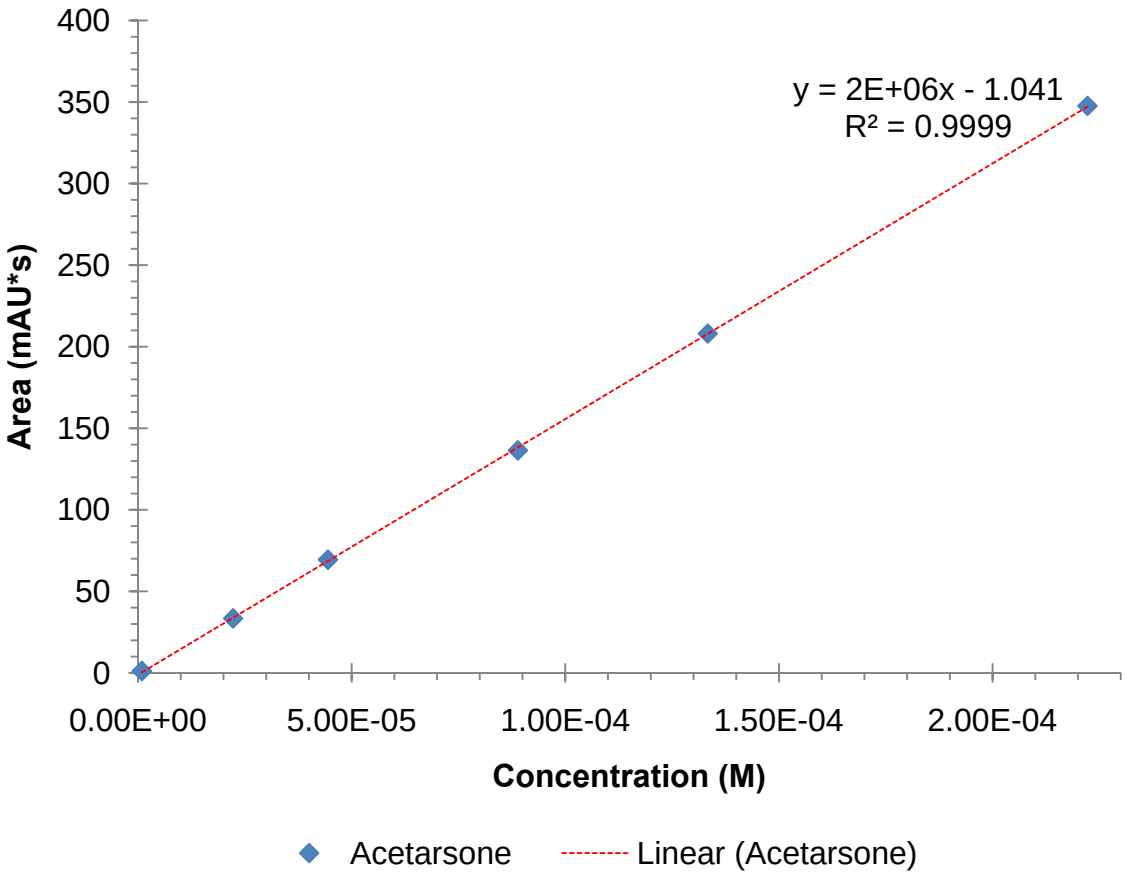

Figure 36: Working curve of acetarsone used for the linearity assessment of the IP-HPLC method; ($F = 67410.31$; p-value = 1.3×10^{-9}).

14.5 LOQ and LOD

The Limit-of-Quantitation (LOQ) and Limit-of-Detection (LOD) were established with experimental data of various chromatographic acquisitions with calculated values as per ICH Q2 (R1). Table 22 depicts the noise determined by the ChemStation software of various DIW blank injections. Refer to Appendix 5 for calibration curve and equipment parameters.

Table 22: Noise data of DIW blanks obtained during the data acquisition of calibration curve #3 (Linearity Assessment, Section 14.4). Noise values were calculated from ChemStation software for the determination of LOQ and LOD values of p-arsanilic acid, acetarsone, and roxarsone. The chromatographic data was obtained as per conditions described in Table 16.

Analyte	p-arsanilic acid	acetarsone	roxarsone
Noise segment Start (min)	0.52	0.87	1.35
Noise segment End (min)	0.62	0.97	1.55
Slope	2.8×10^6	1.5×10^6	4.2×10^6
Noise (mAU)	0.147	0.348	0.134
	0.159	0.315	0.114
	0.179	0.479	0.180
	0.100	0.315	0.146
	0.174	0.148	0.134
	0.113	0.174	0.119
	0.133	0.475	0.175
	0.179	0.211	0.117
	0.117	0.447	0.142
	0.164	0.126	0.190
AVE	0.146	0.304	0.145
s	0.029	0.135	0.028
LOQ (M)	1.02E-07	8.81E-07	6.57E-08
LOQ (ppm)	0.022	0.24	0.017
LOD (M)	3.38E-08	2.91E-07	2.17E-08
LOD (ppm)	0.007	0.08	0.006

14.6 Range

The linear range for the developed method was established with the data obtained from the linearity study (Section 14.4). The concentration ranges confirmed experimentally are described in Table 23. The minimum concentrations quantified with a working curve are in agreement with the LOQ calculated for each analyte as per Table 22 (Section 14.5). Most importantly, the range determined in this investigation adequately covers the FDA approved dosage for roxarsone (0.03 ppm to 0.05 ppm) and p-arsanilic acid (0.05 ppm to 0.10 ppm).[16] Therefore, the method can be considered as a routine analysis for QA/QC. Sections 15-18 discuss the use of the validated method with more complex matrixes (e.g. dissolved ions, potable water, and poultry litter leachates) thereby expanding the applicability to environmental surveillance.

Table 23: Concentration ranges of p-arsanilic acid, acetarsone, and roxarsone as per data obtained for linearity assessment (Section 14.4).

Analyte	Minimum Experimental Concentration		Maximum Experimental Concentration	
	(M)	(ppm)	(M)	(ppm)
p-ASA	1.28×10^{-7}	0.026	1.26×10^{-4}	27.42
acetarsone	8.89×10^{-7}	0.24	2.29×10^{-4}	63.11
roxarsone	9.75×10^{-8}	0.026	1.01×10^{-4}	26.64

14.7 Accuracy

The method is considered accurate as per ICH Q2 (R1) based on the fact that the operational parameters produced chromatographic data that is specific for each compound with a reproducible response throughout the linear range studied. Table 24 depicts the relative error obtained for quality control (QC) samples injected in various experiments. The recovery data of the QC samples confirm the accuracy of the validated method parameters.

Table 24: Quality control samples obtained for p-arsanilic acid, acetarsone, and roxarsone and corresponding relative errors.

Analyte	$Conc_{QC}$		$Conc_{EXP}$		%RE[(b)]
	M	ppm	M[(a)]	ppm[(a)]	
p-arsanilic acid	3.74×10^{-7}	0.081	$3.64 (\pm 0.62) \times 10^{-7}$	0.079 (±0.013)	-2.8
acetarsone	2.25×10^{-5}	6.19	$2.22 (\pm 0.28) \times 10^{-5}$	6.10 (±0.77)	-1.5
roxarsone	2.99×10^{-7}	0.079	$2.90 (\pm 0.74) \times 10^{-7}$	0.076 (±0.019)	-3.1

(a) Error values corresponding to s_{pooled} with n=24

(b) $\%RE = [(Conc_{EXP} - Conc_{QC}) / Conc_{QC}] \times 100$, were $Conc_{EXP}$ is the QC sample's concentration obtained experimentally and $Conc_{QC}$ is the theoretical concentration.

14.8 System Suitability

The peak parameters of the chromatographic data acquired during the linearity study were evaluated for future system suitability assessments. System suitability assessments are an integral part of HPLC analysis. The results are an excellent indicator for the proper equipment operation, the accuracy of reference standard preparations, and integrity of the analytical results. Tables 25-27 depict the result summary of the peak parameters evaluated at the two validated mobile phase pH levels for roxarsone, p-arsanilic acid, and acetarsone respectively.

Table 25: Roxarsone peak parameters of chromatographic data acquired during the linearity study (Section 14.4).

pH	6.3		6.5	
Parameter	AVE	s_{pooled}	AVE	s_{pooled}
T_f	1.5	0	1.5	0
N	5081	396	8216	888
R [(a)]	6.06	0.46	8.31	0.31
k	2.38	0.19	2.14	0.05

(a) Resolution calculated relative acetarsone (the previous eluting peak).

Table 26: p-Arsanilic acid peak parameters of chromatographic data acquired during the linearity study (Section 14.4).

pH	6.3		6.5	
Parameter	AVE	s_{pooled}	AVE	s_{pooled}
T_f	1.4	0	1.3	0
N	1553	89	1833	101
k	0.35	0.00	0.37	0.01
α [(a)]	3.05	0.03	2.84	0.08

(a) Selectivity was calculated relative acetarsone (the next eluting peak).

Table 27: Acetarsone peak parameters of chromatographic data acquired during the linearity study (Section 14.4).

pH	6.3		6.5	
Parameter	AVE	s_{pooled}	AVE	s_{pooled}
T_f	1.6	0	1.7	0
N	5081	396	2955	206
R [(a)]	4.43	0.07	5.35	0.12
k	1.06	0.01	1.21	0.02
α [(b)]	1.96	0.02	1.71	0.03

(a) Resolution was calculated relative p-arsanilic acid (the previous eluting peak).
(b) Selectivity was calculated relative roxarsone (the next eluting peak).

15 Analysis of organoarsenic drugs (roxarsone, p-arsanilic acid, and acetarsone) in aqueous solutions containing dissolved ions

15.1 Description of Study

Fresh water contains a diverse amount of dissolved ions including nitrogen, chloride, sodium, bicarbonate, and sulfate.[60] However, run-off water from nearby terrain may add to the amount of ionized species. For example, it has been reported that run-off water from soils amended with fertilizer containing roxarsone have contaminated watersheds.[61] The roxarsone was associated to the poultry manure in the matrix. The basic components of poultry manure are nitrogen, phosphorous, and potassium.[62] Consequently, these components have also been found to leach to surface and groundwater.[63] Based on this, the effects of several dissolved ions in aqueous solutions of roxarsone, p-arsanilic acid, and acetarsone was studied. Individual preparations containing one of three organoarsenic species were analyzed using the validated methodology. Table 28 describes the ions and their solution concentrations used to dissolve p-arsanilic acid, acetarsone, and roxarsone. These were analyzed as blank solutions of each individual ion prior to the samples containing the organoarsenic drug. The HPLC system was allowed to equilibrate (at least 15 minutes) prior to starting the analysis. The initial study was performed with roxarsone and afterwards extended to p-arsanilic acid and acetarsone. The sample preparation schemes are described in Appendix 7 and 8. The equipment operational parameters and calibration curves are described for each corresponding compound throughout the discussion of results.

Table 28: Concentration of aqueous solutions containing dissolved ions common in poultry litter and fertilized soils. These were used to dilute roxarsone, p-arsanilic acid, and acetarsone.

Reagent	Ion	Ion Conc.
		M
NaCl	Cl^-	3.60×10^{-4}
$NaNO_3$	NO_3^-	1.40×10^{-4}
Na_2HPO_4	PO_4^{3-}	1.18×10^{-4}
Na_2SO_4	SO_4^{2-}	3.34×10^{-5}
$NaHCO_3$	CO_3^{2-}	1.53×10^{-4}

Table 29: Concentration of p-arsanilic acid, acetarsone, and roxarsone diluted with aqueous solutions containing dissolved ions. Each organoarsenic was individually prepared with each solution described in Table 28.

Appendix Reference	Analyte	Wk. Std. Conc.
		M
7	roxarsone	3.93×10^{-6}
8	p-ASA	3.74×10^{-6}
	acetarsone	2.70×10^{-6}

15.2 Results and Discussion

A system suitability exercise was performed prior to the acquisition of samples diluted in ionic solutions. A six-level working calibration curve constructed rendered a linear response ($R^2 \geq 0.9990$; $F \geq 3950$; p-value $\leq 2.2 \times 10^{-7}$). Each level was injected triplicate. All peaks corresponding to roxarsone demonstrated adequate symmetry ($T_f \leq 1.7$). The chromatographic data was reproducible for Area (%RSD $\leq$ 0.5%) and t_R (%RSD $\leq$ 0.08%) at all levels.

According to the chromatography acquired, roxarsone was not affected by the presence of the dissolved ions described in Table 27. Roxarsone was reproducible for Area (%RSD $\leq$ 0.52%) and t_R (%RSD $\leq$ 0.16%). In addition, roxarsone t_R compares favorably with the working curve chromatographic data. The roxarsone peak parameter values were acceptable. The %RE (7.9% to 9.2%) suggests acceptable roxarsone recovery levels of 90% to 92%. Table 30 contains a summary of the results.

Table 30: Chromatographic data and recovery results for roxarsone diluted with solutions containing dissolved ions. Data was acquired in triplicate with calibration curve #1 (Appendix 3).

Ion	t_R	Area	T_f	N	k	Conc.	%RE
	min	mAU*s				M	
NO_3^-	1.234 (±0.001)	18.4413 (±0.0267)	1.6 (±0.0)	1988 (±7)	2.07 (±0.01)	4.29 (±0.38) × 10^{-6}	9.2
CO_3^{2-}	1.232 (±0.001)	18.2542 (±0.0086)	1.6 (±0.0)	1977 (±10)	2.07 (±0.01)	4.25 (±0.38) × 10^{-6}	8.1
PO_4^{3-}	1.229 (±0.001)	18.2224 (±0.0063)	1.4 (±0.1)	2234 (±21)	2.06 (±0.01)	4.24 (±0.38) × 10^{-6}	7.9
SO_4^{2-}	1.233 (±0.001)	18.2523 (±0.0138)	1.6 (±0.1)	1998 (±9)	2.07 (±0.00)	4.25 (±0.38) × 10^{-6}	8.1
Cl^-	1.233 (±0.001)	18.2092 (±0.0056)	1.6 (±0.0)	1962 (±22)	2.07 (±0.00)	4.24 (±0.38) × 10^{-6}	7.9

All solutions containing the dissolved ions eluted free of additional artifacts different from DIW with the exception of NO_3^-. Two artifacts eluted in the blank and sample chromatograms related to NO_3^-. The first artifact eluted around 0.542 (±0.001) minutes and the second at 0.754 (±0.001) minutes. The main problem with the first artifact is the elution in the t_R range of p-arsanilic acid (t_R = 0.540 (± 0.005 minutes)). It was later confirmed (during a second study set) that this artifact was most-likely due to a contamination uptake during the sample preparation exercises. However, the second unknown peak was still evident.

The second artifact was observed to be a well formed peak with an area of 12.6709 (±0.873) and k = 0.89. Based on this information, a co-elution between the second NO_3^- unknown and acetarsone ($1.0 < k < 1.2$) is possible. A selectivity factor (α) of 2.36 was calculated for roxarsone relative the second NO_3^- unknown species. This suggests that the second unknown species does not represent an elution problem for roxarsone. Representative chromatography is depicted in figure 37.

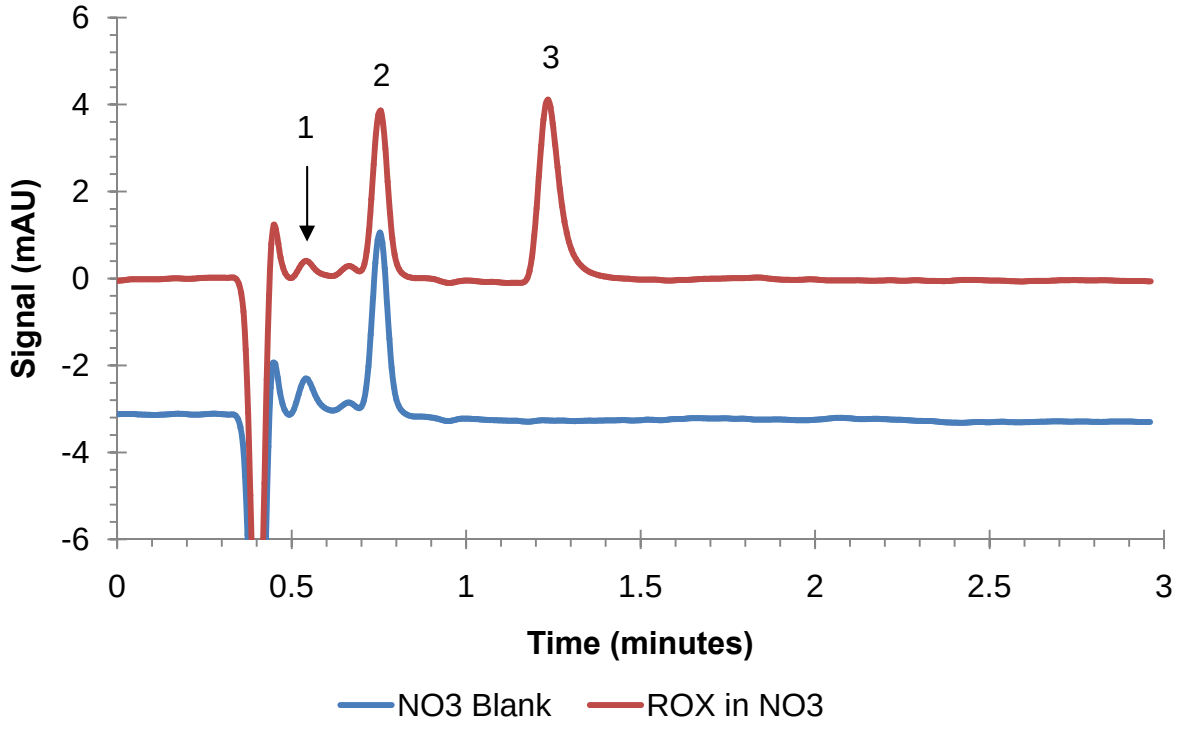

Figure 37: Chromatogram of roxarsone (4.29×10^{-6} M) aqueous solution. The diluent used was $NaNO_3$ (1.40×10^{-4} M). (1) Unknown artifact (attributed to contamination uptake after second study), t_R = 0.541 min.; (2) NO_3^- unknown, t_R = 0.750 min.; (3) roxarsone, t_R = 1.243 min. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.30 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

A new $NaNO_3$ aqueous solution containing 2.99×10^{-6} M roxarsone was analyzed in order to confirm the presence of the unknown peak that may co-elute with acetarsone (Appendix 8). The data demonstrates that the presence of the NO_3^- ion does not have any effect on the elution and response of roxarsone. Table 30 depicts the roxarsone peak parameter values. The low %RE indicates a roxarsone recovery of about 97%.

Table 31: Chromatographic data and recovery results for roxarsone diluted with solutions containing dissolved ions. Data was acquired in triplicate with calibration curve #3 (Appendix 5). The pH of the mobile phase was 6.50.

Ion	t_R	Area	T_f	N	k	Conc.	%RE
	min	mAU*s				M	
NO_3^-	1.425 (± 0.000)	14.9351 (± 0.0517)	1.4 (±0.0)	7581 (±13)	2.54 (±0.00)	3.07 (±1.1) × 10^{-6}	-2.6

The first unknown peak eluting at t_R = 0.542 (±0.001) minutes during the first study was not present in the chromatography obtained in this second study set for the roxarsone aqueous solution containing dissolved NO_3^- ions. This suggests that the most probable cause for this artifact may be attributed to a contamination.

The presence of the second NO_3^- related unknown peak was confirmed. It had a t_R = 0.826 minutes, with k = 1.07, and an area of 13.8979 (±1.0287) mAU*s. The selectivity of α = 2.36 indicates that co-elution problems are not probable between roxarsone and the NO_3^- unknown. However, it still represents a possible co-elution with acetarsone.

The effects of dissolved ions on p-arsanilic acid and acetarsone were evaluated. The aqueous solutions described in Table 29 (Section 15.1) for p-arsanilic acid and acetarsone eluted free of additional artifacts different from DIW (with the exception of $NaNO_3$). The t_R for the organoarsenic drugs was reproducible (%RSD ≤ 0.1%) despite the ions present in the solution. The %RE suggest recovery levels over 94% for p-arsanilic acid and acetarsone (with the exception of the $NaNO_3$ solution). In the presence of NO_3^- ions, the recovery levels for acetarsone decrease to less than 90%.

This is attributed to the poor integration conditions due to the co-elution with the unknown peak. The peak shape of p-arsanilic acid was unaffected as opposed to acetarsone which was altered due to the co-elution with the NO_3^- unknown. The selectivity of acetarsone (α = 1.16) relative the NO_3^- unknown is typical of a co-eluting peak. Figure 38 contains sample chromatograms of each organoarsenic drug understudy diluted in 1.40×10^{-4} M $NaNO_3$. The roxarsone peak in Figure 38 displays a t_R = 1.43 minutes. The mobile phase pH was 6.50 for these chromatograms.

Table 32: Chromatographic data and recovery results for p-arsanilic acid diluted with solutions containing dissolved ions. Data was acquired in triplicate with calibration curve #3 (Appendix 5).

Ion	t_R	Area	T_f	N	k	Conc.	%RE
	min	mAU*s				M	
NO_3^-	0.559 (±0.000)	11.4733 (±0.0006)	1.5 (±0.1)	1729 (±4)	0.39 (±0.00)	$3.51(\pm1.2) \times 10^{-6}$	-6.0
CO_3^{2-}	0.561 (±0.000)	11.7565 (±0.0011)	1.4 (±0.1)	1839 (±4)	0.40 (±0.00)	$3.61 (\pm1.2) \times 10^{-6}$	-3.3
PO_4^{3-}	0.563 (±0.000)	11.7644 (±0.0050)	1.5 (±0.0)	1854 (±0)	0.40 (±0.00)	$3.61 (\pm1.2) \times 10^{-6}$	-3.3
SO_4^{2-}	0.560 (±0.000)	11.4595 (±0.0058)	1.3 (±0.1)	1823 (±4)	0.39 (±0.00)	$3.51 (\pm1.2) \times 10^{-6}$	-6.1
Cl^-	0.560 (±0.000)	11.5445 (±0.0277)	1.3 (±0.0)	1823 (±4)	0.39 (±0.00)	$3.54 (\pm1.2) \times 10^{-6}$	-5.3

Table 33: Chromatographic data and recovery results for acetarsone diluted with solutions containing dissolved ions. Data was acquired in triplicate with calibration curve #3 (Appendix 5).

Ion	t_R	Area	T_f	N	k	Conc.	%RE
	min	mAU*s				M	
NO_3^-	0.894 (±0.000)	3.4198 (±0.0110)	(a)		1.22 (±0.00)	2.41(±1.00) × 10^{-6}	-10.5
CO_3^{2-}	0.899 (±0.000)	3.9084 (±0.0055)	1.4 (±0.0)	3592 (±12)	1.23 (±0.00)	2.73 (±1.00) × 10^{-6}	1.3
PO_4^{3-}	0.897 (±0.001)	3.8601 (±0.0061)	1.4 (±0.0)	3631 (±50)	1.23 (±0.00)	2.70 (±1.00) × 10^{-6}	0.1
SO_4^{2-}	0.897 (±0.000)	3.6515 (±0.0055)	1.5 (±0.1)	3758 (±71)	1.23 (±0.00)	2.57 (±1.00) × 10^{-6}	-4.9
Cl^-	0.897 (±0.000)	3.7720 (±0.0050)	1.4 (±0.0)	3541 (±12)	1.23 (±0.00)	2.64 (±1.00) × 10^{-6}	-2.0

(a) These values were not calculated because of inadequate integration conditions. Refer to discussion of co-elution between acetarsone and the NO_3^- unknown.

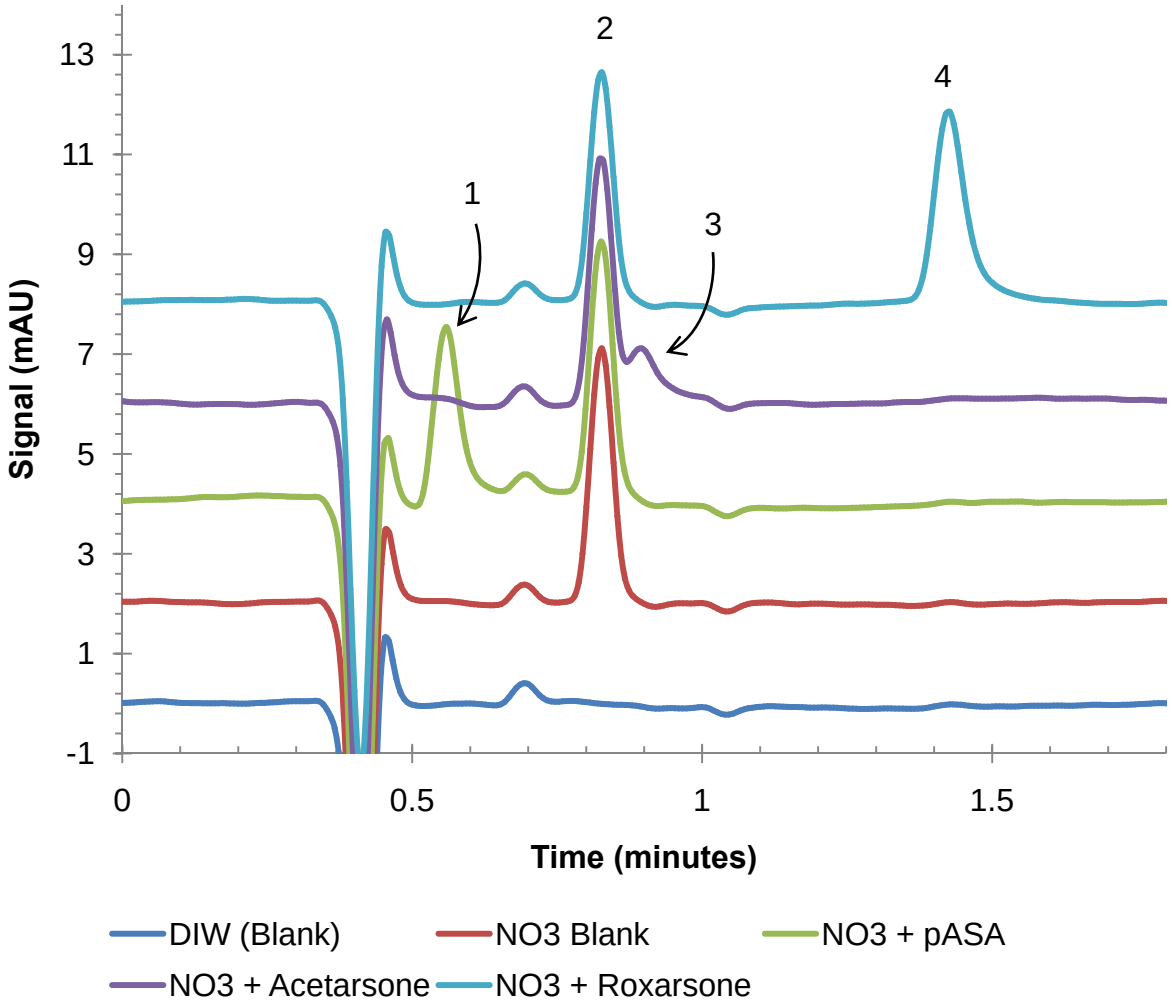

Figure 38: Separation of organoarsenic drugs diluted in aqueous solutions containing dissolved NO_3^- ions. (1) p-arsanilic acid, t_R = 0.56 min. (2) NO_3^- unknown, t_R = 0.82 min. (3) acetarsone, t_R = 0.90 min. (4) roxarsone, t_R = 1.43 min. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

A *spectral purity display* was obtained for the NO_3^- unknown peak (Figure 39). There were several instances in which the spectral similarity plot intercepted the threshold curve. The purity factor obtained was 988.438, which is slightly below the 990 lower limits. Furthermore, the spectra used by the ChemStation® software exceeded the calculated threshold limit. These results are indicators of an impure peak.

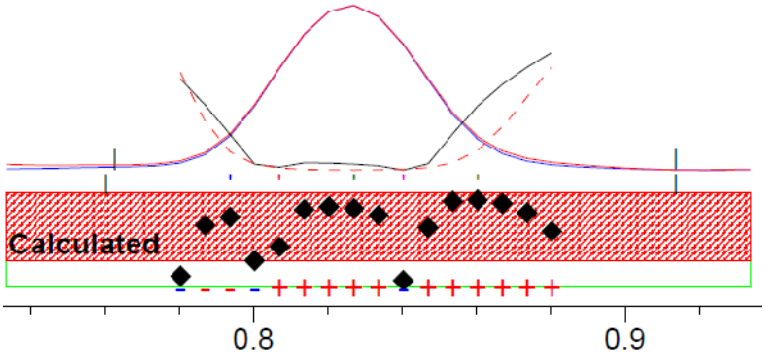

Figure 39: Spectral purity display for unknown peak (t_R = 0.82 minutes) in aqueous solution with dissolved NO_3^- ions.

3D isoplots were obtained of the NO_3^- blank solution to further evaluate peak impurity through its spectral profile of the full chromatographic acquisition (Figure 40). The maximum wavelength for the NO_3^- unknown peak exhibited is in the range of 220 nm to 230 nm. No additional peaks (besides those evident in the DIW and the NO_3^- unknown) were observed. This information suggests that the unknown peak could be composed of more than one species which have co-eluted under the present chromatographic conditions.

(a)

(b)

Figure 40: 3D Isoplot of the blank solution containing dissolved NO_3^- ions. The chromatogram was viewed from (a) the beginning and (b) the end. No additional peaks were detected other than those found in DIW and the NO_3^- unknown.

The presence of the NO_3^- unknown does not represent an elution problem to the main analyte (roxarsone). However, it is a species (or a possible group of species as per the aforementioned purity assessment) which seems to originate in the $NaNO_3$ reagent. Previous studies have used UV spectroscopy to acquire spectra of study $NaNO_3$ aqueous solutions.[64] Strong bandwidths have been observed at 200 nm and much weaker ones at 300 nm. An HPLC method was reported for the analysis of Nitrates and Nitrites in seawater using quaternary ammonium salts, a C_{18} reverse-phase column, and UV detection (λ = 225 nm).[65] Based on this, it is highly probable that the IP-HPLC method developed for the analysis of organoarsenics has the capacity of detecting NO_3^- ions in aqueous solutions.

16 Analysis of organoarsenic drugs diluted with potable water

16.1 Description of Study

The areas in a poultry farm designated for feeding and nesting are mainly cleaned with potable water (PW). The run-off from the PW may carry water-soluble components (such as roxarsone) to other areas of the poultry farm thereby resulting in a cross-contamination. This event may not only result in intoxication of other animals or people, but in substantial disciplinary actions on behalf of the regulatory agencies. The IP-HPLC method's capacity to analyze potable water containing roxarsone, p-arsanilic acid, and acetarsone was tested.

Reference standards containing all three organoarsenic drugs studied were prepared with potable water in order to evaluate matrix interferences. The potable water was obtained from the laboratory sink. Known amounts of roxarsone, p-arsanilic acid, and acetarsone were diluted in PW (Table 34). These were filtered through a 0.45 micron syringe filter prior to analysis. The elution data was acquired with a mobile phase pH of 6.50 with the validated equipment operational parameters. Refer to Appendix 9 for information referent sample preparation. The calibration curve and the equipment operational parameters are located in Appendix 5.

Table 34: Concentrations of roxarsone, p-arsanilic acid, and acetarsone diluted in potable water (PW).

Analyte	Concentration
p-arsanilic acid	3.74×10^{-6} M
acetarsone	2.70×10^{-6} M
roxarsone	2.99×10^{-6} M

16.2 Results and Discussion

A system suitability exercise was performed prior to the acquisition of roxarsone samples diluted in PW. A six-level working calibration curve constructed rendered a linear response ($R^2 \geq 0.9990$; $F \geq 3950.89$; p-value $\leq 2.2 \times 10^{-7}$). Each level was injected triplicate. All peaks corresponding to roxarsone demonstrated adequate symmetry ($T_f \leq 1.4$). The chromatographic data was reproducible for area (%RSD $\leq$ 0.3%) and t_R (%RSD $\leq$ 0.07%) at all levels.

According to the chromatography acquired, roxarsone, p-arsanilic acid, and acetarsone were not affected by the potable water (Table 34). All three organoarsenic drugs exhibited reproducible areas (%RSD $\leq$ 0.8%) and t_R (%RSD $\leq$ 0.1%). The peak parameter values were consistent with studies described in previous sections. The low %RE (-2.6% to 2.7%) suggests roxarsone recovery levels of 97%.

Table 35: Chromatographic data and recovery results for organoarsenic drugs diluted in potable water. Data was acquired in triplicate with calibration curve #3 (Appendix 5).

Analyte	t_R	Area	T_f	N	k	Conc.	%RE
	min	mAU*s				M	
p-arsanilic acid	0.565 (± 0.001)	11.8341 (±0.0896)	1.5 (±0.0)	1818 (±14)	0.40 (±0.00)	3.64 (±1.2) × 10^{-6}	-2.6
acetarsone	0.899 (± 0.001)	3.8030 (±0.0108)	1.4 (±0.1)	3599 (±23)	1.23 (±0.00)	2.67 (±0.98) × 10^{-6}	-1.3
roxarsone	1.420 (± 0.001)	14.9549 (±0.0189)	1.5 (±0.0)	7388 (±52)	2.52 (±0.00)	3.08(±1.1) × 10^{-6}	2.7

The DIW and the PW portrayed identical chromatographic profiles under the validated method parameters. Figure 41 portrays an overlay of DIW, PW, and the organoarsenic drugs in this study. No artifacts that could interfere with the organoarsenic drugs understudy were evident.

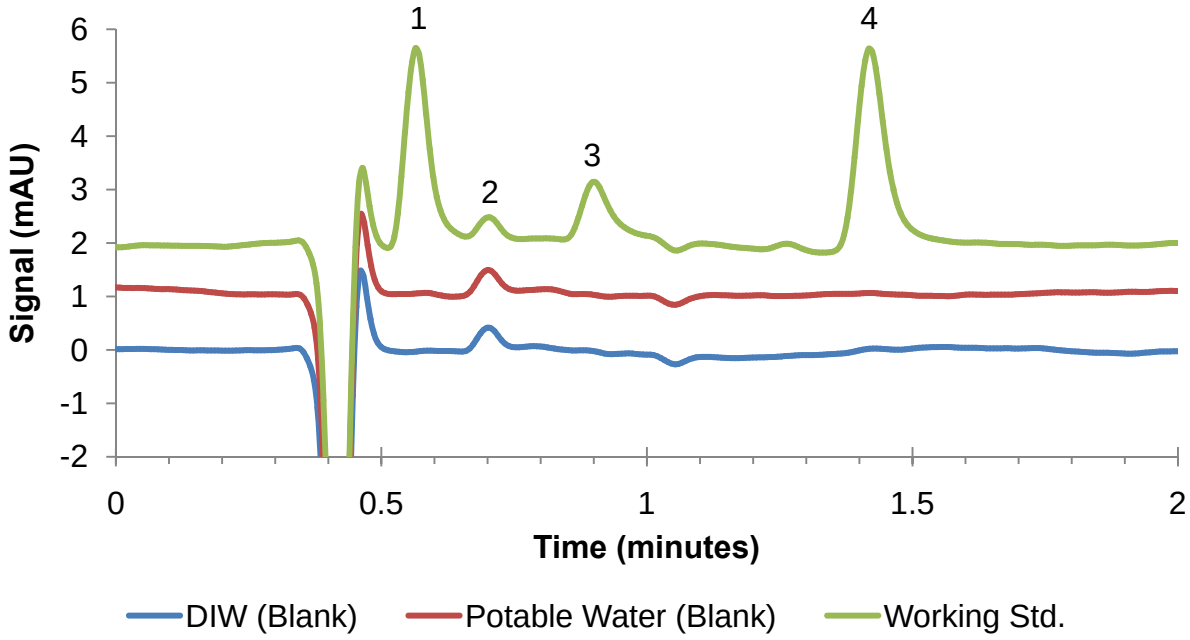

Figure 41: Chromatography of organoarsenic drugs diluted in potable water. (1) p-arsanilic acid, t_R = 0.56 min. (2) IpA-Bkg, t_R = 0.69 min. (3) acetarsone, t_R = 0.89 min. (4) roxarsone, t_R = 1.43 min. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

17 Surveillance of roxarsone degradation in poultry litter leachates.

17.1 Description of Study

Previous investigations indicated that roxarsone is excreted unchanged by poultry.[1, 61] Furthermore, these investigations concluded that the degradation process of roxarsone is initiated once this organoarsenic compound is exposed to poultry litter. A study was performed to assess the validated method's capacity to survey the concentration of roxarsone in the poultry litter. The poultry litter was obtained from a local non-commercial farmer.

The effects of poultry litter on roxarsone were evaluated during a 96 hour period. The sample preparation consisted of two phases. During Phase I, a portion of dried poultry litter was spiked with a known concentration of roxarsone. The amount of water added was maintained at a minimum. The litter was allowed to stand for 30 minutes after addition of roxarsone. Phase II consisted in diluting the amended poultry litter leachates with a substantial amount of water. After an additional 30 minutes of stirring, a portion was filtered through a 0.45 micron syringe filter and analyzed. A litter control sample was prepared in the same manner with the exception that roxarsone was not added. A reference standard control was also prepared in the same manner with the exception that poultry litter was not added. All samples were analyzed in triplicate. Refer to Appendix 10 for the sample preparation schemes and equipment operational parameters.

17.2 Result and Discussion

A system suitability exercise was performed prior to the chromatographic acquisition of poultry littler leachate samples amended with roxarsone at each sampling interval for a total of three (3) calibration curves. Each working curve consisted of a six-level calibration which rendered a linear response ($R^2 \geq 0.9996$; $F \geq 3950$; p-value $\leq 3.8 \times 10^{-7}$). Each level was injected triplicate. All peaks corresponding to roxarsone demonstrated adequate symmetry ($T_f \leq 1.6$). The chromatographic data was reproducible for Area (%RSD $\leq$ 0.8%) and t_R (%RSD $\leq$ 0.07%) at all levels.

A degradation factor was calculated for roxarsone content in samples (19). This formula was used to evaluate the degradation of roxarsone relative the initial concentration.

$$\text{Degradation Factor} = \frac{C}{C_0} \qquad (19)$$

Were C = Experimental concentration, M

C_0 = Theoretical concentration, M

The chromatographic data demonstrated a stable response of the roxarsone control sample throughout the 96 hour surveillance period (Table 36). An unknown peak (identified as unknown 1A) was observed in the poultry litter control sample. However, its elution does not appear to interfere with roxarsone. This will be discussed further on. There weren't any evident peaks in the poultry litter control sample that could interfere with roxarsone.

Table 36: Degradation study results for control samples (roxarsone and poultry litter leachate).

Description	roxarsone control		litter control	
	Conc.	C / C_0 [(a)]	Conc.	C / C_0
Hour	M		M	
1	$3.03 (\pm 0.42) \times 10^{-6}$	0.95	0	0
48	$3.00 (\pm 0.42) \times 10^{-6}$	0.94	0	0
96	$3.07 (\pm 0.42) \times 10^{-6}$	0.97	0	0

(a) $C_0 = 3.17 \times 10^{-6}$ M

The chromatographic data demonstrated a decrease in the roxarsone concentration in the amended poultry litter leachate (Table 37). Roxarsone decreased to levels of almost 50% in 48 hours. The concentration continued decreasing to about 10% after 96 hours from the initial preparation. This behavior was evident in all three sample preparations. This is in agreement with reported findings of previous investigations related to the fate of roxarsone in poultry litter.[1, 21, 61] A plot of C/C_0 vs. time is displayed in Figure 42. Representative chromatograms corresponding to intervals of 1 hour and 48 hours after initial preparation are provided (Figures 43-45). Refer to Appendix 10 for chromatographic data.

Table 37: Degradation study results for poultry litter leachate samples amended with roxarsone (3.17×10^{-6} M). Refer to Appendix 10 for chromatographic data.

Description	roxarsone + litter (sample 1)		roxarsone + litter (sample 2)		roxarsone + litter (Sample 3)	
	Conc.	C/C_0	Conc.	C/C_0	Conc.	C/C_0
Hour	M		M		M	
1	2.82×10^{-6}	0.89	2.82×10^{-6}	0.89	2.82×10^{-6}	0.89
48	1.64×10^{-6}	0.52	1.96×10^{-6}	0.62	2.11×10^{-6}	0.66
96	3.53×10^{-7}	0.11	3.16×10^{-7}	0.10	4.46×10^{-7}	0.14

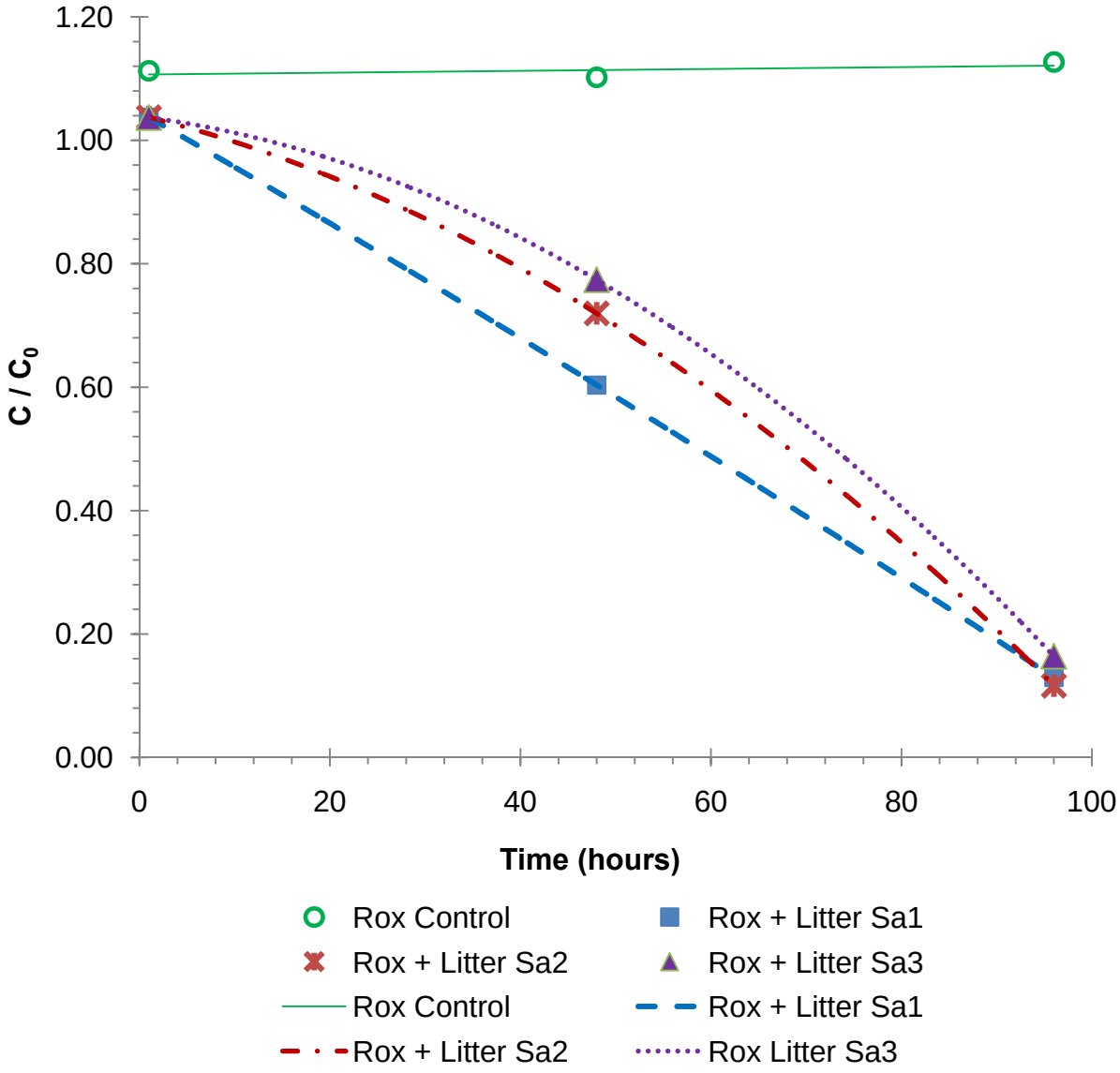

Figure 42: Plot of C/C_0 vs. Time (hours) for the degradation study of poultry litter leachate amended with roxarsone (3.17×10^{-6} M).

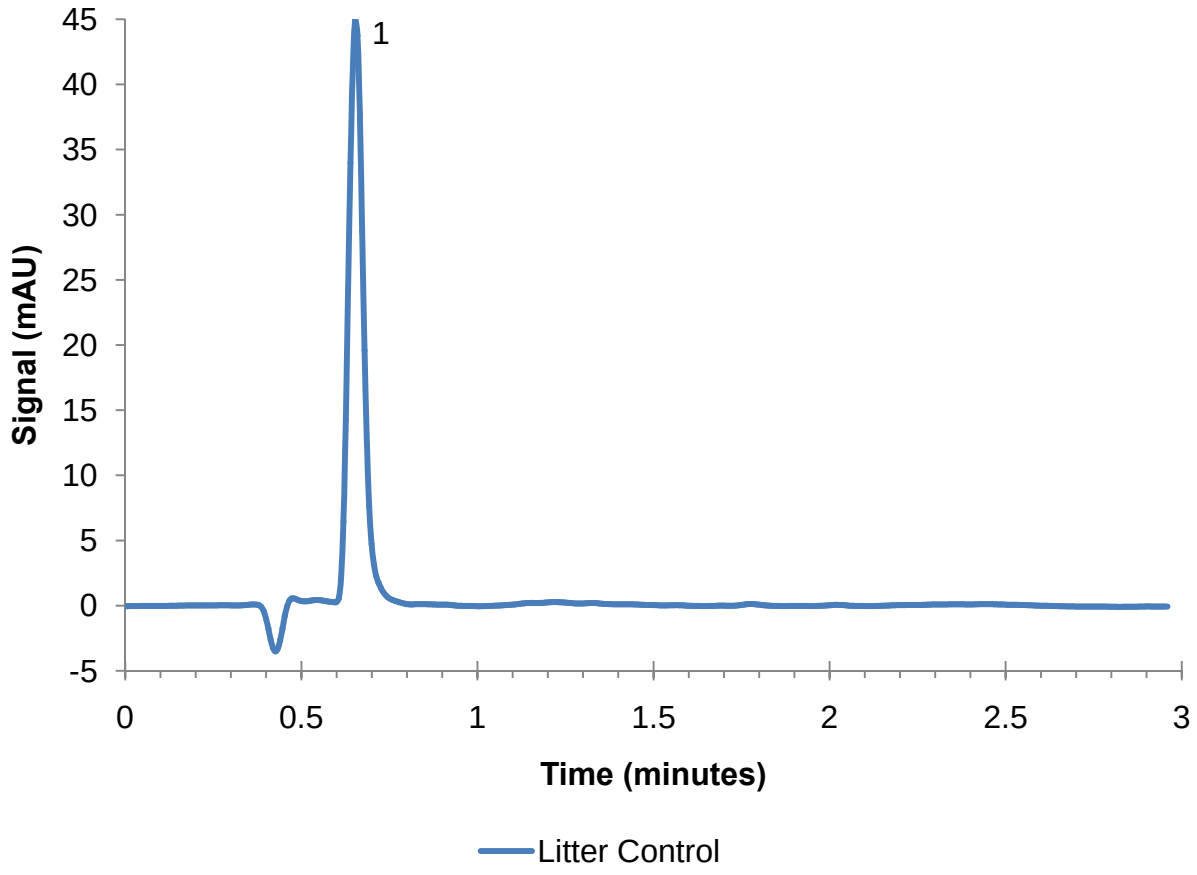

Figure 43: Chromatography of poultry litter leachate control sample acquired 1 hour after initial preparation for the degradation study. (1) unknown 1A, t_R = 0.65 min. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 x 50 mm, 1.8 μm packing.

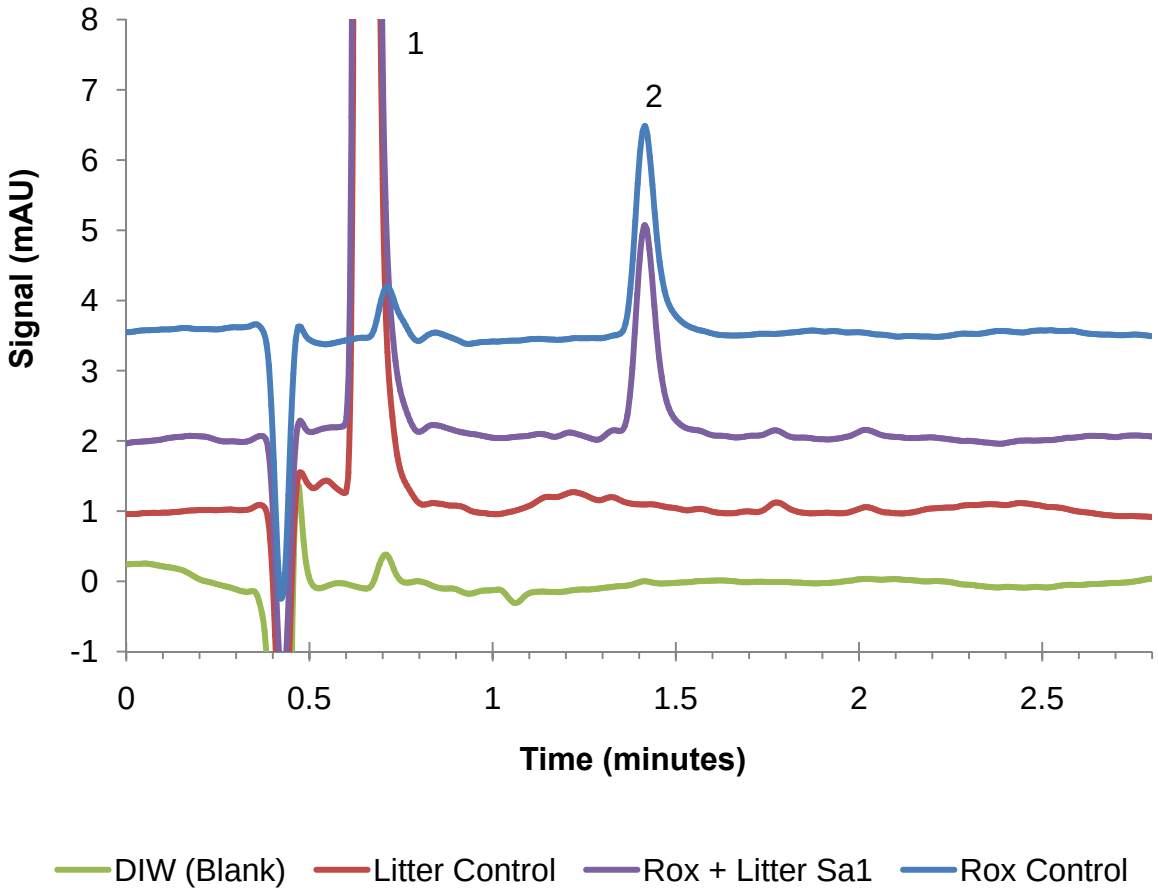

Figure 44: Chromatography of poultry litter leachate amended with roxarsone (3.17×10^{-6} M) 1 hour after initial preparation. (1) unknown 1A, t_R = 0.65 min. (2) roxarsone, t_R = 1.42 min. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50mm, 1.8 μm packing.

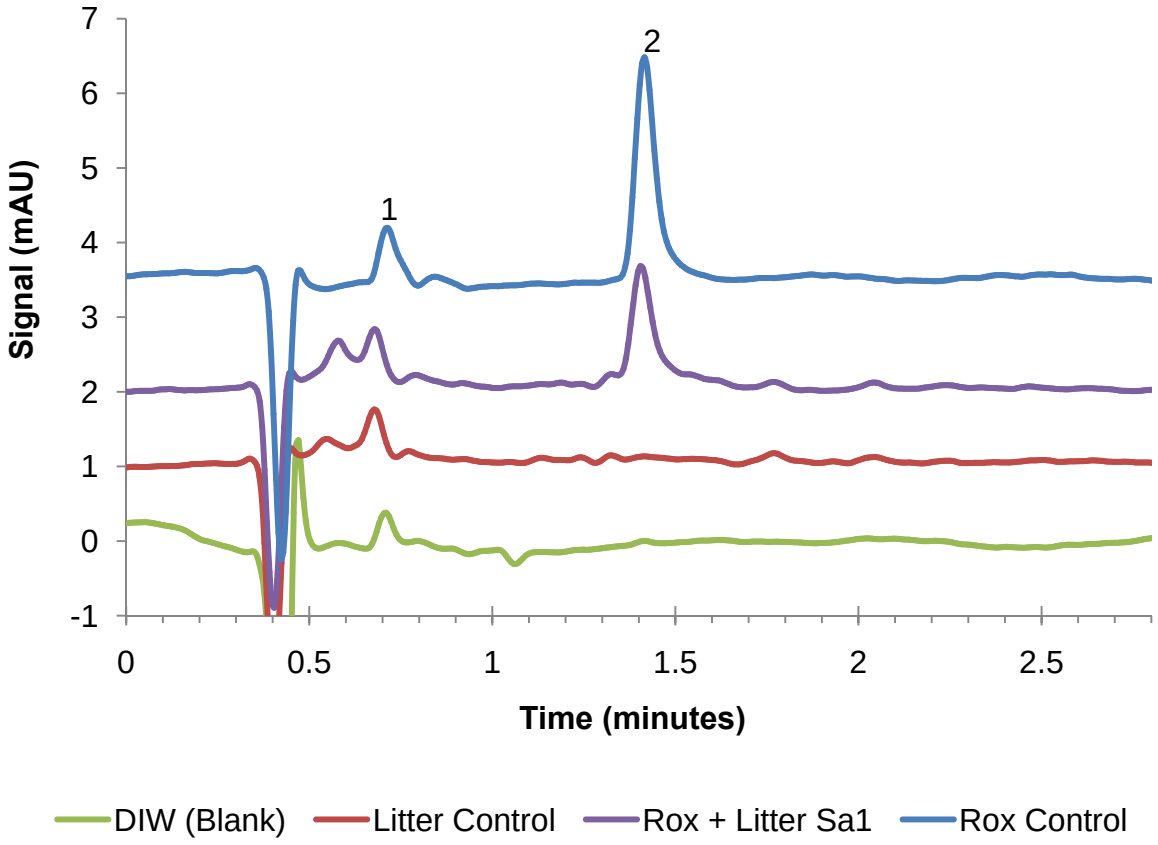

Figure 45: Elution of poultry litter leachate amended with roxarsone, (3.17×10^{-6} M) 48 hours after initial preparation. (1) unknown 1A, t_R = 0.65 min. (2) roxarsone, t_R = 1.42 min. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50 mm, 1.8 μm packing.

An unknown peak eluting at about 0.65 minutes was evident in the poultry litter control and roxarsone-amended samples. For identification purposes, this unknown was named “unknown 1A.” The peak corresponding to unknown 1A elutes about 0.10 minutes later than p-arsanilic acid (t_R = 0.56 minutes). The k values for p-arsanilic acid (k = 0.36) and acetarsone (k = 1.01) differ from unknown 1A (k = 0.61). In addition, the relative retention time (r_R) of p-arsanilic acid (the peak with the closest t_R value to unknown 1A) referent roxarsone is given by 0.56 min / 1.43 min = 0.39. The r_R of unknown 1A relative to roxarsone is given by 0.65 min/1.43 min = 0.45. Therefore, it is not probable that unknown 1A corresponds to p-arsanilic acid.

A significant decrease was observed for unknown 1A in the samples containing poultry litter leachate. About 0.4% was left after 48 hours of the initial preparation. The chromatography corresponding to the roxarsone-amended poultry litter also contained unknown 1A in the samples analyzed after 1 hour of preparation. However, it decreased by 99% on all roxarsone-amended litter samples after 48 hours of preparation. Most importantly, the peak corresponding to unknown 1A did not represent a problem with the identification and quantitation of roxarsone in the amended poultry samples.

Unknown 1A may correspond to a species containing nitrogen. Poultry litter is known to contains large amounts of nitrogen and phosphorous.[66] It has been reported that the nitrogen content of poultry litter is generally lost in the form of ammonia when the poultry manure is moist.[63] Ammonia is a water-soluble, nitrogen-based compound present in the poultry litter with a volatile nature. For this reason, poultry farms place a bed of absorbent material where the broilers excrete manure.

The poultry litter used in this study was collected freshly and stored in a sealed, amber glass bottle prior to sample preparation. However, the prepared samples of poultry litter leachate were not stored in an air-tight container, thereby being exposed to an aerobic environment. It is highly probable that nitrogen-based compounds found in the poultry litter may have been lost as ammonia (due to its volatile nature). This event further confirms the possibility of detecting nitrogen-based compounds under the validated chromatographic conditions as discussed in Section 15 for aqueous solutions of $NaNO_3$.

18 Surveillance of roxarsone degradation in TiO_2 suspensions

18.1 Description of Study

The toxic effects of the organoarsenic drug roxarsone and its degradation products are introduced to the natural bodies of water through soil amended with contaminated litter.[67, 68] This represents an important environmental problem since these agents can be readily absorbed by plants thus becoming a potential risk to public safety.[21] Remediation procedures with titanium dioxide (TiO_2) have been investigated for methylated arsenic compounds, As^V, and As^{III}.[69, 70] TiO_2 has been used extensively due to its photocatalytic and adsorbent properties.[71, 72] The use of the developed IP-HPLC method for surveillance a TiO_2 remediation process for roxarsone was studied.

A simple procedure was adapted for the degradation of roxarsone in water.[71] A 10 mL sample of 3.99×10^{-5} M roxarsone was remediated with a TiO_2 suspension of 1 mg/mL. The sample was prepared in duplicate. One sample was exposed to UV radiation (λ = 321 nm) for one hour. The second sample remained under normal lighting conditions in a laboratory hood. Each was accompanied by a roxarsone control sample of the same concentration. A portion of the aliquot was removed from the reaction vessel and centrifuged for about 2 minutes. The supernatant was filtered through a 0.45 micron syringe filter and transferred to a suitable HPLC vial. Refer to Appendix 11 for the detailed sample preparation scheme and Appendix 5 for equipment operational parameters.

18.2 Results and Discussion

A system suitability exercise was performed prior to the acquisition of roxarsone samples diluted in PW. A six-level working calibration curve constructed rendered a linear response ($R^2 \geq 0.9990$; $F \geq 3950.89$; p-value $\leq 2.2 \times 10^{-7}$). Each level was injected triplicate. All peaks corresponding to roxarsone demonstrated adequate symmetry ($T_f \leq 1.4$). The chromatographic data was reproducible for area (%RSD $\leq$ 0.3%) and t_R (%RSD $\leq$ 0.07%) at all levels.

The peak corresponding to roxarsone depicted consistency in shape and elution parameters in all the samples were it was detected (Table 38). The lighting conditions and exposure time did not affect roxarsone control samples being that no significant differences were evident in between concentrations determined by the analysis (Table 38). The low %RE results obtained for the control samples suggest a roxarsone recovery of about 96%.

Roxarsone's concentration in the sample containing the 1 mg/mL TiO_2 suspension decreased about 60% after one hour of exposition to normal laboratory lighting conditions (Table 39). However, roxarsone was not detected in the sample containing the TiO_2 after exposition to UV radiation for an hour.

Table 38: Chromatographic peak parameters for 10 mL of roxarsone (3.99×10^{-5} M) with TiO_2 suspension (1 mg/mL).

Sample	t_R	Area	T_f	N	k
	min	mAU*s			
Rox. Control (Non-UV, 0Hr)	1.417	163.5791	1.5	7301	2.50
Rox. Control (Non-UV, 1Hr)	1.417	163.6796	1.5	7312	2.50
Rox. Control (UV, 1Hr)	1.419	163.6899	1.5	7326	2.50
Rox. + TiO_2 (Non-UV, 1Hr)	1.420	69.4644	1.5	7321	2.51
Rox. + TiO_2 (UV, 1Hr)	ND	ND	ND	ND	ND

Table 39: Photo-degradation study results for a 10 mL sample of roxarsone (3.99×10^{-5} M) with TiO_2 suspension (1 mg/mL).

Sample	roxarsone conc.	%RE
	M	
roxarsone control (Non-UV, 0Hr)	$3.83 (\pm 0.10) \times 10^{-5}$	-3.9
roxarsone control (Non-UV, 1Hr)	$3.84 (\pm 0.10) \times 10^{-5}$	-3.9
roxarsone control (UV, 1Hr)	$3.84 (\pm 0.10) \times 10^{-5}$	-3.9
roxarsone + TiO_2 (Non-UV, 1Hr)	$1.60 (\pm 0.10) \times 10^{-5}$	-59.9
roxarsone + TiO_2 (UV, 1Hr)	ND	NA

The chromatography obtained for this particular study demonstrated the capacity of the validated method parameters for identification and quantification of roxarsone during a remediation process involving TiO_2. All the chromatographic profiles of the control samples compared favorably between them. No additional peaks were observed in the chromatography (other than those found in the DIW blank) for all sample chromatograms. Figures 46 and 47 display the chromatography for both lighting conditions (Non-UV and UV respectively).

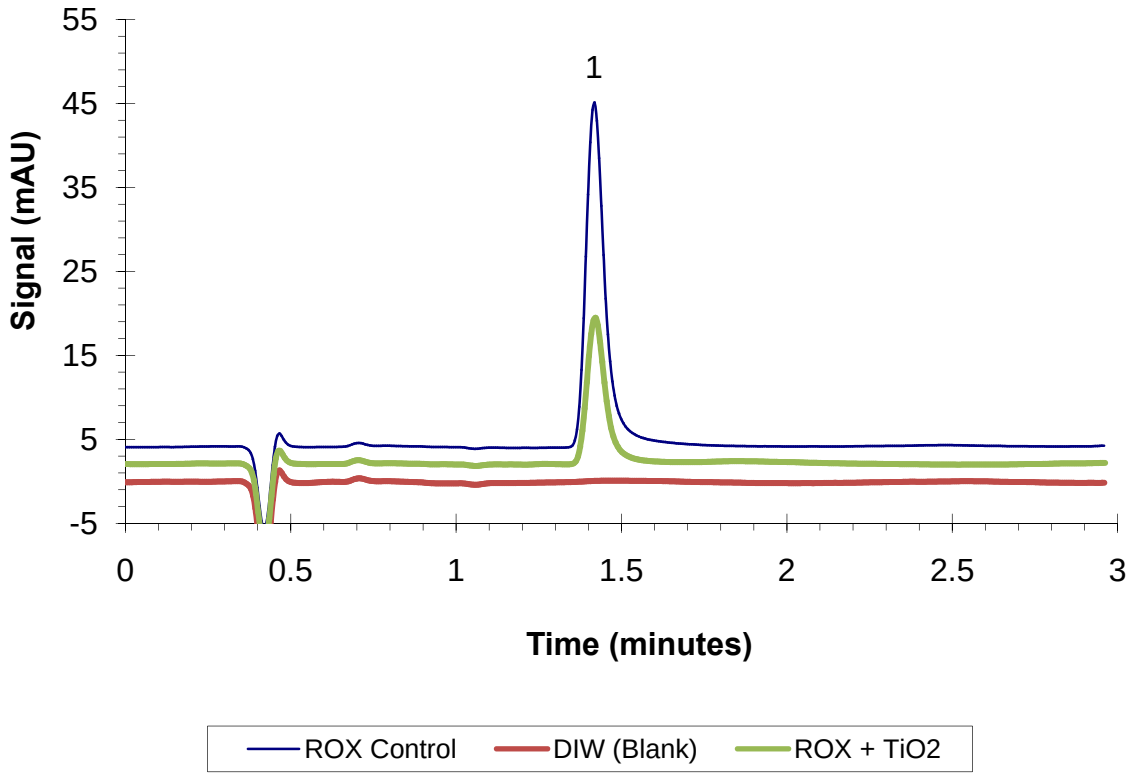

Figure 46: Elution data of roxarsone (3.99×10^{-5} M) in a TiO_2 suspension (1 mg/mL) exposed for 1 hour to normal laboratory lighting conditions. (1) roxarsone, t_R = 1.42 minutes. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 x 50 mm, 1.8 μm packing.

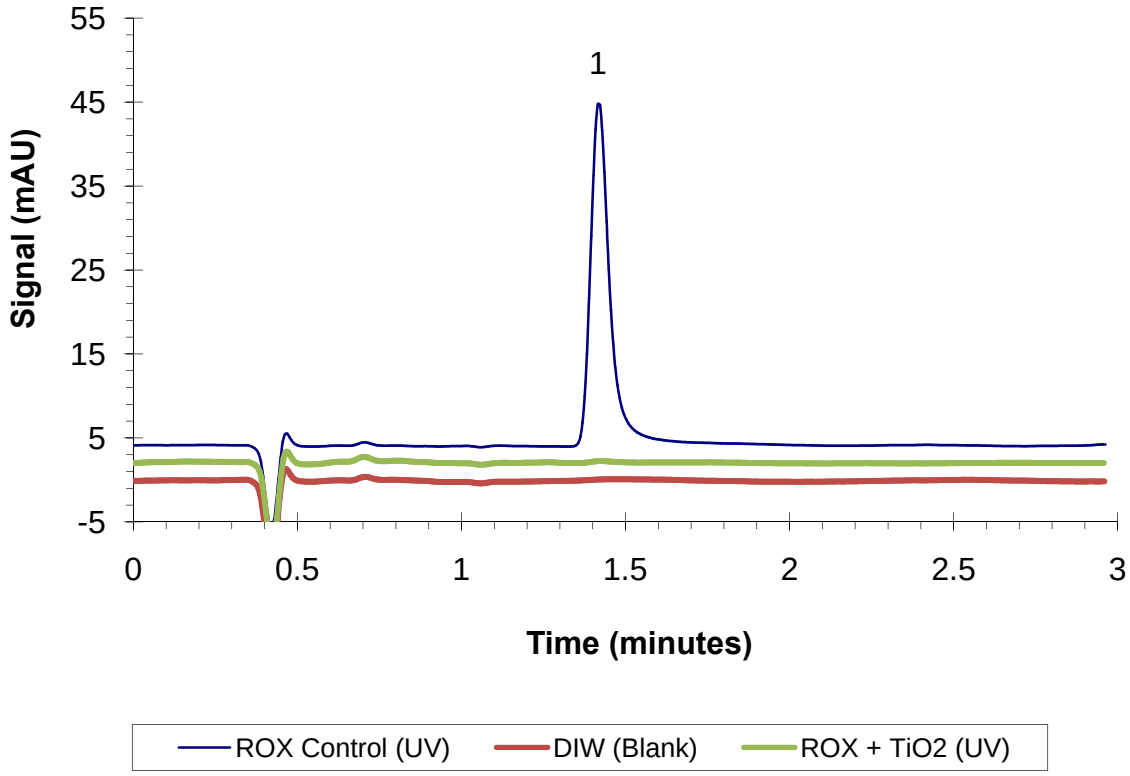

Figure 47: Elution data of roxarsone (3.99×10^{-5} M) in a TiO_2 suspension (1 mg/mL) exposed for 1 hour to a UV lamp (λ = 312 nm). (1) roxarsone, t_R = 1.42 minutes. Mobile phase: (85:15) 18.3 mM MTBA-OH with 1% TEA, pH = 6.50 / methanol. Flow: 1.4 mL/min. Column temperature: 40ºC. Detector wavelength: 245 nm. Injection volume: 5 μL. Column: Zorbax Eclipse Plus, 4.6 × 50mm, 1.8 μm packing.

The decrease of Roxarsone's concentration in each sample could be visually appreciated. The pale-yellow color was evident in all samples in which Roxarsone was detected by the IP-HPLC method. The color was still visible despite the turbidity of the samples containing the 1 mg/mL TiO_2 suspension. The appearance of the solutions after one hour of the initial preparation is portrayed in Figure 48.

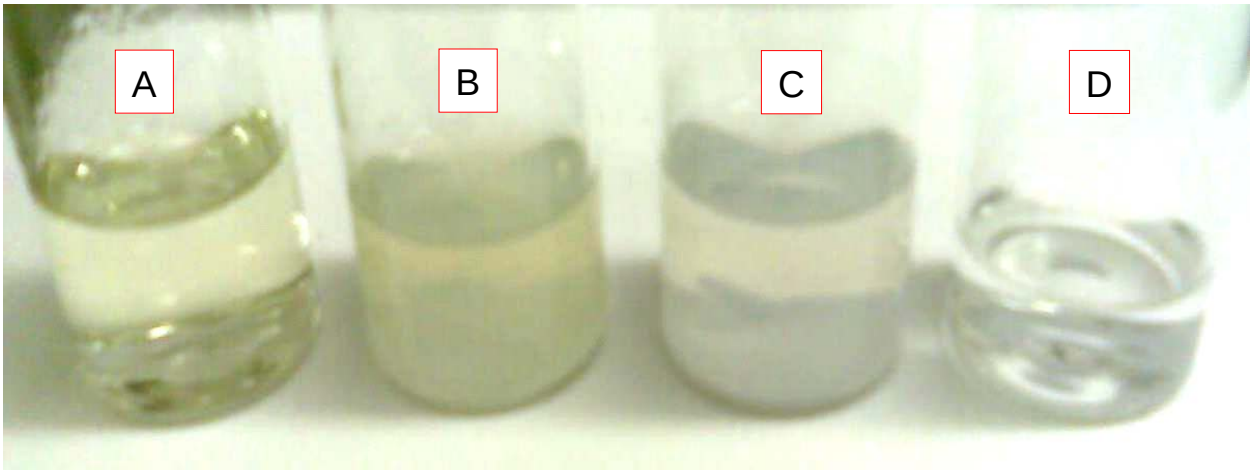

Figure 48: Sample aqueous solutions monitored during a photo-degradation study using the validated IP-HPLC method. The picture was taken one hour after the initial preparation. The solution description / roxarsone concentrations (as determined by the IP-HPLC method) are as follows: (A) roxarsone control sample (UV) / 3.84×10^{-5} M; (B) roxarsone in a 1 mg/mL TiO_2 suspension (Non-UV) / 1.60×10^{-5} M; (C) roxarsone in a 1 mg/mL TiO_2 suspension after one hour UV exposition (λ = 312 nm) / roxarsone not detected; (D) Supernatant of solution C after centrifuging for 2 minutes and filtering through a 0.45 micron syringe filter / roxarsone was not detected.

19 Concluding Remarks

Roxarsone (3-nitro-4-hydroxyphenylarsonic acid), p-arsanilic acid, and acetarsone are only three of a large family of organoarsenic animal feed additives. Roxarsone has found wide acceptance in the poultry farming industry since its introduction in the early 1940's regardless of the toxic implications that accompany its use. The fact that arsenic-based 19th century therapeutics such as Fowler's solution are re-introduced as Trisenox® in the beginning of the 21st century suggests that anthropogenic sources of this toxic element will continue to be present in the future. An additional effort is required in research to completely eradicate the use of such harmful substances. However, there are over two centuries worth of evidence that suggests that the detrimental effects introduced by organoarsenics (such as roxarsone) in the environment must be addressed in a more effective manner. The fact that it is excreted unchanged in poultry litter (which is later amended to agricultural soils as fertilizer), and that there are documented reports related to plant uptake and wide-spread contamination through run-off water, gives rise to many questions as to the extent of its toxic effects . Efficient remediation studies are in process for contaminated agricultural fields, but the use of this organoarsenic does not seem to diminish.

High-performance liquid chromatography (HPLC) is a method that has found wide acceptance in many industries due to its capacity of analyzing a wide variety of compounds. The introduction of the ion-pairing (IP) mode of chromatography provided additional tools for the HPLC method development scientist looking to design versatile analysis of aqueous solutions.

This investigation presented the development of an IP-HPLC method for the analysis of the organoarsenic drugs roxarsone, p-arsanilic acid, and acetarsone. After variation of a diverse amount of parameters and solution preparations, the final mobile phase consisted of a 85:15 composition of a 18.3 mM methyl-tributyl ammonium hydroxide aqueous phase (pH = 6.30 to 6.50; adjusted with glacial acetic acid) and methanol as the organic phase. The combination of the mobile phase, the Eclipse Plus, 4.6mm × 50 mm, 1.8 μm packing, Rapid-Resolution column, UV detection of λ = 245 nm, and a column temperature of 40°C provided the isocratic chromatographic conditions for the separation, identification, and quantitation of the organoarsenic drugs roxarsone, p-arsanilic acid, and acetarsone.

The method was subjected to USP and ICH validation protocols which have been accepted as standard guidelines by a wide array of regulatory agencies such as the Food and Drug Administration (FDA). A linear response ($R^2 \geq 0.9990$, *p*-values < 0.05) was obtained for all organoarsenic drugs analyzed. The developed procedure demonstrated reproducibility for area (%RSD < 1.0%), and elution time (%RSD <0.1%). An adequate range was achieved with an LOQ below the 0.03 ppm minimum level of roxarsone required by the FDA. Furthermore, all organoarsenicals eluted in less than 1.5 minutes.

The method was capable of eluting roxarsone free from co-eluting species in DIW, Potable Water, and aqueous solutions containing dissolved ions common in fresh water and fertilized soils. However, peaks corresponding to concentrations of p-arsanilic acid greater than 1.29×10^{-4} M co-eluted with an artifact attributed to the ion-pairing agent background (IpA-Bkg). This is not a major problem because dilutions can

be performed of samples exhibiting high concentrations of p-arsanilic acid. However, it is important that the dilution factor be applied in the final concentration calculations. Acetarsone eluted free from all artifacts with the exception of the aqueous solution containing NO_3 ions. An unknown peak (attributed to nitrate) co-eluted in a manner which compromised the peak integration and quantification. However, all other dissolved ions did not present any interference.

The method's applicability as a surveillance tool for analysis of roxarsone in complex matrixes was tested with poultry litter. Poultry samples obtained from a local non-commercial farmer were spiked with known amounts of roxarsone. The resulting chromatography demonstrated the successful separation of roxarsone from matrix interferences. The basic figures of merit for the evaluation of an adequate separation and appropriate elution of a chromatographic peak (e.g. symmetry, response) were unaffected by the poultry litter matrix.

The developed IP-HPLC method can be used for routine surveillance for remediation studies. Roxarsone-contaminated water was exposed to TiO_2 suspensions and UV-radiation. The method was capable of providing sample results in a three (3) minute time range while assuring the quality and integrity of the analysis. Furthermore, the rate of producing results was rapid enough to survey accelerated photo-degradation studies. Roxarsone was not detected in TiO_2 suspensions after UV-exposition an hour.

Further studies should be performed to enhance the methods applicability. Other organoarsenic drugs should be studied and validated with this method. The operational parameters and solutions required to perform this technique opens the opportunity of coupling to more sophisticated hyphenated equipment set-ups such as ICP-MS for

structure elucidation studies and arsenic speciation. Additional investigations should be directed towards the elucidation of mechanisms involved in the photo-remediation process using TiO_2 suspensions with UV exposure.

The chromatographic data obtained during this investigation demonstrated the robustness of the analytical method developed for the detection and quantitation of roxarsone, p-arsanilic acid, and acetarsone in aqueous solutions and complex matrixes. The results indicate that the IP-HPLC validated procedure is a promising tool for surveillance activities of agricultural fields and poultry litter. The low-cost, rapid analysis time and simplified methodology make this technique attractive for a modern QA/QC laboratory. In conclusion, this investigation presents a promising alternative for the analysis of the organoarsenic drugs roxarsone, p-arsanilic acid, and acetarsone.

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APPENDIXES

Appendix 1: Potentiometric titration of 9.45×10^{-5} M roxarsone with NaOH

Table A1-1: Information required for calculations of the potentiometric titration of roxarsone with and NaOH.

C_{ROX} (M)	9.45×10^{-5}	C_{NaOH} (M)	1.00×10^{-3}
V_{ROX} (mL)	50.00	K_w	1.00×10^{-14}

Table A1-2: Potentiometric titration of 9.45×10^{-5} M roxarsone with 1.0×10^{-3} M NaOH.

V_{NaOH}	pH	V_{NaOH}	pH	V_{NaOH}	pH	V_{NaOH}	pH	V_{NaOH}	pH
0.0	2.654	11.5	4.645	23.0	8.09	34.5	8.586	46.0	8.747
0.5	2.715	12.0	4.854	23.5	8.135	35.0	8.589	46.5	8.754
1.0	2.776	12.5	5.099	24.0	8.182	35.5	8.616	47.0	8.752
1.5	2.824	13.0	5.438	24.5	8.181	36.0	8.628	47.5	8.752
2.0	2.853	13.5	5.753	25.0	8.195	36.5	8.628	48.0	8.768
2.5	2.896	14.0	6.138	25.5	8.218	37.0	8.635	48.5	8.768
3.0	2.928	14.5	6.556	26.0	8.225	37.5	8.651	49.0	8.775
3.5	3.000	15.0	6.85	26.5	8.28	38.0	8.655	49.5	8.775
4.0	3.047	15.5	7.072	27.0	8.301	38.5	8.66	50.0	8.782
4.5	3.093	16.0	7.195	27.5	8.331	39.0	8.671	50.5	8.784
5.0	3.117	16.5	7.319	28.0	8.35	39.5	8.68	51.0	8.779
5.5	3.290	17.0	7.414	28.5	8.365	40.0	8.69	51.5	8.788
6.0	3.423	17.5	7.488	29.0	8.393	40.5	8.694	52.0	8.786
6.5	3.554	18.0	7.531	29.5	8.416	41.0	8.692	52.5	8.791
7.0	3.689	18.5	7.616	30.0	8.432	41.5	8.693	53.0	8.805
7.5	3.819	19.0	7.735	30.5	8.446	42.0	8.692	53.5	8.804
8.0	3.97	19.5	7.798	31.0	8.465	42.5	8.713	54.0	8.807
8.5	4.076	20.0	7.818	31.5	8.473	43.0	8.724	54.5	8.807
9.0	4.169	20.5	7.887	32.0	8.494	43.5	8.713	55.0	8.816
9.5	4.298	21.0	7.904	32.5	8.531	44.0	8.72		
10.0	4.388	21.5	7.968	33.0	8.545	44.5	8.731		
10.5	4.412	22.0	8.002	33.5	8.554	45.0	8.735		
11.0	4.486	22.5	8.035	34.0	8.57	45.5	8.738		

Appendix 2: Potentiometric titration of 1.0×10^{-3} M roxarsone with NaOH

Table 2-1: Information required for calculations of the potentiometric titration of roxarsone with and NaOH.

C_{ROX} (M)	1.00×10^{-3}	C_{NaOH} (M)	1.00×10^{-3}
V_{ROX} (mL)	50.00	K_w	1.00×10^{-14}

Table A2-2: Potentiometric titration of 1.0×10^{-3} M roxarsone with 1.0×10^{-3} M NaOH.

V_{NaOH}	pH	First Derivative				Second Derivative			
		ΔpH	ΔV	ΔpH/ΔV	V_{AVE1}	$\Delta(\Delta pH/\Delta V)$	ΔV^2	$\Delta^2 pH/\Delta V^2$	V_{AVE2}
0.0	2.97	0.00	0.5	0.00	0.25	0.00	0.5	0.00	0.50
0.5	2.97	0.00	0.5	0.00	0.75	0.00	0.5	0.00	1.00
1.0	2.97	0.00	0.5	0.00	1.25	0.02	0.5	0.04	1.50
1.5	2.97	0.01	0.5	0.02	1.75	-0.02	0.5	-0.04	2.00
2.0	2.98	0.00	0.5	0.00	2.25	0.00	0.5	0.00	2.50
2.5	2.98	0.00	0.5	0.00	2.75	0.02	0.5	0.04	3.00
3.0	2.98	0.01	0.5	0.02	3.25	-0.02	0.5	-0.04	3.50
3.5	2.99	0.00	0.5	0.00	3.75	0.00	0.5	0.00	4.00
4.0	2.99	0.00	0.5	0.00	4.25	0.00	0.5	0.00	4.50
4.5	2.99	0.00	0.5	0.00	4.75	0.02	0.5	0.04	5.00
5.0	2.99	0.01	0.5	0.02	5.25	-0.02	0.5	-0.04	5.50
5.5	3.00	0.00	0.5	0.00	5.75	0.02	0.5	0.04	6.00
6.0	3.00	0.01	0.5	0.02	6.25	-0.02	0.5	-0.04	6.50
6.5	3.01	0.00	0.5	0.00	6.75	0.00	0.5	0.00	7.00
7.0	3.01	0.00	0.5	0.00	7.25	0.02	0.5	0.04	7.50
7.5	3.01	0.01	0.5	0.02	7.75	-0.02	0.5	-0.04	8.00
8.0	3.02	0.00	0.5	0.00	8.25	0.02	0.5	0.04	8.50
8.5	3.02	0.01	0.5	0.02	8.75	-0.02	0.5	-0.04	9.00
9.0	3.03	0.00	0.5	0.00	9.25	0.00	0.5	0.00	9.50
9.5	3.03	0.00	0.5	0.00	9.75	0.00	0.5	0.00	10.00
10.0	3.03	0.00	0.5	0.00	10.25	0.02	0.5	0.04	10.50
10.5	3.03	0.01	0.5	0.02	10.75	-0.02	0.5	-0.04	11.00
11.0	3.04	0.00	0.5	0.00	11.25	0.02	0.5	0.04	11.50
11.5	3.04	0.01	0.5	0.02	11.75	-0.02	0.5	-0.04	12.00
12.0	3.05	0.00	0.5	0.00	12.25	0.02	0.5	0.04	12.50
12.5	3.05	0.01	0.5	0.02	12.75	0.00	0.5	0.00	13.00
13.0	3.06	0.01	0.5	0.02	13.25	0.00	0.5	0.00	13.50
13.5	3.07	0.01	0.5	0.02	13.75	-0.02	0.5	-0.04	14.00
14.0	3.08	0.00	0.5	0.00	14.25	0.02	0.5	0.04	14.50
14.5	3.08	0.01	0.5	0.02	14.75	0.00	0.5	0.00	15.00
15.0	3.09	0.01	0.5	0.02	15.25	0.00	0.5	0.00	15.50

Table A2-2: Potentiometric titration of 1.0×10^{-3} M roxarsone with 1.0×10^{-3} M NaOH.

V_{NaOH}	pH	First Derivative				Second Derivative			
		ΔpH	ΔV	ΔpH/ΔV	V_{AVE1}	$\Delta(\Delta pH/\Delta V)$	ΔV^2	$\Delta^2 pH/\Delta V^2$	V_{AVE2}
15.5	3.10	0.01	0.5	0.02	15.75	0.00	0.5	0.00	16.00
16.0	3.11	0.01	0.5	0.02	16.25	0.00	0.5	0.00	16.50
16.5	3.12	0.01	0.5	0.02	16.75	0.00	0.5	0.00	17.00
17.0	3.13	0.01	0.5	0.02	17.25	0.00	0.5	0.00	17.50
17.5	3.14	0.01	0.5	0.02	17.75	0.00	0.5	0.00	18.00
18.0	3.15	0.01	0.5	0.02	18.25	0.00	0.5	0.00	18.50
18.5	3.16	0.01	0.5	0.02	18.75	0.02	0.5	0.04	19.00
19.0	3.17	0.02	0.5	0.04	19.25	-0.02	0.5	-0.04	19.50
19.5	3.19	0.01	0.5	0.02	19.75	0.00	0.5	0.00	20.00
20.0	3.20	0.01	0.5	0.02	20.25	0.02	0.5	0.04	20.50
20.5	3.21	0.02	0.5	0.04	20.75	-0.02	0.5	-0.04	21.00
21.0	3.23	0.01	0.5	0.02	21.25	0.00	0.5	0.00	21.50
21.5	3.24	0.01	0.5	0.02	21.75	0.02	0.5	0.04	22.00
22.0	3.25	0.02	0.5	0.04	22.25	0.00	0.5	0.00	22.50
22.5	3.27	0.02	0.5	0.04	22.75	-0.02	0.5	-0.04	23.00
23.0	3.29	0.01	0.5	0.02	23.25	0.02	0.5	0.04	23.50
23.5	3.30	0.02	0.5	0.04	23.75	0.00	0.5	0.00	24.00
24.0	3.32	0.02	0.5	0.04	24.25	0.00	0.5	0.00	24.50
24.5	3.34	0.02	0.5	0.04	24.75	-0.02	0.5	-0.04	25.00
25.0	3.36	0.01	0.5	0.02	25.25	0.06	0.5	0.12	25.50
25.5	3.37	0.04	0.5	0.08	25.75	-0.04	0.5	-0.08	26.00
26.0	3.41	0.02	0.5	0.04	26.25	0.02	0.5	0.04	26.50
26.5	3.43	0.03	0.5	0.06	26.75	0.02	0.5	0.04	27.00
27.0	3.46	0.04	0.5	0.08	27.25	-0.04	0.5	-0.08	27.50
27.5	3.50	0.02	0.5	0.04	27.75	0.02	0.5	0.04	28.00
28.0	3.52	0.03	0.5	0.06	28.25	-0.02	0.5	-0.04	28.50
28.5	3.55	0.02	0.5	0.04	28.75	0.00	0.5	0.00	29.00
29.0	3.57	0.02	0.5	0.04	29.25	0.02	0.5	0.04	29.50
29.5	3.59	0.03	0.5	0.06	29.75	0.00	0.5	0.00	30.00
30.0	3.62	0.03	0.5	0.06	30.25	0.02	0.5	0.04	30.50
30.5	3.65	0.04	0.5	0.08	30.75	-0.02	0.5	-0.04	31.00
31.0	3.69	0.03	0.5	0.06	31.25	0.00	0.5	0.00	31.50
31.5	3.72	0.03	0.5	0.06	31.75	0.02	0.5	0.04	32.00
32.0	3.75	0.04	0.5	0.08	32.25	0.00	0.5	0.00	32.50
32.5	3.79	0.04	0.5	0.08	32.75	-0.02	0.5	-0.04	33.00
33.0	3.83	0.03	0.5	0.06	33.25	0.02	0.5	0.04	33.50
33.5	3.86	0.04	0.5	0.08	33.75	0.00	0.5	0.00	34.00
34.0	3.90	0.04	0.5	0.08	34.25	0.00	0.5	0.00	34.50
34.5	3.94	0.04	0.5	0.08	34.75	0.02	0.5	0.04	35.00
35.0	3.98	0.05	0.5	0.10	35.25	-0.02	0.5	-0.04	35.50
35.5	4.03	0.04	0.5	0.08	35.75	0.00	0.5	0.00	36.00
36.0	4.07	0.04	0.5	0.08	36.25	0.00	0.5	0.00	36.50
36.5	4.11	0.04	0.5	0.08	36.75	0.00	0.5	0.00	37.00
37.0	4.15	0.04	0.5	0.08	37.25	0.00	0.5	0.00	37.50

Table A2-2: Potentiometric titration of 1.0×10^{-3} M roxarsone with 1.0×10^{-3} M NaOH.

V_{NaOH}	pH	First Derivative				Second Derivative			
		ΔpH	ΔV	ΔpH/ΔV	V_{AVE1}	$\Delta(\Delta pH/\Delta V)$	ΔV^2	$\Delta^2 pH/\Delta V^2$	V_{AVE2}
37.5	4.19	0.04	0.5	0.08	37.75	0.02	0.5	0.04	38.00
38.0	4.23	0.05	0.5	0.10	38.25	-0.02	0.5	-0.04	38.50
38.5	4.28	0.04	0.5	0.08	38.75	0.00	0.5	0.00	39.00
39.0	4.32	0.04	0.5	0.08	39.25	0.00	0.5	0.00	39.50
39.5	4.36	0.04	0.5	0.08	39.75	-0.02	0.5	-0.04	40.00
40.0	4.40	0.03	0.5	0.06	40.25	0.00	0.5	0.00	40.50
40.5	4.43	0.03	0.5	0.06	40.75	0.02	0.5	0.04	41.00
41.0	4.46	0.04	0.5	0.08	41.25	0.00	0.5	0.00	41.50
41.5	4.50	0.04	0.5	0.08	41.75	-0.04	0.5	-0.08	42.00
42.0	4.54	0.02	0.5	0.04	42.25	0.02	0.5	0.04	42.50
42.5	4.56	0.03	0.5	0.06	42.75	0.00	0.5	0.00	43.00
43.0	4.59	0.03	0.5	0.06	43.25	0.02	0.5	0.04	43.50
43.5	4.62	0.04	0.5	0.08	43.75	-0.04	0.5	-0.08	44.00
44.0	4.66	0.02	0.5	0.04	44.25	0.02	0.5	0.04	44.50
44.5	4.68	0.03	0.5	0.06	44.75	-0.02	0.75	-0.03	45.13
45.0	4.71	0.04	1.0	0.04	45.5	0.01	1	0.01	46.00
46.0	4.75	0.05	1.0	0.05	46.5	0.00	1	0.00	47.00
47.0	4.80	0.05	1.0	0.05	47.5	0.00	1	0.00	48.00
48.0	4.85	0.05	1.0	0.05	48.5	-0.01	1	-0.01	49.00
49.0	4.90	0.04	1.0	0.04	49.5	0.00	1	0.00	50.00
50.0	4.94	0.04	1.0	0.04	50.5	-0.03	1	-0.03	51.00
51.0	4.98	0.01	1.0	0.01	51.5	0.02	1	0.02	52.00
52.0	4.99	0.03	1.0	0.03	52.5	-0.01	1	-0.01	53.00
53.0	5.02	0.02	1.0	0.02	53.5	0.00	1	0.00	54.00
54.0	5.04	0.02	1.0	0.02	54.5	0.02	1	0.02	55.00
55.0	5.06	0.04	1.0	0.04	55.5	0.00	1	0.00	56.00
56.0	5.10	0.04	1.0	0.04	56.5	0.00	1	0.00	57.00
57.0	5.14	0.04	1.0	0.04	57.5	0.00	1	0.00	58.00
58.0	5.18	0.04	1.0	0.04	58.5	-0.01	1	-0.01	59.00
59.0	5.22	0.03	1.0	0.03	59.5	0.00	1	0.00	60.00
60.0	5.25	0.03	1.0	0.03	60.5	0.02	1	0.02	61.00
61.0	5.28	0.05	1.0	0.05	61.5	-0.01	1	-0.01	62.00
62.0	5.33	0.04	1.0	0.04	62.5	-0.01	1	-0.01	63.00
63.0	5.37	0.03	1.0	0.03	63.5	0.01	1	0.01	64.00
64.0	5.40	0.04	1.0	0.04	64.5	-0.02	1	-0.02	65.00
65.0	5.44	0.02	1.0	0.02	65.5	0.03	1	0.03	66.00
66.0	5.46	0.05	1.0	0.05	66.5	-0.01	1	-0.01	67.00
67.0	5.51	0.04	1.0	0.04	67.5	0.01	1	0.01	68.00
68.0	5.55	0.05	1.0	0.05	68.5	-0.01	1	-0.01	69.00
69.0	5.60	0.04	1.0	0.04	69.5	-0.01	1	-0.01	70.00
70.0	5.64	0.03	1.0	0.03	70.5	0.00	1	0.00	71.00
71.0	5.67	0.03	1.0	0.03	71.5	0.03	1	0.03	72.00
72.0	5.70	0.06	1.0	0.06	72.5	0.00	1	0.00	73.00
73.0	5.76	0.06	1.0	0.06	73.5	-0.02	1	-0.02	74.00

Table A2-2: Potentiometric titration of 1.0×10^{-3} M roxarsone with 1.0×10^{-3} M NaOH.

V_{NaOH}	pH	First Derivative				Second Derivative			
		ΔpH	ΔV	ΔpH/ΔV	V_{AVE1}	$\Delta(\Delta pH/\Delta V)$	ΔV^2	$\Delta^2 pH/\Delta V^2$	V_{AVE2}
74.0	5.82	0.04	1.0	0.04	74.5	-0.03	1	-0.03	75.00
75.0	5.86	0.01	1.0	0.01	75.5	0.02	1	0.02	76.00
76.0	5.87	0.03	1.0	0.03	76.5	-0.02	1	-0.02	77.00
77.0	5.90	0.01	1.0	0.01	77.5	0.04	1	0.04	78.00
78.0	5.91	0.05	1.0	0.05	78.5	-0.01	1	-0.01	79.00
79.0	5.96	0.04	1.0	0.04	79.5	-0.03	1	-0.03	80.00
80.0	6.00	0.01	1.0	0.01	80.5	0.03	1	0.03	81.00
81.0	6.01	0.04	1.0	0.04	81.5	0.02	1	0.02	82.00
82.0	6.05	0.06	1.0	0.06	82.5	0.00	1	0.00	83.00
83.0	6.11	0.06	1.0	0.06	83.5	-0.01	1	-0.01	84.00
84.0	6.17	0.05	1.0	0.05	84.5	0.02	1	0.02	85.00
85.0	6.22	0.07	1.0	0.07	85.5	0.00	1	0.00	86.00
86.0	6.29	0.07	1.0	0.07	86.5	-0.01	1	-0.01	87.00
87.0	6.36	0.06	1.0	0.06	87.5	0.02	1	0.02	88.00
88.0	6.42	0.08	1.0	0.08	88.5	-0.01	1	-0.01	89.00
89.0	6.50	0.07	1.0	0.07	89.5	0.00	1	0.00	90.00
90.0	6.57	0.07	1.0	0.07	90.5	0.01	1	0.01	91.00
91.0	6.64	0.08	1.0	0.08	91.5	0.05	1	0.05	92.00
92.0	6.72	0.13	1.0	0.13	92.5	-0.03	1	-0.03	93.00
93.0	6.85	0.10	1.0	0.10	93.5	0.03	1	0.03	94.00
94.0	6.95	0.13	1.0	0.13	94.5	0.00	1	0.00	95.00
95.0	7.08	0.13	1.0	0.13	95.5	-0.03	0.75	-0.04	95.88
96.0	7.21	0.05	0.5	0.10	96.25	0.00	0.5	0.00	96.50
96.5	7.26	0.05	0.5	0.10	96.75	0.02	0.5	0.04	97.00
97.0	7.31	0.06	0.5	0.12	97.25	-0.04	0.5	-0.08	97.50
97.5	7.37	0.04	0.5	0.08	97.75	0.02	0.5	0.04	98.00
98.0	7.41	0.05	0.5	0.10	98.25	0.00	0.5	0.00	98.50
98.5	7.46	0.05	0.5	0.10	98.75	-0.04	0.5	-0.08	99.00
99.0	7.51	0.03	0.5	0.06	99.25	0.04	0.5	0.08	99.50
99.5	7.54	0.05	0.5	0.10	99.75	-0.04	0.5	-0.08	100.00
100.0	7.59	0.03	0.5	0.06	100.25	0.00	0.5	0.00	100.50
100.5	7.62	0.03	0.5	0.06	100.75	0.00	0.5	0.00	101.00
101.0	7.65	0.03	0.5	0.06	101.25	0.02	0.5	0.04	101.50
101.5	7.68	0.04	0.5	0.08	101.75	-0.02	0.5	-0.04	102.00
102.0	7.72	0.03	0.5	0.06	102.25	0.00	0.5	0.00	102.50
102.5	7.75	0.03	0.5	0.06	102.75	0.00	0.5	0.00	103.00
103.0	7.78	0.03	0.5	0.06	103.25	-0.02	0.5	-0.04	103.50
103.5	7.81	0.02	0.5	0.04	103.75	0.02	0.5	0.04	104.00
104.0	7.83	0.03	0.5	0.06	104.25	-0.02	0.5	-0.04	104.50
104.5	7.86	0.02	0.5	0.04	104.75	-0.02	0.5	-0.04	105.00
105.0	7.88	0.01	0.5	0.02	105.25	0.02	0.5	0.04	105.50
105.5	7.89	0.02	0.5	0.04	105.75	0.00	0.5	0.00	106.00
106.0	7.91	0.02	0.5	0.04	106.25	0.00	0.5	0.00	106.50
106.5	7.93	0.02	0.5	0.04	106.75	-0.01	0.75	-0.01	107.13

Table A2-2: Potentiometric titration of 1.0×10^{-3} M roxarsone with 1.0×10^{-3} M NaOH.

V_{NaOH}	pH	First Derivative				Second Derivative			
		ΔpH	ΔV	ΔpH/ΔV	V_{AVE1}	$\Delta(\Delta pH/\Delta V)$	ΔV^2	$\Delta^2 pH/\Delta V^2$	V_{AVE2}
107.0	7.95	0.03	1.0	0.03	107.5	0.00	1	0.00	108.00
108.0	7.98	0.03	1.0	0.03	108.5	0.02	1	0.02	109.00
109.0	8.01	0.05	1.0	0.05	109.5	-0.02	1	-0.02	110.00
110.0	8.06	0.03	1.0	0.03	110.5	0.01	1	0.01	111.00
111.0	8.09	0.04	1.0	0.04	111.5	0.00	1	0.00	112.00
112.0	8.13	0.04	1.0	0.04	112.5	-0.01	1	-0.01	113.00
113.0	8.17	0.03	1.0	0.03	113.5	0.01	1	0.01	114.00
114.0	8.20	0.04	1.0	0.04	114.5	-0.01	1	-0.01	115.00
115.0	8.24	0.03	1.0	0.03	115.5	0.00	1	0.00	116.00
116.0	8.27	0.03	1.0	0.03	116.5	0.00	1	0.00	117.00
117.0	8.30	0.03	1.0	0.03	117.5	0.00	1	0.00	118.00
118.0	8.33	0.03	1.0	0.03	118.5	-0.01	1	-0.01	119.00
119.0	8.36	0.02	1.0	0.02	119.5	0.01	1	0.01	120.00
120.0	8.38	0.03	1.0	0.03	120.5	-0.01	1	-0.01	121.00
121.0	8.41	0.02	1.0	0.02	121.5	0.01	1	0.01	122.00
122.0	8.43	0.03	1.0	0.03	122.5	0.01	1	0.01	123.00
123.0	8.46	0.04	1.0	0.04	123.5	-0.01	1	-0.01	124.00
124.0	8.50	0.03	1.0	0.03	124.5	0.00	1	0.00	125.00
125.0	8.53	0.03	1.0	0.03	125.5	-0.01	1	-0.01	126.00
126.0	8.56	0.02	1.0	0.02	126.5	0.01	1	0.01	127.00
127.0	8.58	0.03	1.0	0.03	127.5	-0.01	1	-0.01	128.00
128.0	8.61	0.02	1.0	0.02	128.5	0.00	1	0.00	129.00
129.0	8.63	0.02	1.0	0.02	129.5	0.00	1	0.00	130.00
130.0	8.65	0.02	1.0	0.02	130.5	0.00	1	0.00	131.00
131.0	8.67	0.02	1.0	0.02	131.5	0.00	1	0.00	132.00
132.0	8.69	0.02	1.0	0.02	132.5	0.00	1	0.00	133.00
133.0	8.71	0.02	1.0	0.02	133.5	-0.01	1	-0.01	134.00
134.0	8.73	0.01	1.0	0.01	134.5	0.00	1	0.00	135.00
135.0	8.74	0.01	1.0	0.01	135.5	0.02	1	0.02	136.00
136.0	8.75	0.03	1.0	0.03	136.5	-0.01	1	-0.01	137.00
137.0	8.78	0.02	1.0	0.02	137.5	0.00	1	0.00	138.00
138.0	8.80	0.02	1.0	0.02	138.5	0.01	1	0.01	139.00
139.0	8.82	0.03	1.0	0.03	139.5	-0.02	1	-0.02	140.00
140.0	8.85	0.01	1.0	0.01	140.5	0.00	1	0.00	141.00
141.0	8.86	0.01	1.0	0.01	141.5	0.01	1	0.01	142.00
142.0	8.87	0.02	1.0	0.02	142.5	0.00	1	0.00	143.00
143.0	8.89	0.02	1.0	0.02	143.5	0.00	1	0.00	144.00
144.0	8.91	0.02	1.0	0.02	144.5	-0.01	1	-0.01	145.00
145.0	8.93	0.01	1.0	0.01	145.5	0.01	1.5	0.00	146.25
146.0	8.94	0.03	2.0	0.02	147	0.00	2	0.00	148.00
148.0	8.97	0.03	2.0	0.01	149	0.00	2	0.00	150.00
150.0	9.00	0.03	2.0	0.01	151	0.00	2	0.00	152.00
152.0	9.03	0.03	2.0	0.02	153	-0.01	2	0.00	154.00
154.0	9.06	0.02	2.0	0.01	155	0.00	3	0.00	156.50

Table A2-2: Potentiometric titration of 1.0×10^{-3} M roxarsone with 1.0×10^{-3} M NaOH.

V_{NaOH}	pH	First Derivative				Second Derivative			
		ΔpH	ΔV	ΔpH/ΔV	V_{AVE1}	$\Delta(\Delta pH/\Delta V)$	ΔV^2	$\Delta^2 pH/\Delta V^2$	V_{AVE2}
156.0	9.08	0.05	4.0	0.01	158	0.00	4	0.00	160.00
160.0	9.13	0.04	4.0	0.01	162	0.00	4	0.00	164.00
164.0	9.17	0.04	4.0	0.01	166	-0.01	4	0.00	168.00
168.0	9.21	0.02	4.0	0.00	170				
172.0	9.23								

Appendix 3: Calibration curve #1 for roxarsone, p-arsanilic acid, and acetarsone.

Table A3-1: Reagents used for 85:15 mobile phase consisting of (A) 18.3 mM methyl-tributylammonium hydroxide (MTBA-OH), 1% triethylamine (TEA), pH = 6.30 and (B) methanol HPLC grade.

Reagent	Manuf.	Catalog No.	Purity
DIW	Barnstead	N/A	18.4 MΩ-cm
MTBA-OH	Aldrich	522287	20% (w/w)
TEA	J.T. Baker	911-07	99.0%
acetic acid	Fisher	A507	99.0%
methanol	Fisher	A452	99.0%

Table A3-2: Preparation scheme of stock standard solutions for calibration curve #1.

Analyte	p-ASA	acetarsone	roxarsone
Manuf.	Sigma Aldrich	Sigma Aldrich	Riedel de Haen
Catalog No.	A9258	A0259	46726
Lot No.	047K0135	075K1465	2326X
MW (g/mol)	217.05	275.09	263.04
Purity	99.0	99.0	98.3%
Weight (g)	0.0280	0.0247	0.0263
Volume (mL)	100	100	100
Conc. (M)	1.28×10^{-3}	8.89×10^{-4}	9.83×10^{-4}
Conc. (ppm)	277.20	244.53	258.53

Table A3-3: Preparation scheme of p-ASA working standard solutions in DIW for calibration curve #1.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	1.28×10^{-4}	27.72
2	500	10	6.39×10^{-5}	13.86
3	250	10	3.19×10^{-5}	6.93
4	10	10	1.28×10^{-6}	0.28
5	3000 [(a)]	10	3.83×10^{-7}	0.083
6	1000 [(a)]	10	1.28×10^{-7}	0.028

(a) Aliquot taken from level 4 working standard.

Table A3-4: Preparation scheme of acetarsone working standard solutions in DIW for calibration curve #1.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	2500	10	2.22×10^{-4}	61.13
2	1500	10	1.33×10^{-4}	36.68
3	1000	10	8.89×10^{-5}	24.45
4	500	10	4.44×10^{-5}	12.23
5	250	10	2.22×10^{-5}	6.11
6	10	10	8.89×10^{-7}	0.24

Table A3-5: Preparation scheme of roxarsone working standard solutions in DIW for calibration curve #1.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	9.83×10^{-5}	25.85
2	500	10	4.91×10^{-5}	12.93
3	250	10	2.46×10^{-5}	6.46
4	10	10	9.83×10^{-7}	0.26
5	3000 [(a)]	10	2.95×10^{-7}	0.078
6	1000 [(a)]	10	9.83×10^{-8}	0.026

(a) Aliquot taken from level 4 working standard.

Table A3-6: Chromatographic data for p-ASA calibration curve #1. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	0.540 (± 0.00)	341.7081 (± 1.704)	0.402	0.34	3.10	1.4	0.0555	1513	N/A
2	0.541 (± 0.00)	174.4967 (± 1.251)	0.402	0.35	3.09	1.4	0.0570	1440	N/A
3	0.541 (± 0.00)	83.4977 (± 0.050)	0.401	0.35	3.06	1.4	0.0564	1474	N/A
4	0.541 (± 0.00)	3.0400 (± 0.006)	0.400	0.35	3.06	1.5	0.0591	1340	N/A
5	0.542 (± 0.00)	2.1691 (± 0.050)	0.402	0.35	3.08	1.5	0.0542	1596	N/A
6	0.542 (± 0.00)	1.4067 (± 0.011)	0.402	0.35	3.06	1.5	0.0535	1640	N/A

(a) Selectivity calculated relative acetarsone.
(b) Resolution was not calculated since no peaks elute before p-ASA.

Table A3-7: Chromatographic data for acetarsone calibration curve #1. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	0.831 (±0.00)	347.6863 (± 1.125)	0.402	1.07	1.94	1.6	0.0735	2046	4.51
2	0.831 (±0.00)	208.0734 (± 1.329)	0.402	1.07	1.94	1.6	0.0730	2070	4.46
3	0.831 (±0.00)	136.4377 (± 0.296)	0.401	1.07	1.94	1.6	0.0732	2066	4.48
4	0.832 (±0.00)	69.39447 (± 0.531)	0.400	1.08	1.94	1.6	0.0731	2072	4.40
5	0.832 (±0.00)	33.37283 (± 0.168)	0.402	1.07	1.94	1.6	0.0747	1985	4.50
6	0.831 (±0.00)	1.1094 (± 0.011)	0.402	1.07	1.94	1.5	0.0738	2029	4.54

(a) Selectivity calculated relative roxarsone.
(b) Resolution calculated relative p-ASA.

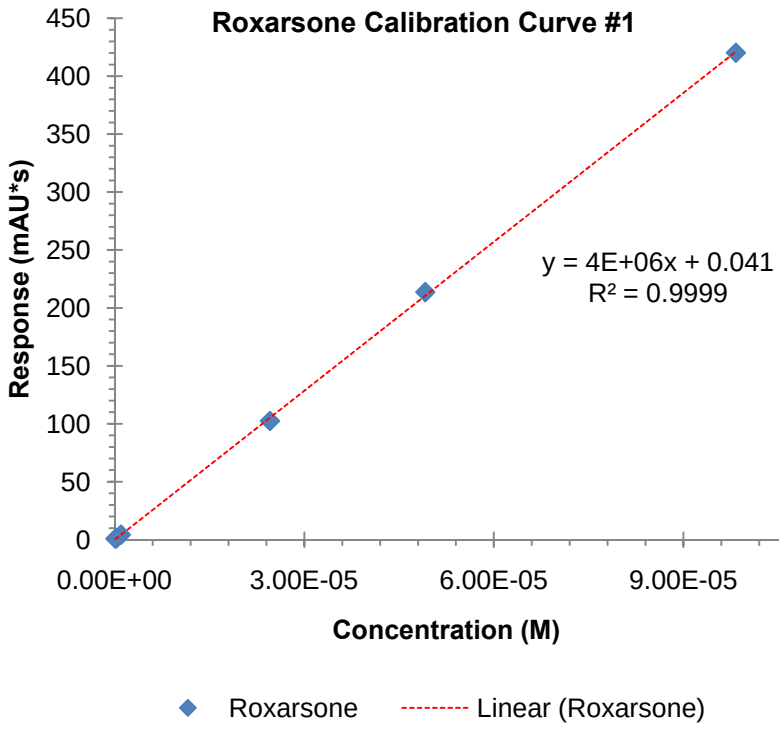
Table A3-8: Chromatographic data for roxarsone calibration curve #1. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	1.235 (± 0.00)	420.2885 (± 0.838)	0.402	2.08	N/A	1.5	0.0726	4637	5.54
2	1.235 (± 0.00)	213.6609 (± 0.702)	0.402	2.08	N/A	1.5	0.0727	4616	5.55
3	1.236 (± 0.00)	102.5323 (± 0.528)	0.401	2.08	N/A	1.6	0.0733	4550	5.53
4	1.237 (± 0.00)	4.2474 (± 0.012)	0.400	2.09	N/A	1.6	0.0735	4532	5.52
5	1.238 (± 0.00)	1.4145 (± 0.011)	0.402	2.08	N/A	1.5	0.0682	5276	5.68
6	1.237 (± 0.00)	0.9070 (± 0.004)	0.402	2.08	N/A	1.4	0.0646	5870	5.87

(a) Selectivity was not calculated since no peaks elute after roxarsone.
(b) Resolution calculated relative acetarsone.

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	5
Column Serial Number	
SN: USUXG03355	

Regression Statistics	
Multiple R	0.9999
R Square	0.9999
Adjusted R Square	0.9998
Standard Error	2.13
Observations	6

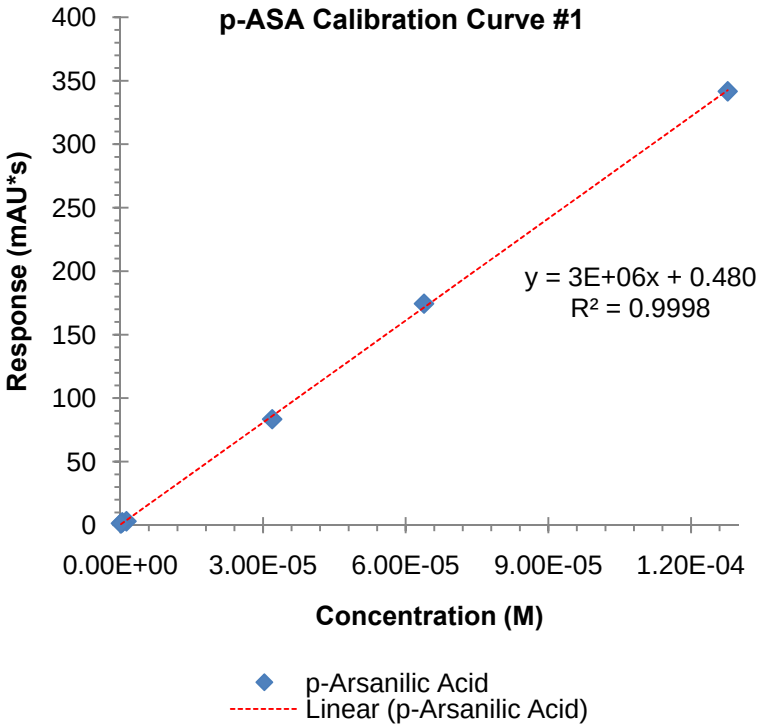


ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	140788.36	140788.36	31095.64	6.2E-09
Residual	4	18.11	4.53		
Total	5	140806.47			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.0414	1.12	0.04	0.97	-3.06	3.14
X Variable 1	4284312.90	24295.81	176.34	0.00	4216856.90	4351768.89

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	5
Column Serial Number	
SN: USUXG03355	

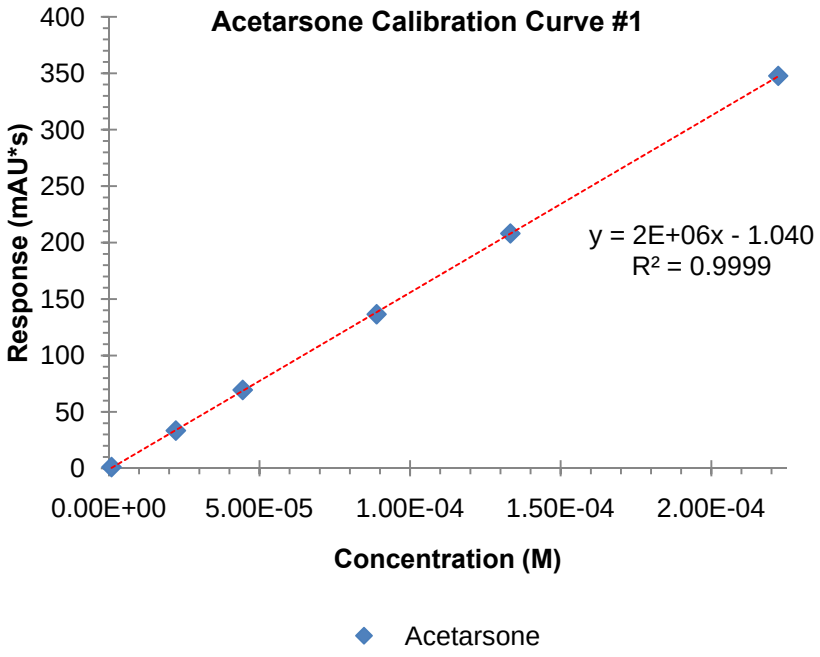
Regression Statistics	
Multiple R	0.9999
R Square	0.9998
Adjusted R Square	0.9998
Standard Error	2.08
Observations	6



ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	92913.61	92913.61	21396.13	1.3E-08
Residual	4	17.37	4.34		
Total	5	92930.98			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.4807	1.09	0.44	0.68	-2.56	3.52
X Variable 1	2678536.75	18311.75	146.27	0.00	2627695.17	2729378.33

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	5
Column Serial Number	
SN: USUXG03355	



Regression Statistics	
Multiple R	1.0000
R Square	0.9999
Adjusted R Square	0.9999
Standard Error	1.11
Observations	6

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	83098.86	83098.86	67410.31	1.3E-09
Residual	4	4.93	1.23		
Total	5	83103.79			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-1.0405	0.69	-1.52	0.20	-2.95	0.86
X Variable 1	1566989.72	6035.36	259.63	0.00	1550232.88	1583746.55

Appendix 4: Calibration curve #2 for roxarsone, p-arsanilic acid, and acetarsone.

Table A4-1: Reagents used for 85:15 mobile phase consisting of (A) 18.3 mM methyl-tributylammonium hydroxide (MTBA-OH), 1% triethylamine (TEA), pH = 6.30 and (B) methanol HPLC grade.

Reagent	Manuf.	Catalog No.	Purity
DIW	Barnstead	N/A	18.4 MΩ-cm
MTBA-OH	Aldrich	522287	20% (w/w)
TEA	J.T. Baker	911-07	99.0%
acetic acid	Fisher	Fisher	99.0%
methanol	Fisher	Fisher	99.0%

Table A4-2: Preparation scheme of stock standard solutions of for calibration curve #2.

Analyte	p-ASA	acetarsone	roxarsone
Manuf.	Sigma Aldrich	Sigma Aldrich	Riedel de Haen
Catalog No.	A9258	A0259	46726
Lot No.	047K0135	075K1465	2326X
MW (g/mol)	217.05	275.09	263.04
Purity	99.0	99.0	98.3%
Weight (g)	0.0277	0.0255	0.0271
Volume (mL)	100	100	100
Conc. (M)	1.26×10^{-3}	9.18×10^{-4}	9.83×10^{-3}
Conc. (ppm)	274.23	252.45	266.39

Table A4-3: Preparation scheme of p-ASA working standard solutions in DIW for calibration curve #2.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	1.26×10^{-4}	27.42
2	500	10	6.32×10^{-5}	13.71
3	250	10	3.16×10^{-5}	6.86
4	10	10	1.26×10^{-6}	0.27
5	3000 [(a)]	10	3.79×10^{-7}	0.082
6	1000 [(a)]	10	1.26×10^{-7}	0.027

(a) Aliquot taken from level 4 working standard.

Table A4-4: Preparation scheme of acetarsone working standard solutions in DIW for calibration curve #2.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	2500	10	2.29×10^{-4}	63.11
2	1500	10	1.38×10^{-4}	37.87
3	1000	10	9.18×10^{-5}	25.25
4	500	10	4.59×10^{-5}	12.62
5	250	10	2.29×10^{-5}	6.31
6	10	10	9.18×10^{-7}	0.25

Table A4-5: Preparation scheme of roxarsone working standard solutions in DIW for calibration curve #2.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	1.01×10^{-4}	26.64
2	500	10	5.06×10^{-5}	13.32
3	250	10	2.53×10^{-5}	6.66
4	10	10	1.01×10^{-6}	0.27
5	3000 [(a)]	10	3.04×10^{-7}	0.080
6	1000 [(a)]	10	1.01×10^{-7}	0.027

(a) Aliquot taken from level 4 working standard.

Table A4-6: Chromatographic data for p-ASA calibration curve #2. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	0.540 (± 0.000)	360.1939 (± 3.518)	0.402	0.34	3.05	1.3	0.0547	1561	N/A
2	0.540 (± 0.000)	177.1200 (± 0.501)	0.400	0.35	3.02	1.4	0.0540	1600	N/A
3	0.541 (± 0.001)	86.4482 (± 0.363)	0.400	0.35	3.02	1.4	0.0543	1588	N/A
4	0.541 (± 0.001)	3.2883 (± 0.031)	0.401	0.35	3.03	1.2	0.0537	1628	N/A
5	0.541 (± 0.001)	2.5839 (± 0.010)	0.401	0.35	3.03	1.3	0.0536	1630	N/A
6	0.542 (± 0.001)	1.5275 (± 0.020)	0.401	0.35	3.01	1.4	0.0538	1622	N/A

(a) Selectivity calculated relative acetarsone.
(b) Resolution was not calculated since no peaks elute before p-ASA.

Table A4-7: Chromatographic data for acetarsone calibration curve #2. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	0.823 (± 0.000)	348.2395 (± 1.189)	0.402	1.05	1.98	1.6	0.0677	2362	4.39
2	0.824 (± 0.000)	213.5035 (± 0.454)	0.400	1.05	1.97	1.5	0.0696	2241	4.36
3	0.825 (± 0.001)	139.5292 (± 0.278)	0.400	1.06	1.97	1.6	0.0713	2139	4.38
4	0.824 (± 0.000)	71.2511 (± 0.577)	0.401	1.06	1.98	1.6	0.0705	2184	4.28
5	0.825 (± 0.001)	33.22553 (± 0.238)	0.401	1.05	1.98	1.5	0.0681	2347	4.39
6	0.825 (± 0.001)	1.010233 (± 0.001)	0.401	1.06	1.97	1.6	0.0660	2506	4.45

(a) Selectivity calculated relative roxarsone.
(b) Resolution calculated relative p-ASA.

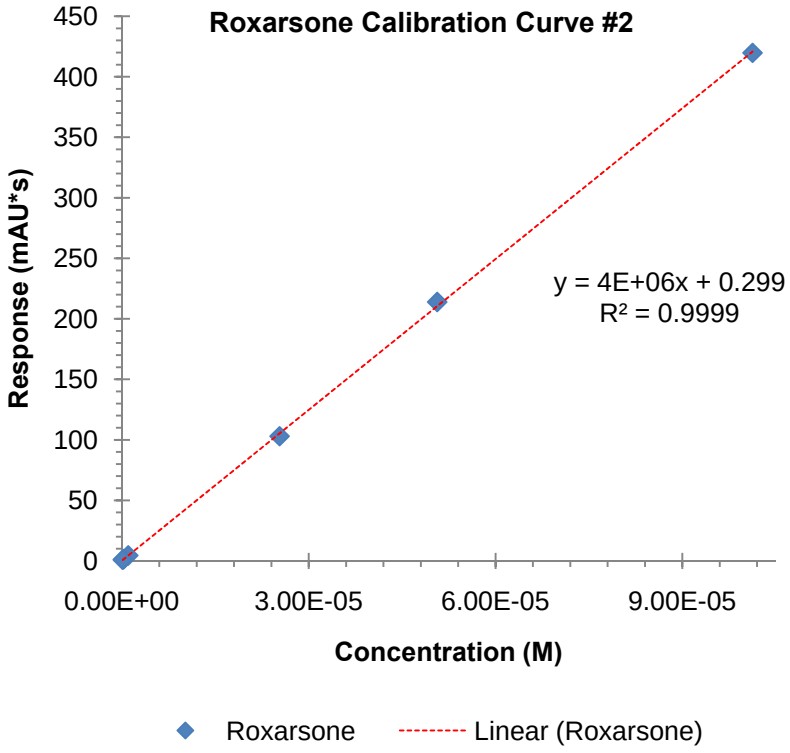
Table A4-8: Chromatographic data for roxarsone calibration curve #2. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	1.278 ± 0.001)	419.7256 (± 0.427)	0.402	2.18	N/A	1.5	0.0714	5123	6.54
2	1.278 ± 0.001)	213.8515 (± 0.964)	0.400	2.20	N/A	1.5	0.0714	5129	6.44
3	1.278 ± 0.001)	103.0474 (± 0.406)	0.400	2.19	N/A	1.5	0.0707	5236	6.39
4	1.279 ± 0.001)	4.5282 (± 0.038)	0.401	2.19	N/A	1.6	0.0697	5385	6.48
5	1.279 ± 0.001)	1.4621 (± 0.015)	0.401	2.19	N/A	1.4	0.0703	5298	6.56
6	1.278 ± 0.001)	0.9214 (± 0.013)	0.401	2.19	N/A	1.4	0.0701	5326	6.66

(a) Selectivity was not calculated since no peaks elute after roxarsone.
(b) Resolution calculated relative acetarsone.

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03364	

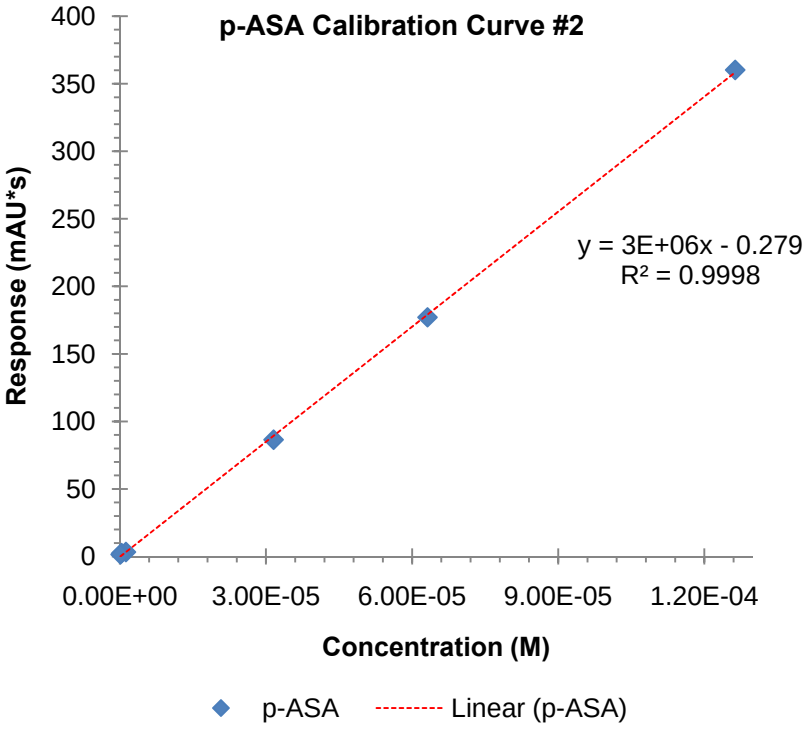
Regression Statistics	
Multiple R	0.9999
R Square	0.9999
Adjusted R Square	0.9998
Standard Error	2.11
Observations	6
Multiple R	0.9999



ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	140385.52	140385.52	31622.03	6.0E-09
Residual	4	17.76	4.44		
Total	5	140403.28			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.2995	1.11	0.27	0.80	-2.77	3.37
X Variable 1	4151886.12	23348.05	177.83	0.00	4087061.55	4216710.68

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03364	

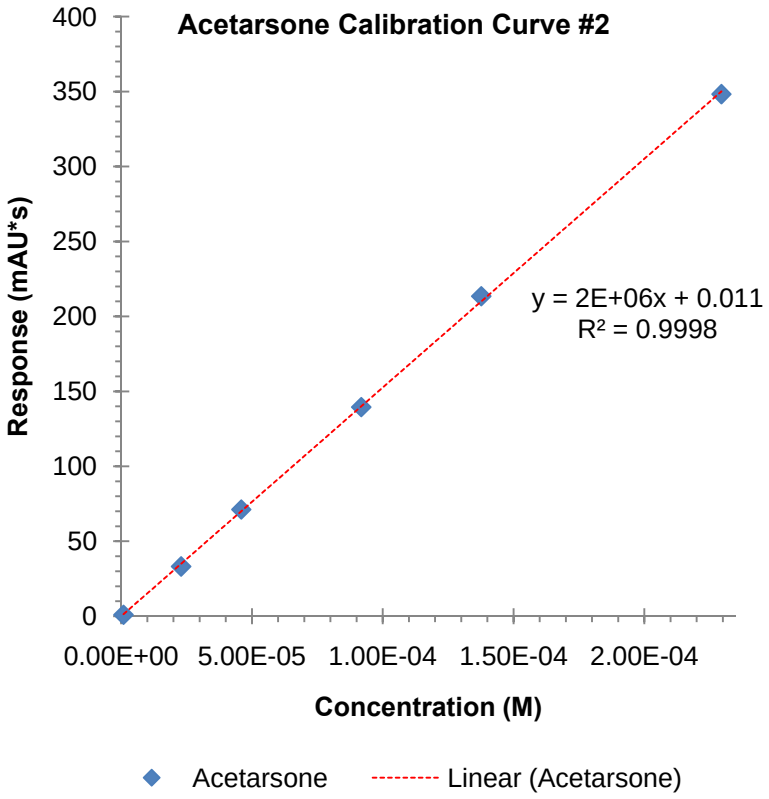


Regression Statistics	
Multiple R	0.9999
R Square	0.9998
Adjusted R Square	0.9997
Standard Error	2.29
Observations	6

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	102189.10	102189.10	19534.78	1.6E-08
Residual	4	20.92	5.23		
Total	5	102210.03			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-0.2793	1.20	-0.23	0.83	-3.61	3.05
X Variable 1	2839477.99	20315.82	139.77	0.00	2783072.24	2895883.74

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03364	



Regression Statistics	
Multiple R	0.9999
R Square	0.9998
Adjusted R Square	0.9997
Standard Error	2.26
Observations	6

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	84007.45	84007.45	16448.96	2.2E-08
Residual	4	20.43	5.11		
Total	5	84027.88			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.0113	1.40	0.01	0.99	-3.87	3.89
X Variable 1	1526104.56	11899.13	128.25	0.00	1493067.29	1559141.83

Appendix 5: Calibration curve #3 for roxarsone, p-arsanilic acid, and acetarsone.

Table A5-1: Reagents used for 85:15 mobile phase consisting of (A) 18.3 mM methyl-tributylammonium hydroxide (MTBA-OH), 1% triethylamine (TEA), pH = 6.50 and (B) methanol HPLC grade.

Reagent	Manuf.	Catalog No.	Purity
DIW	Barnstead	N/A	18.4 MΩ-cm
MTBA-OH	Aldrich	522287	20% (w/w)
TEA	J.T. Baker	911-07	99.0%
acetic acid	Fisher	Fisher	99.0%
methanol	Fisher	Fisher	99.0%

Table A5-2: Preparation scheme of stock standard solutions in DIW for calibration curve #3.

Analyte	p-ASA	acetarsone	roxarsone
Manuf.	Sigma Aldrich	Sigma Aldrich	Riedel de Haen
Catalog No.	A9258	A0259	46726
Lot No.	047K0135	075K1465	2326X
MW (g/mol)	217.05	275.09	263.04
Purity	99.0	99.0	98.3%
Weight (g)	0.0273	0.0250	0.0267
Volume (mL)	100	100	100
Conc. (M)	1.25×10^{-3}	9.18×10^{-4}	9.98×10^{-4}
Conc. (ppm)	270.27	247.50	262.46

Table A5-3: Preparation scheme of p-ASA working standard solutions in DIW for calibration curve #3.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	1.25×10^{-4}	27.03
2	500	10	6.23×10^{-5}	13.51
3	250	10	3.11×10^{-5}	6.76
4	10	10	1.25×10^{-6}	0.27
5	3000 [(a)]	10	3.74×10^{-7}	0.081
6	1000 [(a)]	10	1.25×10^{-7}	0.027

(a) Aliquot taken from level 4 working standard.

Table A5-4: Preparation scheme of acetarsone working standard solutions in DIW for calibration curve #3.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	2500	10	2.25×10^{-4}	61.88
2	1500	10	1.35×10^{-4}	37.13
3	1000	10	9.00×10^{-5}	24.75
4	500	10	4.50×10^{-5}	12.38
5	250	10	2.25×10^{-5}	6.19
6	10	10	9.00×10^{-7}	0.25

Table 5-5: Preparation scheme of roxarsone working standard solutions in DIW for calibration curve #3.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	9.98×10^{-5}	26.25
2	500	10	4.99×10^{-5}	13.12
3	250	10	2.49×10^{-5}	6.56
4	10	10	9.98×10^{-7}	0.23
5	3000 [(a)]	10	2.99×10^{-7}	0.079
6	1000 [(a)]	10	9.98×10^{-8}	0.026

(a) Aliquot taken from level 4 working standard.

Table 5-6: Chromatographic data for p-ASA calibration curve #3. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	a [(a)]	T_f	w (min)	N	R [(b)]
1	0.551 (± 0.000)	352.4846 (± 0.094)	0.400	0.38	2.79	1.3	0.0517	1820	N/A
2	0.552 (± 0.000)	186.3005 (± 0.098)	0.401	0.38	2.81	1.3	0.0522	1787	N/A
3	0.553 (± 0.000)	88.0593 (± 0.033)	0.400	0.38	2.77	1.4	0.0524	1782	N/A
4	0.555 (± 0.001)	3.4999 (± 0.002)	0.401	0.38	2.76	1.3	0.0512	1878	N/A
5	0.555 (± 0.000)	2.155± (±0.012)	0.400	0.39	2.74	1.4	0.0507	1920	N/A
6	0.557 (± 0.001)	1.3995 (± 0.004)	0.400	0.39	2.71	1.4	0.0510	1905	N/A

(a) Selectivity calculated relative acetarsone.
(b) Resolution was not calculated since no peaks elute before p-ASA.

Table 5-7: Chromatographic data for acetarsone calibration curve #3. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	a [(a)]	T_f	w (min)	N	R [(b)]
1	0.893 (±0.001)	344.4946 (±0.235)	0.400	1.23	1.69	1.6	0.0624	3274	5.47
2	0.894 (±0.000)	206.6446 (±0.695)	0.401	1.23	1.69	1.7	0.0633	3188	5.43
3	0.894 (±0.001)	134.8168 (±0.129)	0.400	1.24	1.69	1.7	0.0645	3076	5.46
4	0.895 (±0.000)	71.0555 (±0.023)	0.401	1.23	1.70	1.7	0.0648	3049	5.35
5	0.896 (±0.001)	33.6987 (±0.021)	0.400	1.24	1.68	1.7	0.0655	2995	5.49
6	0.893 (±0.001)	1.0046 (±0.000)	0.400	1.23	1.69	1.4	0.0624	3278	5.51

(a) Selectivity calculated relative roxarsone.
(b) Resolution calculated relative p-ASA.

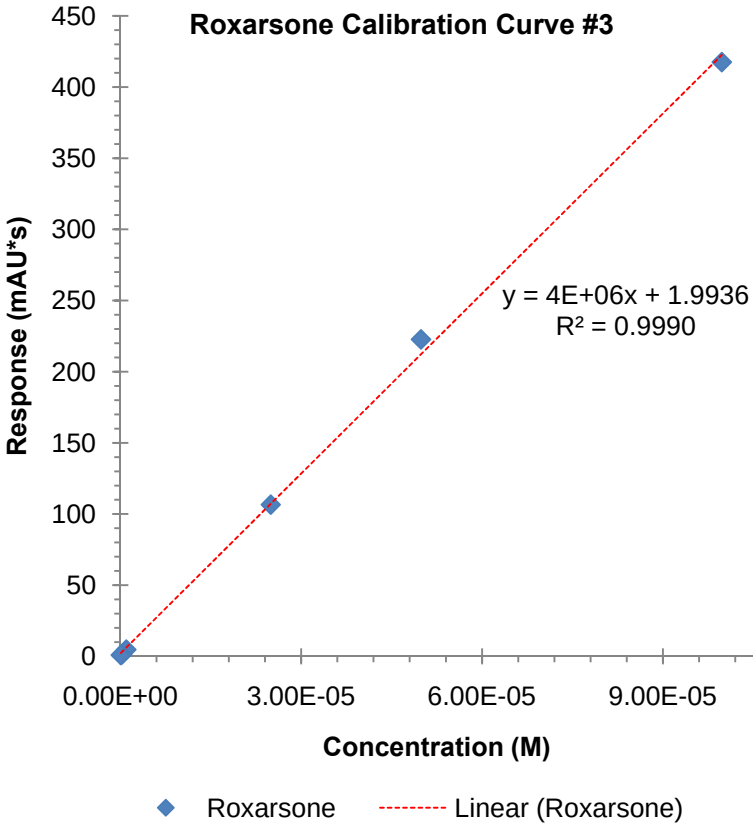
Table 5-8: Chromatographic data for roxarsone calibration curve #3. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	a [(a)]	T_f	w (min)	N	R [(b)]
1	1.430 (±0.001)	417.5209 (±0.289)	0.400	2.57	N/A	1.4	0.0624	8398	8.61
2	1.430 (±0.001)	222.7046 (±0.176)	0.401	2.57	N/A	1.4	0.0632	8195	8.48
3	1.430 (±0.001)	106.5466 (±0.035)	0.400	2.58	N/A	1.4	0.0635	8109	8.37
4	1.429 (±0.001)	4.7049 (±0.001)	0.401	2.56	N/A	1.6	0.0606	8893	8.52
5	1.430 (±0.001)	1.4675 (±0.003)	0.400	2.57	N/A	1.4	0.0591	9357	8.58
6	1.427 (±0.001)	0.9041 (±0.003)	0.400	2.57	N/A	1.3	0.0592	9282	8.78

(a) Selectivity was not calculated since no peaks elute after roxarsone.
(b) Resolution calculated relative acetarsone.

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03355	

Regression Statistics	
Multiple R	0.9995
R Square	0.9990
Adjusted R Square	0.9987
Standard Error	5.96
Observations	6

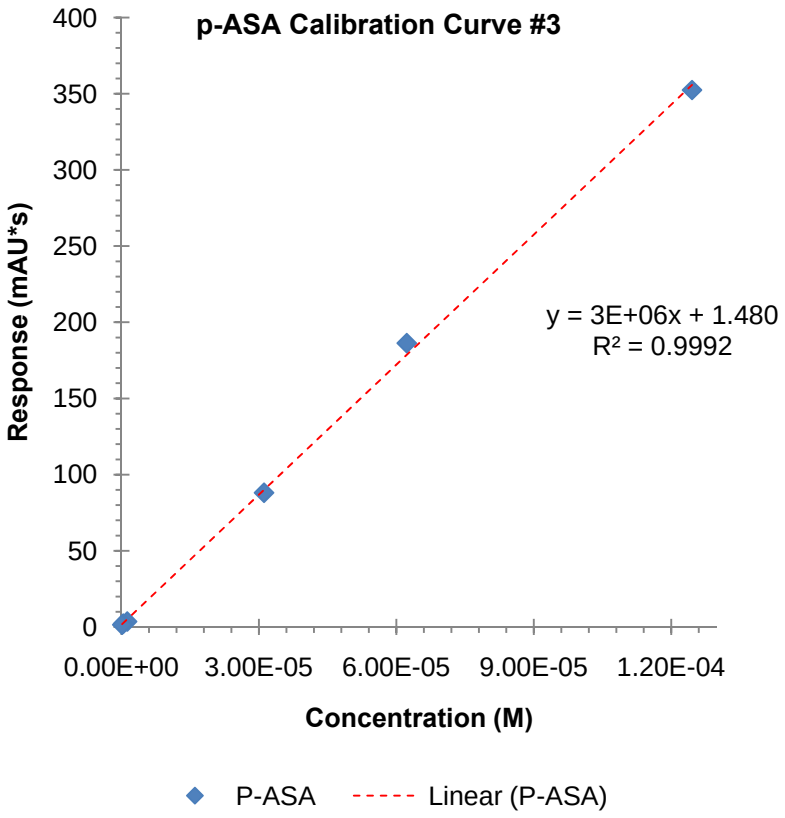


ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	140441.55	140441.55	3950.89	3.8E-07
Residual	4	142.19	35.55		
Total	5	140583.73			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1.9936	3.13	0.64	0.56	-6.70	10.68
X Variable 1	4214927.42	67056.80	62.86	0.00	4028747.90	4401106.93

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03355	

Regression Statistics	
Multiple R	0.9996
R Square	0.9992
Adjusted R Square	0.9990
Standard Error	4.37
Observations	6

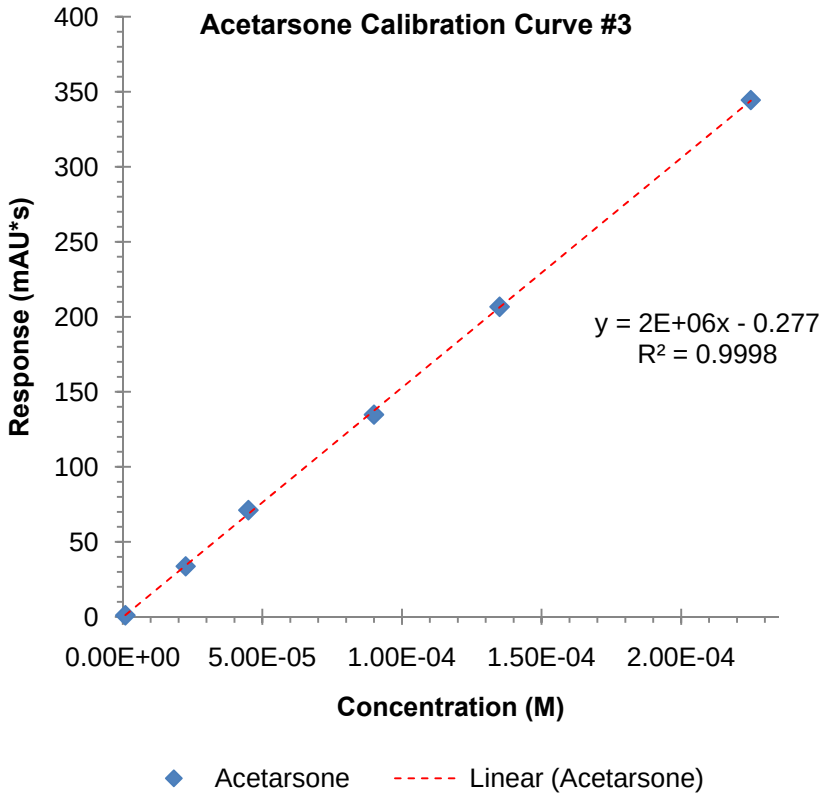


ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	99678.80	99678.80	5223.04	2.2E-07
Residual	4	76.34	19.08		
Total	5	99755.14			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1.4805	2.29	0.65	0.55	-4.89	7.85
X Variable 1	2845474.77	39372.51	72.27	0.00	2736159.17	2954790.37

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03355	

Regression Statistics	
Multiple R	0.9999
R Square	0.9998
Adjusted R Square	0.9998
Standard Error	1.84
Observations	6



ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	81257.31	81257.31	23967.46	1.0E-08
Residual	4	13.56	3.39		
Total	5	81270.88			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-0.2771	1.14	-0.24	0.82	-3.44	2.88
X Variable 1	1530935.16	9888.85	154.81	0.00	1503479.31	1558391.00

Appendix 6: Calibration curve #4 for roxarsone, p-arsanilic acid, and acetarsone.

Table A6-1: Reagents used for 85:15 mobile phase consisting of (A) 18.3mM Methyl-tributylammonium hydroxide (MTBA-OH), 1% triethylamine (TEA), pH = 6.50 and (B) methanol HPLC grade.

Reagent	Manuf.	Catalog No.	Purity
DIW	Barnstead	N/A	18.4 MΩ-cm
MTBA-OH	Aldrich	522287	20% (w/w)
TEA	J.T. Baker	911-07	99.0%
acetic acid	Fisher	Fisher	99.0%
methanol	Fisher	Fisher	99.0%

Table A6-2: Preparation scheme of stock standard solutions in DIW for calibration curve #4.

Analyte	p-ASA	acetarsone	roxarsone
Manuf.	Sigma Aldrich	Sigma Aldrich	Riedel de Haen
Catalog No.	A9258	A0259	46726
Lot No.	047K0135	075K1465	2326X
MW (g/mol)	217.05	275.09	263.04
Purity	99.0	99.0	98.3%
Weight (g)	0.0274	0.0252	0.0261
Volume (mL)	100	100	100
Conc. (M)	1.25×10^{-3}	9.07×10^{-4}	9.74×10^{-4}
Conc. (ppm)	271.26	249.48	256.56

Table A6-3: Preparation scheme of p-ASA working standard solutions in DIW for calibration curve #4.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	1.25×10^{-4}	27.13
2	500	10	6.25×10^{-5}	13.56
3	250	10	3.12×10^{-5}	6.78
4	10	10	1.25×10^{-6}	0.27
5	3000 [(a)]	10	3.75×10^{-7}	0.081
6	1000 [(a)]	10	1.25×10^{-7}	0.027

(a) Aliquot taken from level 4 working standard.

Table A6-4: Preparation scheme of acetarsone working standard solutions in DIW for calibration curve #4.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	2500	10	2.27×10^{-4}	62.37
2	1500	10	1.36×10^{-4}	37.42
3	1000	10	9.07×10^{-5}	24.95
4	500	10	4.53×10^{-5}	12.47
5	250	10	2.27×10^{-5}	6.24
6	10	10	9.07×10^{-7}	0.25

Table A6-5: Preparation scheme of roxarsone working standard solutions in DIW for calibration curve #4.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	9.75×10^{-5}	25.66
2	500	10	4.88×10^{-5}	12.83
3	250	10	2.49×10^{-5}	6.41
4	10	10	9.75×10^{-7}	0.26
5	3000 [(a)]	10	2.93×10^{-7}	0.077
6	1000 [(a)]	10	9.75×10^{-8}	0.026

(a) Aliquot taken from level 4 working standard.

Table A6-6: Chromatographic data for p-ASA calibration curve #4. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	0.546 (± 0.001)	345.0543 (± 0.884)	0.401	0.36	2.89	1.4	0.0528	1711	N/A
2	0.547 (± 0.001)	180.4203 (± 0.224)	0.401	0.36	2.92	1.4	0.0528	1713	N/A
3	0.546 (± 0.001)	86.4465 (± 0.027)	0.401	0.36	2.92	1.4	0.0530	1700	N/A
4	0.547 (± 0.000)	3.3270 (± 0.014)	0.401	0.36	2.90	1.3	0.0518	1787	N/A
5	0.547 (± 0.001)	2.0049 (± 0.005)	0.401	0.36	2.91	1.3	0.0487	2016	N/A
6	0.548 (± 0.001)	1.2534 (± 0.010)	0.401	0.37	2.90	1.4	0.0493	1977	N/A

(a) Selectivity calculated relative acetarsone.
(b) Resolution was not calculated since no peaks elute before p-ASA.

Table A6-7: Chromatographic data for acetarsone calibration curve #4. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	0.880 (± 0.000)	342.8813 (± 0.963)	0.401	1.20	1.73	1.8	0.0665	2805	5.27
2	0.880 (± 0.001)	203.2613 (± 0.017)	0.401	1.20	1.74	1.8	0.0677	2705	5.22
3	0.880 (± 0.001)	129.7613 (± 0.305)	0.401	1.20	1.74	1.7	0.0671	2757	5.24
4	0.881 (± 0.000)	67.6224 (± 0.217)	0.401	1.20	1.75	1.7	0.0665	2805	5.14
5	0.880 (± 0.001)	32.4632 (± 0.057)	0.401	1.20	1.74	1.7	0.0672	2749	5.25
6	0.880 (± 0.000)	1.0120 (± 0.001)	0.401	1.19	1.74	1.7	0.0668	2777	5.31

(a) Selectivity calculated relative roxarsone.
(b) Resolution calculated relative p-ASA.

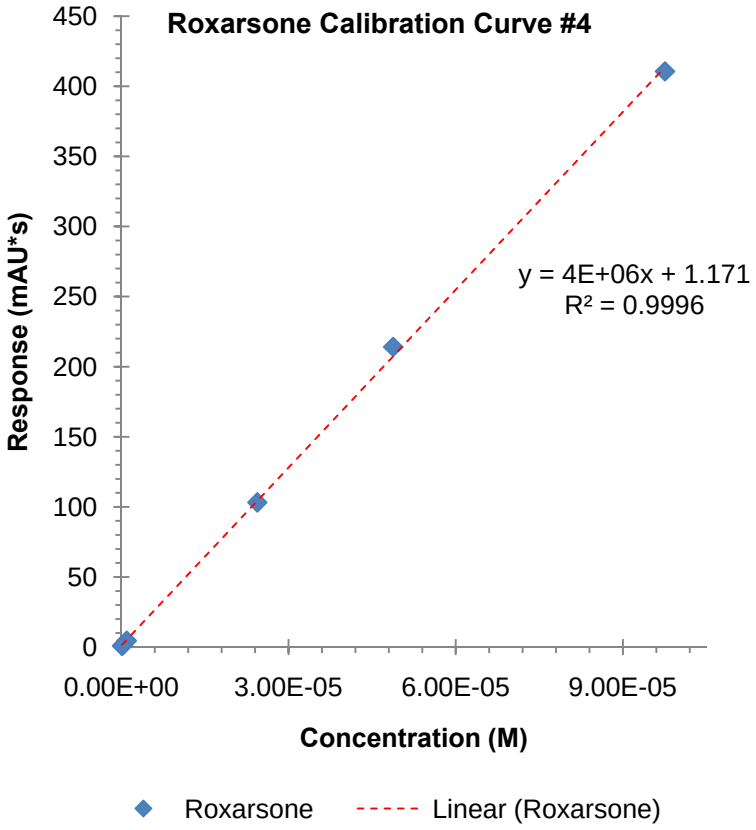
Table A6-8: Chromatographic data for roxarsone calibration curve #4. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	α [(a)]	T_f	W (min)	N	R [(b)]
1	1.410 ± 0.001	410.6819 ± 0.433	0.401	2.52	N/A	1.6	0.0675	6978	7.92
2	1.410 ± 0.001	214.1813 ± 0.206	0.401	2.52	N/A	1.5	0.0668	7125	7.88
3	1.410 ± 0.000	103.1639 ± 0.040	0.401	2.52	N/A	1.5	0.0673	7023	7.88
4	1.410 ± 0.001	4.6111 ± 0.009	0.401	2.52	N/A	1.6	0.0655	7410	8.02
5	1.409 ± 0.001	1.3316 ± 0.000	0.401	2.51	N/A	1.5	0.0619	8294	8.20
6	1.409 ± 0.000	0.8391 ± 0.001	0.401	2.51	N/A	1.4	0.0577	9530	8.50

(a) Selectivity was not calculated since no peaks elute after roxarsone.
(b) Resolution calculated relative acetarsone.

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03364	

Regression Statistics	
Multiple R	0.9998
R Square	0.9996
Adjusted R Square	0.9995
Standard Error	3.81
Observations	6

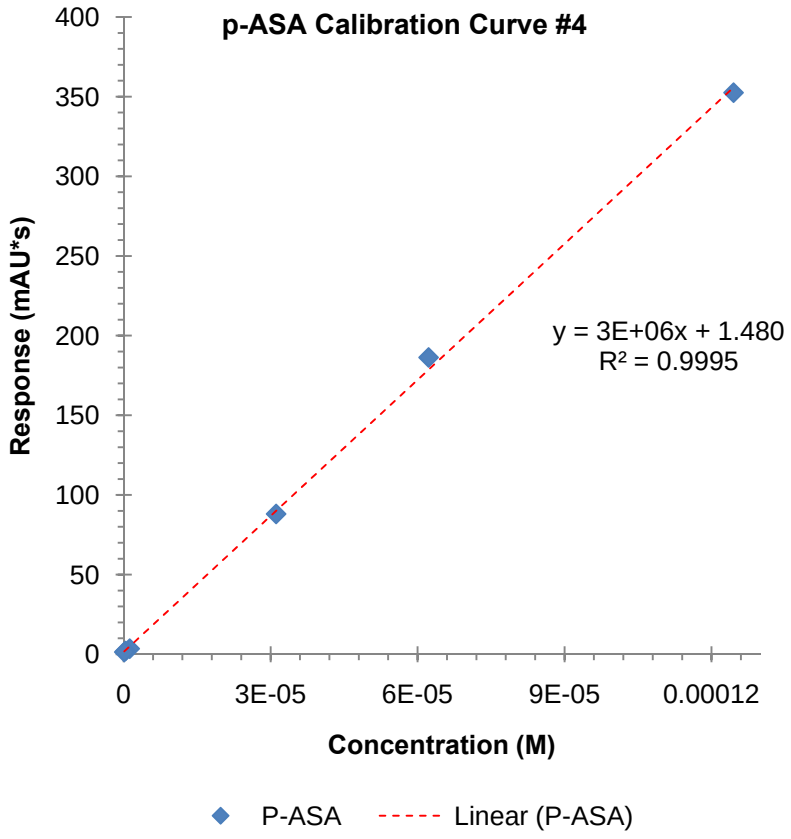


ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	135150.98	135150.98	9305.68	6.9E-08
Residual	4	58.09	14.52		
Total	5	135209.08			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1.1717	2.00	0.59	0.59	-4.38	6.73
X Variable 1	4229827.48	43847.88	96.47	0.00	4108086.26	4351568.70

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03364	

Regression Statistics	
Multiple R	0.9998
R Square	0.9995
Adjusted R Square	0.9994
Standard Error	3.41
Observations	6

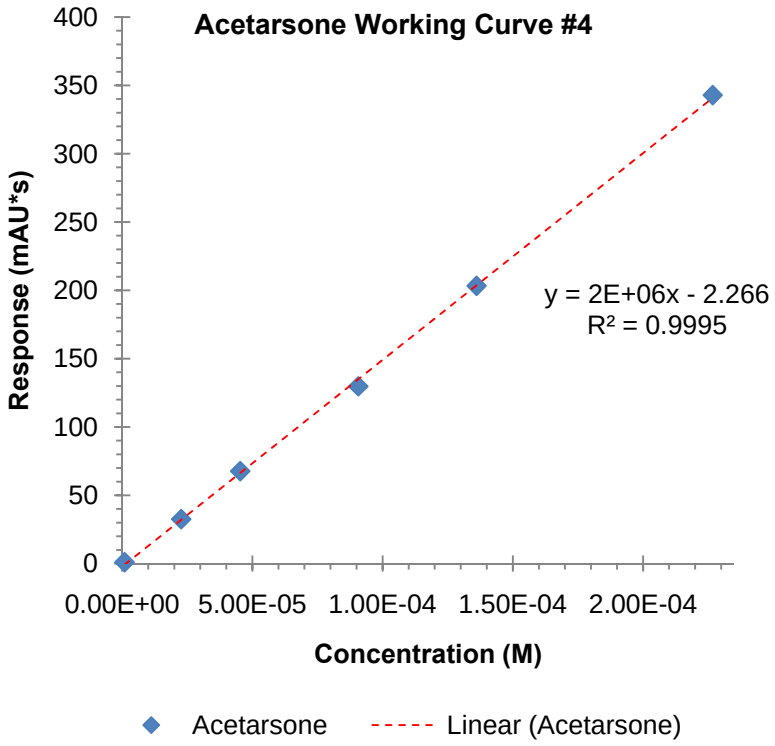


ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	95298.77	95298.77	8202.62	8.9E-08
Residual	4	46.47	11.62		
Total	5	95345.24			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1.2294	1.79	0.69	0.53	-3.74	6.20
X Variable 1	2772101.09	30607.85	90.57	0.00	2687120.06	2857082.12

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03364	

Regression Statistics	
Multiple R	0.9998
R Square	0.9995
Adjusted R Square	0.9994
Standard Error	3.04
Observations	6



ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	80689.64	80689.64	8749.94	7.8E-08
Residual	4	36.89	9.22		
Total	5	80726.53			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-2.2666	1.88	-1.21	0.29	-7.48	2.94
X Variable 1	1513470.34	16179.73	93.54	0.00	1468548.20	1558392.49

Appendix 7: Organoarsenic drugs (roxarsone) in aqueous solutions containing dissolved ions.

Table A7-1: Preparation of aqueous solutions with dissolved ions.

Description	MW	Purity	Weight	Final Vol.	Conc.
	g/mol	%	g	mL	M
NaCl	58.44	100.0	0.0421	200	3.60×10^{-4}
$NaNO_3$	84.99	99.0	0.024	200	1.40×10^{-4}
Na_2HPO_4	119.98	100.7	0.028	200	1.18×10^{-4}
Na_2SO_4	322.20	99.7	0.0216	200	3.34×10^{-4}
$NaHCO_3$	105.99	99.7	0.0325	200	1.53×10^{-4}

Table A7-2: Sample preparation scheme for roxarsone diluted with solutions containing dissolved ions.

Roxarsone Spike Vol. (mL)	0.040
Roxarsone Spike Conc. (M)	3.93×10^{-4}
Roxarsone Spike mmol	3.95×10^{-5}
Diluent Vol. (mL)	10.0
Total Vol. (mL)	10
Final Roxarsone Conc. (M)	3.93×10^{-6}

Appendix 8: Organoarsenic drugs (p-arsanilic acid, acetarsone) in aqueous solutions containing dissolved ions.

Table A8-1: Sample preparation scheme for organoarsenic drugs diluted with solutions containing dissolved ions described in Table A7-1.

Analyte	p-ASA	acetarsone	roxarsone
Spike Vol. (mL)	0.03	0.03	0.03
Spike Conc. (M)	1.25×10^{-3}	9.00×10^{-4}	9.98×10^{-4}
Spike amount (mmol)	3.74×10^{-5}	2.70×10^{-3}	2.99×10^{-3}
Diluent Vol. (mL)	10.0	10.0	10.0
Total Vol. (mL)	10	10	10
Final Conc. (M)	3.74×10^{-6}	2.70×10^{-6}	2.99×10^{-6}

Appendix 9: Analysis of organoarsenic drugs diluted with potable water (PW)

Table A9-1: Preparation scheme for organoarsenic drugs diluted with potable water.

Analyte	p-ASA	acetarsone	roxarsone
Spike Vol. (mL)	0.03	0.03	0.03
Spike Conc. (M)	1.25×10^{-3}	9.00×10^{-4}	9.98×10^{-4}
Spike amount (mmol)	3.74×10^{-5}	2.70×10^{-3}	2.99×10^{-3}
Total Vol. (mL)	10	10	10
Final Conc. (M)	3.74×10^{-6}	2.70×10^{-6}	2.99×10^{-6}

Appendix 10: Surveillance of roxarsone degradation in poultry litter leachate

Phase I of the sample preparation process consisted of transferring 250 mg of dried poultry litter to an erlenmeyer and accurately adding 300 μL of roxarsone (9.98×10^{-4} M). Approximately 10.0 mL of DIW was added to the amended poultry litter thus resulting in a roxarsone concentration of 2.91×10^{-5} M at the end of Phase I. The amended poultry litter was allowed to stand at ambient temperature for about 30 minutes to allow any interaction between roxarsone and the sample to initiate. The process was repeated with two additional samples in order to complete a total of three samples amended with roxarsone at the end of Phase I.

Control samples were prepared. A poultry litter control sample was prepared by adding 10.0 mL of DIW to 250 mg sample of dried poultry litter contained in a 500 mL erlenmeyer flask. The sample was allowed to stand for 30 minutes prior to continuing with Phase II. A roxarsone control solution was prepared by accurately transferring 300 μL of roxarsone (9.98×10^{-4} M) to a 500 mL erlenmeyer flask. 10.0 mL of DIW was added to the erlenmeyer and allowed to stand for 30 minutes. The final roxarsone concentration in an erlenmeyer flask at the end of Phase I was 2.91×10^{-5} M.

Phase II consisted of adding 100 mL of DOW to all the samples (amended poultry litter leachate and control samples) after the 30 minutes settling time. The samples were lightly agitated with a magnetic stirrer. The roxarsone final concentration was 2.72×10^{-6} M for all samples (with the exception of the poultry litter control).

A 5 mL aliquot was obtained from each sample and filtered through a 0.45 micron syringe filter for analysis immediately after Phase II was completed. A total of 60 minutes incurred from the moment in which the poultry litter was initially exposed to roxarsone until the sample aliquot was obtained for analysis. The sample solutions were prepared and stored in erlenmeyer flasks which were lightly covered with paraffin. They were placed inside a laboratory hood under ambient temperature and normal lighting conditions. A 5 mL aliquot was also obtained from each sample upon completion of 48 hours and 96 hours. The samples were filtered through a 0.45 micron syringe filter and transferred to a suitable HPLC vial. Samples corresponding to 96 hours were acquired with calibration curve #5 (which is detailed subsequently). Only roxarsone standard preparations were used to construct calibration curve #5.

Table A10-1: Reagents used for 85:15 mobile phase consisting of (A) 18.3mM methyl-tributylammonium hydroxide (MTBA-OH), 1% triethylamine (TEA), pH = 6.50 and (B) methanol HPLC grade.

Reagent	Manuf.	Catalog No.	Purity
DIW	Barnstead	N/A	18.4 MΩ-cm
MTBA-OH	Aldrich	522287	20% (w/w)
TEA	J.T. Baker	911-07	99.0%
acetic acid	Fisher	Fisher	99.0%
methanol	Fisher	Fisher	99.0%

Table A10-2: Preparation scheme of stock standard solutions in DIW for calibration curve #5.

Analyte	roxarsone
Manuf.	Riedel de Haen
Catalog No.	46726
Lot No.	2326X
MW (g/mol)	263.04
Purity	98.3%
Weight (g)	0.0265
Volume (mL)	100
Conc. (M)	9.90×10^{-4}
Conc. (ppm)	260.50

Table A10-3: Preparation scheme of roxarsone working standard solutions in DIW for calibration curve #5.

Level	Stock Aliquot (μL)	Final Vol. (mL)	Conc. (M)	Conc. (ppm)
1	1000	10	9.90×10^{-5}	26.05
2	500	10	4.95×10^{-5}	13.02
3	250	10	2.48×10^{-5}	6.51
4	10	10	9.90×10^{-7}	0.26
5	3000 [(a)]	10	2.48×10^{-7}	0.078
6	1000 [(a)]	10	9.90×10^{-8}	0.026

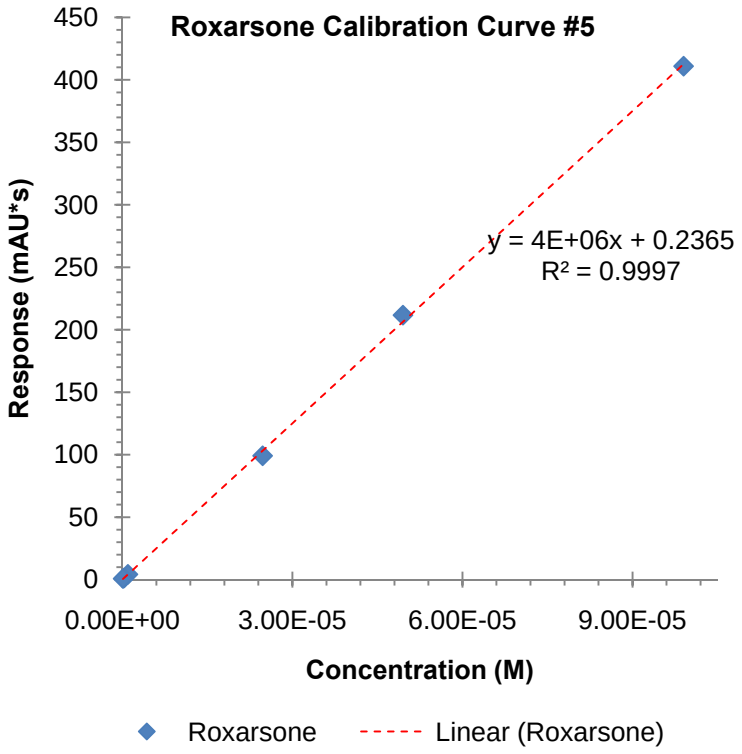
(a) Aliquot taken from level 4 working standard.

Table A10-4: Chromatographic data for roxarsone calibration curve #5. The results displayed correspond to the average of triplicate injections.

Level	t_R (min)	Area (mAU*s)	t_M (min)	k	T_f	W (min)	N
1	1.392 (± 0.001)	410.9586 (± 0.437)	0.400	2.48	1.6	0.0710	6147
2	1.392 (± 0.000)	211.7328 (± 0.136)	0.400	2.48	1.6	0.0701	6303
3	1.393 (± 0.000)	99.1587 (± 0.666)	0.400	2.48	1.6	0.0697	6398
4	1.393 (± 0.001)	4.2503 (± 0.005)	0.400	2.48	1.6	0.0652	7303
5	1.393 (± 0.001)	1.3026 (± 0.011)	0.400	2.48	1.6	0.0647	7420
6	1.393 (± 0.001)	0.8305 (± 0.002)	0.400	2.48	1.4	0.0602	8581

HPLC Parameters	
Flow (mL/min)	1.4
Temp. (ºC)	40
λ (nm)	245
Inj. Vol. (µL)	2
Column Serial Number	
SN: USUXG03364	

Regression Statistics	
Multiple R	0.9998
R Square	0.9997
Adjusted R Square	0.9996
Standard Error	3.36
Observations	6



ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	134847.29	134847.29	11928.86	4.2E-08
Residual	4	45.22	11.30		
Total	5	134892.51			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.2365	1.76	0.13	0.90	-4.66	5.14
X Variable 1	4161297.78	38100.38	109.22	0.00	4055514.16	4267081.40

Table A10-5: Initial preparation of poultry litter leachate with roxarsone (Phase 1).

Description	Litter wt.	Rox. Vol.	Rox. Conc.	Amount Rox.	DIW Vol.	Final Vol.	Rox. Conc.	Litter Conc.
	g	mL	M	mmol	mL	mL	M	mg / mL
Rox. Control	0	0.30	9.98×10^{-4}	2.99×10^{-4}	9.7	10	2.99×10^{-5}	0
Litter Control	0.2490	0	N/A	0	10	10	0	24.90
Rox. + Litter (Sa. 1)	0.2503	0.30	9.98×10^{-4}	2.99×10^{-4}	9.7	10	2.99×10^{-5}	25.03
Rox. + Litter (Sa. 2)	0.2511	0.30	9.98×10^{-4}	2.99×10^{-4}	9.7	10	2.99×10^{-5}	25.11
Rox. + Litter (Sa. 3)	0.2547	0.30	9.98×10^{-4}	2.99×10^{-4}	9.7	10	2.99×10^{-5}	25.47

Table A10-6: Final dilution of poultry litter leachate spiked with roxarsone 30 minutes after initial preparation (Phase 2).

Description	DIW Vol. Added	Final Vol.	Final Rox. Conc.	Litter Conc.
	mL	mL	M	mg / mL
Rox. Control	100	110	2.72×10^{-6}	0
Litter Control	100	110	0	2.26
Rox. + Litter (Sa. 1)	100	110	2.72×10^{-6}	2.28
Rox. + Litter (Sa. 2)	100	110	2.72×10^{-6}	2.28
Rox. + Litter (Sa. 3)	100	110	2.72×10^{-6}	2.32

Table A10-7: Chromatographic data and recovery results for roxarsone degradation poultry litter leachate (1 hour after initial preparation). Data was acquired in triplicate with calibration curve #3 (Appendix 5).

Sample	Analyte	t_R	Area	T_f	N	k	Rox. Conc.	%RE
		min	mAU*s				M	
Litter Control	Unk. 1A	0.653	251.6384	N/A	3010	0.61	N/A	
Rox. Control	Rox.	1.415	14.7566	1.4	7641	2.49	$3.03 (\pm 1.1) \times 10^{-6}$	11.3
Poultry Litter (Sa. 1)	Unk. 1A	0.654	330.0611	N/A	2930	0.61	N/A	
	Rox.	1.417	13.8918	1.3	8489	2.50	$2.82 (\pm 1.1) \times 10^{-6}$	3.7
Poultry Litter (Sa. 2)	Unk. 1A	0.655	219.3639	N/A	2955	0.62	N/A	
	Rox.	1.416	13.8923	1.4	8359	2.50	$2.82 (\pm 1.1) \times 10^{-6}$	3.7
Poultry Litter (Sa. 3)	Unk. 1A	0.657	210.3151	N/A	2960	0.62	N/A	
	Rox.	1.415	13.8870	1.3	8475	2.49	$2.82 (\pm 1.1) \times 10^{-6}$	3.7

Table A10-8: Chromatographic data and recovery results for roxarsone degradation poultry litter leachate (48 hours after initial preparation). Data was acquired in triplicate with calibration curve #4 (Appendix 6).

Sample	Analyte	t_R	Area	T_f	N	k	Rox. Conc.	%RE
		min	mAU*s				M	
Litter Control	Unk. 1A	0.679	0.8741	N/A	3748	0.69	N/A	
Rox. Control	Rox.	1.407	13.8560	1.6	7123	2.51	$3.00 (\pm 0.69) \times 10^{-6}$	10.2
Poultry Litter (Sa. 1)	Unk. 1A	0.678	1.2488	N/A	2592	0.69	N/A	
	Rox.	1.407	8.1162	1.6	6993	2.51	$1.64 (\pm 0.70) \times 10^{-6}$	-39.7
Poultry Litter (Sa. 2)	Unk. 1A	0.678	0.8403	N/A	3574	0.69	N/A	
	Rox.	1.406	9.4534	1.5	7638	2.51	$1.96 (\pm 0.69) \times 10^{-6}$	-28.1
Poultry Litter (Sa. 3)	Unk. 1A	0.679	0.9535	N/A	3001	0.69	N/A	
	Rox.	1.405	10.0807	1.6	7457	2.50	$2.11 (\pm 0.69) \times 10^{-6}$	-22.6

Table A10-9: Chromatographic data and recovery results for roxarsone degradation poultry litter leachate (96 hours after initial preparation). Data was acquired in triplicate with calibration curve #5.

Sample	Analyte	t_R	Area	T_f	N	k	Rox. Conc.	%RE
		min	mAU*s				M	
Litter Control	Unk. 1A	0.675	0.7828	N/A	3376	0.69	N/A	
Rox. Control	Rox.	1.393	12.9956	1.6	6270	2.48	$3.07 (\pm 0.62) \times 10^{-6}$	12.6
Poultry Litter (Sa. 1)	Unk. 1A	0.674	1.7265	N/A	1993	0.68	N/A	
	Rox.	1.398	1.7036	1.4	5345	2.49	$3.53 (\pm 0.63) \times 10^{-6}$	-87.0
Poultry Litter (Sa. 2)	Unk. 1A	0.673	1.5097	N/A	1920	0.68	N/A	
	Rox.	1.395	1.5522	1.5	6711	2.49	$3.16 (\pm 0.63) \times 10^{-6}$	-88.4
Poultry Litter (Sa. 3)	Unk. 1A	0.673	1.4695	N/A	2181	0.68	N/A	
	Rox.	1.392	2.0927	1.6	7296	2.48	$4.46 (\pm 0.63) \times 10^{-6}$	-83.6

Appendix 11: Surveillance of Roxarsone Degradation in TiO_2 suspensions

A 1 mg/mL suspension of TiO_2 was used to effectively remove roxarsone from a 10 mL water sample. The sample was prepared in duplicate. One sample was exposed to UV radiation (λ = 321 nm) for one (1) hour. The second sample remained under normal lighting conditions in the laboratory hood. Each was accompanied by a roxarsone control sample of the same concentration. A 5 mL aliquot was removed from the reaction vessel and centrifuged for about two (2) minutes. The supernatant was filtered through a 0.45 micron syringe filter and transferred to a suitable HPLC vial. The equipment parameters and calibration curve are described in Appendix 3.

Table A11-1: Sample preparation scheme for roxarsone in TiO_2 suspensions for surveillance.

Description	Rox. Vol.	Rox. Conc.	Amount Rox.	Final Vol.	TiO_2 Wt.	TiO_2 Conc.	Rox. Conc.	
	mL	M	mmol	mL	mg	mg / mL	M	ppm
Rox. Control (Non-UV)	0.4	9.98×10^{-4}	3.99×10^{-4}	10	0	0.00	3.99×10^{-5}	10.5
Rox. Control (UV)	0.4	9.98×10^{-4}	3.99×10^{-4}	10	0	0.00	3.99×10^{-5}	10.5
Rox. + TiO_2 (Non-UV)	0.4	9.98×10^{-4}	3.99×10^{-4}	10	10.1	1.01	3.99×10^{-5}	10.5
Rox. + TiO_2 (UV)	0.4	9.98×10^{-4}	3.99×10^{-4}	10	10.2	1.02	3.99×10^{-5}	10.5