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SEPARATION OF PHENYLARSONIC COMPOUNDS BY ION PAIRING-REVERSED PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract. Phenylarsonic compounds are extensively used as feed additives to promote growth and to control disease in the poultry industry. A method was conducted that allowed the analysis of arsenic animal feed additives by ion pairing-reversed phase HPLC (IP-RP-HPLC) with a UV detection using an octadecylsilylsilica column. The separation of anionic arsenic compounds [3-nitro-4-hydroxyphenylarsonic acid (3-NHPAA), *p*-arsanilic acid (*p*-ASA) and phenylarsonic acid (PA)] was accomplished using 5 mM tetrabutylammonium chloride as the ion pairing reagent in aqueous mobile phase containing 3 mM malonic acid and 5% methanol at a flow rate of 1.4 mL min^{-1}. The influence of mobile phase pH on the separation was studied; the optimum pH found was 5.85. The detection limit obtained for 3-NHPAA was 0.43 ng As.

Keywords: arsenic, animal feed additives, ion pairing-reversed phase-high performance liquid chromatography

Abstrak. Sebatian fenilarsonik digunakan dengan meluas sebagai bahan tambah makanan ternakan bagi membantu proses tumbesaran dan mengawal penyakit di dalam industri ternakan ayam. Satu kaedah dijalankan bagi analisis arsenik di dalam bahan tambah makanan ternakan secara HPLC fasa terbalik-pasangan ion dengan pengesan ultralembayung. Dengan menggunakan turus oktadekilsililsilika, pemisahan sebatian arsenik anion [asid 3-nitro-4-hidroksifenilarsonik (3-NHPAA), asid *p*-arsanilik (*p*-ASA) dan asid fenilarsonik (PA)] telah diperoleh menggunakan tetrabutilammonium klorida 5 mM sebagai reagen pasangan ion di dalam fasa bergerak yang mengandungi asid malonik 3 mM dan 5% metanol pada kadar alir 1.4 mL min^{-1}. Pengaruh pH fasa bergerak ke atas pemisahan dikaji dan didapati pH optimum adalah 5.85. Had pengesanan yang diperoleh bagi 3-NHPAA ialah 0.43 ng As.

Kata kunci: arsenik, bahan tambah makanan ternakan, HPLC fasa terbalik-pasangan ion

1.0 INTRODUCTION

A number of phenylarsonic compounds have been used as feed additives to control coccidial intestinal parasites which causes considerable economic loss in the poultry industry. 3-nitro-4-hydroxyphenylarsonic acid (roxarsone, 3-NHPAA) and *p*-arsanilic acid (*p*-ASA) have been shown to have these therapeutic properties and also act as growth promoters [1–2]. Other phenylarsonic compounds that have not been used

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as feed additives are phenylarsonic acid (PA) and *o*-arsanilic acid (*o*-ASA), which can serve as the chromatographic internal standard. Figure 1 shows the structures of these arsenic compounds.

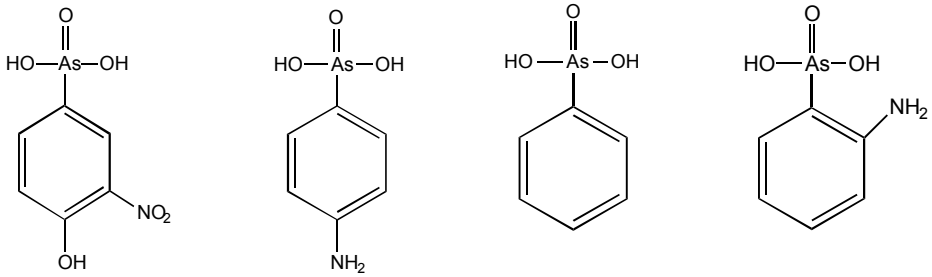


Figure 1 Structure of Four Arsenic Compounds Studied: 3-nitro-4-hydroxyphenylarsonic acid (3-NHPAA), *p*-arsanilic acid (*p*-ASA), phenylarsonic acid (PA) and *o*-arsanilic acid (*o*-ASA)

Roxarsone (3-NHPAA) is the most commonly used organic arsenic feed additive. Very little is retained in chicken meat, and most of the fed roxarsone is excreted unchanged [3]. Arsenic in the poultry litter is added to the environment during disposal of the chicken litter and being used as fertilizer on fields. The degradation pathways of this arsenic compound in the soils and natural waters have indicated the presence of roxarsone, arsenite [As^{3+}] and dimethylarsinate [DMAA].

Little work on arsenic separation has dealt with the phenylarsonic compounds; the majority of reported works were on separation of arsenite, arsenate, monomethylarsonic acid [MMAA] and dimethylarsinic acid [DMAA]. Le and Ma [4] reported a significant advancement whereby they were able to separate arsenite, arsenate, MMAA and DMAA within 2–4 mins by using HPLC guard columns coupled with hydride generation-atomic fluorescence spectrometry.

A limited number of methods have been developed and used for the determination of roxarsone and their metabolites. A liquid chromatography method has been developed for the determination of roxarsone in poultry feed using solid phase extraction in combination with reversed phase liquid chromatography with UV detection [1]. Hyphenated techniques involving high performance liquid chromatography (HPLC) and element sensitive detectors, such as ICP-MS and atomic spectrometry have been used for the determination of roxarsone and other arsenical animal feed additives [2, 5]. Most of these reported works require long analysis time. Most recently, a high speed separation of arsenic compounds has been reported using narrow bore HPLC column coupled with ICP-MS detection [6]. The arsenic compounds are mostly present in the ionic forms at neutral pH, thus HPLC in the ion-pair [7–8] and suppressed ionization [5-11] modes have been studied for the separation of ionic arsenic compounds. Extensive studies were done on the optimization of

HPLC parameters, including the composition, pH and the flow rate of the mobile phase.

Conventional HPLC column packing of 5 μm particle size can take more than 30 min based on our previous study [12]. This study reports on a method for the rapid separation of 3-NHPAA, *p*-ASA and PA using ion pair-reversed phase HPLC 3 μm particle size column.

2.0 EXPERIMENTAL

2.1 Instrumentation

The HPLC system used consisted of a Shimadzu LC-9A pump (Tokyo, Japan), a Rheodyne six-port sample injector (Model 7125, Cotati USA) with a 20- μL sample loop. A stainless steel reversed-phase C_{18} silica column (60 mm × 4.6 mm, 3 μm particle size)(Hewlett-Packard) was used for the separation. A UV detector (Shimadzu SPD-6A) was used for the detection at 254 nm wavelength. The chromatograms were recorded using a Waters 746 Data Module (Waters Millipore USA).

All chromatographic elutions were isocractic and carried out at room temperature.

2.2 Reagents

Standards of 3-nitro-4-hydroxyphenylarsonic acid (3-NHPAA) (TCI-GR, Japan), *p*-arsanilic acid (*p*-ASA) (TCI-EP, Japan), *o*-arsanilic acid (*o*-ASA) (Sigma, Germany) and phenylarsonic acid (PA) (TCI-GR, Japan) were prepared in 18.4 MΩ-cm distilled deionized water (Barnsted Easypure RF). Standard solutions containing less than 1 μg/mL As were prepared fresh daily by serial dilution with distilled deionized water from 10 μg/mL As standard solutions. Standard solution containing 500 ng/mL 3-NHPAA, 500 ng/mL *p*-ASA and 20 μg/mL PA (which was added as the internal standard) was injected onto the HPLC system.

For the IP-RP-HPLC separations, a HPLC grade methanol (BDH, Poole, England) and distilled deionized water was used as the eluent. Tetrabutylammonium chloride (TBACl) (Fluka Chemika, Switzerland) was used as the ion-pairing reagent. Malonic acid (Merck, Germany) was also added to the mobile phase. The mobile phase pH was adjusted using dilute 3M NH_4OH solution.

Commercial roxarsone [3-NHPAA], marketed under the tradename of Nitro-ten, imported by Vet Visions (M) Sdn. Bhd., was obtained by courtesy of Veterinary Research Institute Ipoh, Malaysia.

2.3 Procedures

Mobile phase containing 5 mM TBACl, 3 mM malonic acid and 5% methanol were prepared in distilled deionized water, using a modified version of Le *et al.* [4]. The mobile phase pH was adjusted in the pH range of 5.49 – 6.66 with dilute ammonium

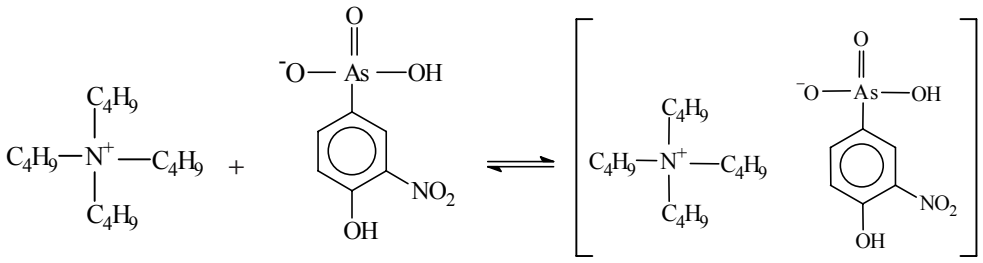
hydroxide. The mobile phase was filtered through a 0.45 μm filter (Millipore, USA) and degassed by the simultaneous application of vacuum and ultrasound, prior to use.

The C_{18} column was equilibrated with the ion-pairing reagent prior to sample injection. The equilibration process took up approximately 500 mL of mobile phase.

Commercial Nitro-ten tablet was analyzed by dissolving one tablet in 2.3 L of distilled deionized water (18.4 MΩ-cm). The binder for the tablet was insoluble and the solution was filtered through a 0.45 μm disposable filter (Millipore, USA) prior to injection onto the HPLC system.

3.0 RESULTS AND DISCUSSION

The separation of phenylarsonic compounds by HPLC is pH dependent. The pK_a values reported for some of these arsenic compounds were as follows: pK_a [3-NHPAA] = 3.41, pK_a [p-ASA] = 2, 4.02, 8.92 and pK_a [PA] = 3.47, 8.48 [13]. At neutral pH, these arsenic compounds are present as anions. Thus, reversed-phase HPLC in the ion-pairing mode can be used to separate anionic, neutral and cationic arsenic species with suitable counterions, e.g. tetramethylammonium cation or tetrabutylammonium cation in the mobile phase [3, 6]. The counterion used, tetrabutylammonium cation, forms an ion pair with the anionic arsenic compound, shown in the equation as follows:



counterion arsenic compound ion pair

The resulting ion pair gave additional interactions with the C_{18} stationary phase for a better separation.

Studies were performed to achieve optimum separation by adjusting mobile phase parameters, including composition of organic modifier, pH, flow rate, and ion-pairing reagent type and concentration. Long separation times and equilibration times are often required for the separation of a mixture of arsenic compounds.

3.1 Mobile Phase Flow Rate

A conventional HPLC column (4.6 mm i.d. column packed with 5 μm particle size)

normally operates at mobile phase volumetric flows ranging from 0.8 to 1.1 mL min^{-1}. The mobile phase flow rate influences the efficiency of the separation of the arsenic compounds. Using a 3 μm particle packing, a mixture of two phenylarsonic acids (*p*-ASA and 3-NHPAA) were tested at three different flow rates of 1.0, 1.2 and 1.4 mL min^{-1}. Higher flow rates were not investigated to avoid excessive back pressure due to the smaller 3 μm particle packing of the HPLC column. Figure 2 shows the effect of flow rate on the separation.

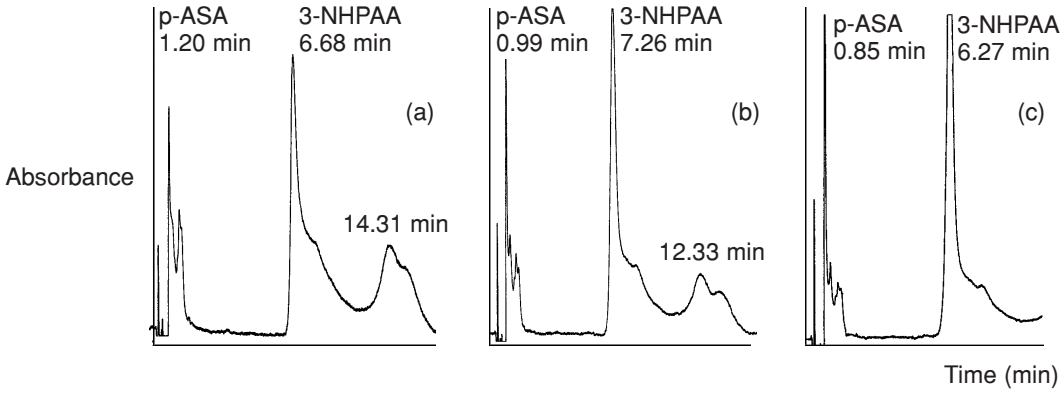


Figure 2 IP-RP-HPLC Chromatograms of p-ASA and 3-NHPAA Obtained Using 5 mM TBACl, 3 mM malonic acid and 5% methanol mobile phase at flow rates of (a) 1.0, (b) 1.2 and (c) 1.4 mL min^{-1}.

At a low flow rate of 1.0 mL min^{-1}, several peaks appeared instead of just two peaks corresponding to the two phenylarsonic acids standard. Peak tailing was evident for both arsenic standards indicating extensive retention of the phenylarsenicals by the stationary phase. Changing the flow rate to 1.2 mL min^{-1} showed a reduction in the separation times. However, peak tailings were still present. At a flow rate of 1.4 mL min^{-1}, better peaks shape were obtained, and the separation times were reduced further and elution completed in 7 min. Therefore, a mobile phase flow rate of 1.4 mL min^{-1} was used for the remainder of the study.

3.2 Effect of pH

Tetrabutylammonium chloride at a concentration of 5 mM was used as the ion-pairing reagent for the separation of two phenylarsonic acids. The mobile phase also contained 3 mM malonic acid. The mobile phase pH was adjusted between 5.49 and 6.66 by using dilute 3M ammonium hydroxide solution.

The chromatograms shown in Figure 3 exhibits the effect of pH on the separation of phenylarsonic acids. Present in the mixture was phenylarsonic acid (PA). This compound served as an internal standard and has not been used as an animal feed additives.

p-ASA, PA and 3-NHPAA are all dissociated at the pH range studied. These compounds contain acidic functional groups and their dissociations depend on the pK_a and mobile phase pH. The chromatograms presented in Figure 3 show good separation for the three arsenic compound studied. The elution order was in agreement with that previously reported by Spiros *et al.* [5], i.e. *p*-ASA eluted first, followed by PA and 3-NHPAA. A compound with high apparent charge is expected to interact strongly with the ion-pairing reagent, resulting in longer retention times. The retention behavior of *p*-ASA differed slightly from PA and 3-NHPAA. The peak for *p*-ASA indicated that the standard consisted of several minor components. In all the chromatograms a sharp peak immediately after injection was obtained which did not corresponds to any of the injected standards.

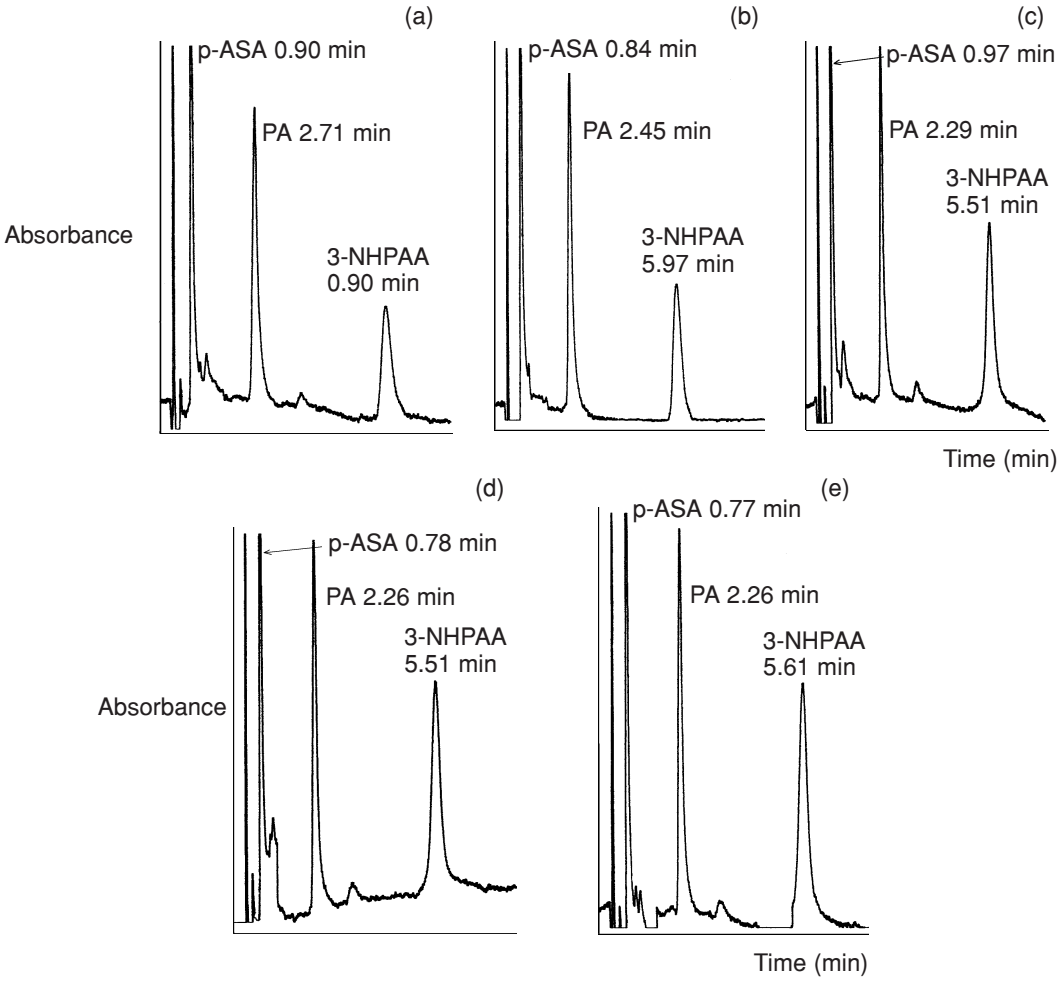


Figure 3 Effect of pH on the Separation of a Mixture of Three Arsenic Compounds Obtained Using 5 mM TBACl, 3 mM malonic acid and 5% methanol mobile phase pH at (a) 5.49, (b) 5.85, (c) 6.02, (d) 6.27 and (e) 6.66

It was observed that an increase in the mobile phase pH resulted in reduced retention times for the three phenylarsonic compounds studied. The best chromatogram was observed using a mobile phase of pH 5.85.

Another phenylarsonic compound tested, which is not an animal feed additives, was *o*-arsanilic acid (*o*-ASA). Figure 4 shows the chromatograms for *o*-ASA at the pH range studied. It was observed that *o*-ASA elute at the same time as PA. Therefore, as a mixture, these two arsenic compounds will not be separated under the above conditions.

As can be observed in Figure 3 and 4, the sharp peak immediately after injection appeared on all the chromatograms corresponds to the distilled deionized water which was used in the standards and mobile phase preparation.

The limit of detection for 3-NHPAA, PA and *o*-ASA are listed in Table 1. The value for *p*-ASA was undetermined. The limit of detection for the arsenic species were determined based on the calculation of S/N = 2.

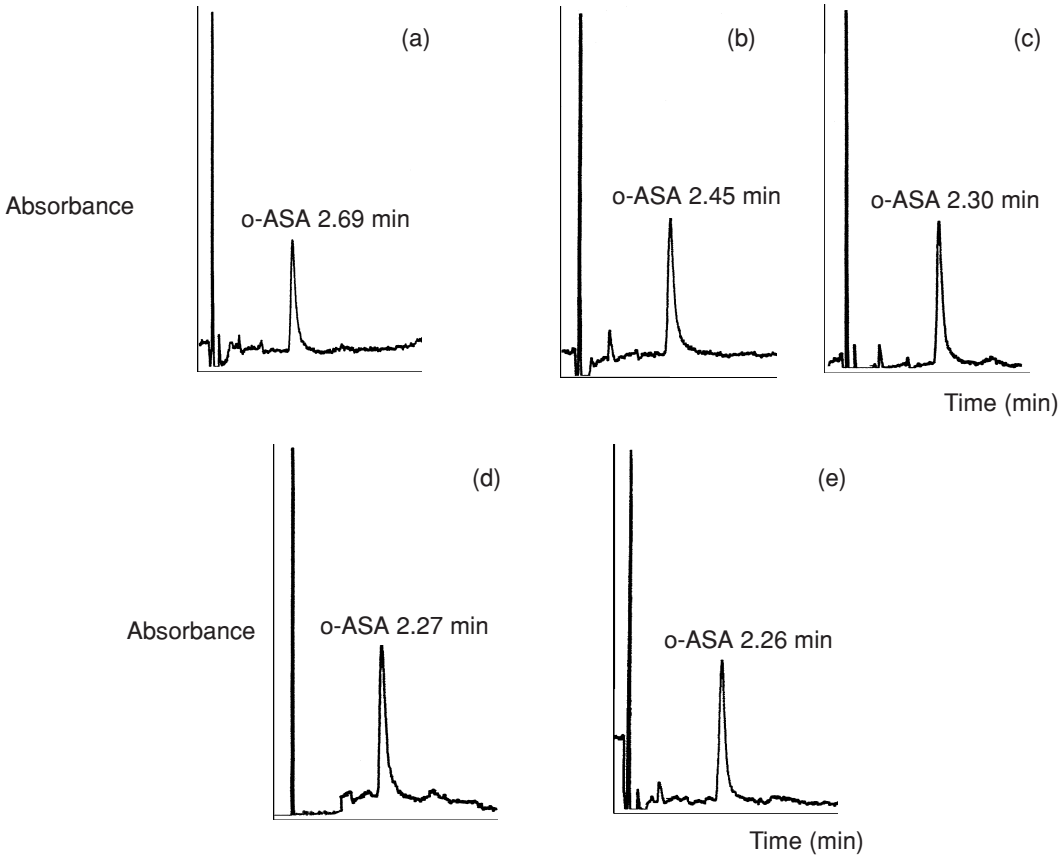


Figure 4 IP-RP-HPLC Separation of o-ASA Using Mobile Phase Containing 5 mM TBACl, 3 mM malonic acid and 5% methanol of pH (a) 5.49 (b) 5.85 (c) 6.02 (d) 6.27 (e) 6.66

Table 1 Limit of Detection for the Arsenic Compounds

	As, μg/L	As, ng
3-NHPAA	22	0.43
PA	870	17.4
o-ASA	44	0.87

The limit of detection for 3-nitro-4-hydroxyphenylarsonic acid is fairly good and this value is below the limit for the current maximum contaminant level of arsenic in drinking water which is 50 μg/L in the United States [13].

3.3 Analysis of Real Sample

The arsenical feed additive 3-NHPAA in Nitro-ten was also analyzed using IP-RP-HPLC. One tablet of Nitro-ten was dissolved in 2.3 L of water according to the procedure recommended by the manufacturer. PA was added as the internal standard prior to injection. 3-NHPAA was the major arsenic containing species found in the feed additive (Figure 5). This is in agreement with results previously reported on the analysis of Nitro-ten tablet.

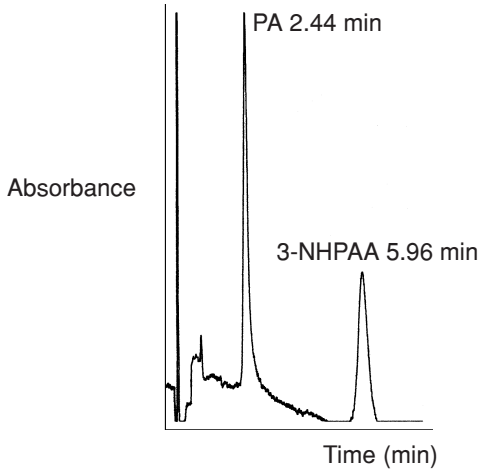


Figure 5 Chromatogram of Nitro-ten tablet, an arsenical feed additive. IP-RP-HPLC Using 5 mM TBACl, 3 mM malonic acid and 5% methanol mobile phase at pH 5.85 other HPLC Condition as in Text.

4.0 CONCLUSION

Phenylarsonic compounds were separated and determined by IP-RP-HPLC using a mobile phase of 5 mM TBACl, 3 mM malonic acid and 5% methanol at a pH of

5.85. The analysis time was relatively fast thus facilitating more sample throughput.

The analytical technique will be applied to the analysis of chicken litter which are being used as manures in vegetable farms. The fate of roxarsone and its degradation pathways in the soils and natural waters need further investigation.

5.0 ACKNOWLEDGEMENT

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