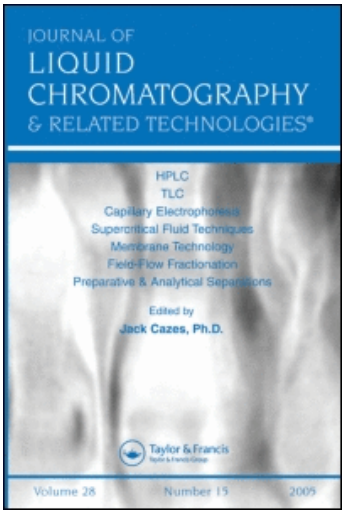

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION FOLLOWING MICROWAVE ASSISTED EXTRACTION OF 3-NITRO-4-HYDROXYPHENYLARSONIC ACID FROM SWINE LIVER, KIDNEY, AND MUSCLE

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ABSTRACT

A simple and rapid analytical method is described for the determination of 3-nitro-4-hydroxyphenylarsonic acid (roxarsone, 3-nitro) in tissues. It involves extraction of 3-nitro from swine tissues by microwave-assisted process (MAP™) followed by high performance liquid chromatography using a PRP-1 column. The compound is detected in visible region at 410 nm after post-column reaction with 0.15 N sodium hydroxide. The detection

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limit is estimated to be 0.25 $\mu g\ g^{-1}$ for 3-nitro, equivalent to about 0.2 $\mu g\ g^{-1}$ of arsenic. Official tolerances for arsenic in liver and muscle are set at 2.0 $\mu g\ g^{-1}$ and 0.5 $\mu g\ g^{-1}$, respectively. The method was employed to detect 3-nitro residues in incurred tissue samples from pigs fed 150 mg kg^{-1} of 3-nitro for a 28-d period.

INTRODUCTION

3-Nitro (3-nitro-4-hydroxyphenylarsonic acid; roxarsone) is recommended at a feed level of 25-50 mg kg^{-1} as a general growth promotant for swine (1). The compound has been used in Canada in swine production for over 25 years and is considered one of the "old" drugs which was registered without sufficient nutritional and toxicological data.

Edmonds and Baker noted an improvement in the growth of young pigs (2) when raised on diet supplemented with 3-nitro. However, Akhtar et al. (3,4) did not observe any beneficial effect of 3-nitro on the growth of young as well as growing-finishing pigs.

It is possible that swine fed a 3-nitro medicated diet may accumulate the arsenical compound as well as its metabolites in liver, kidney and muscle. A 5-day withdrawal is regulated prior to slaughter of treated animals. Official tolerances for arsenic in liver and muscles are set at 2.0 $\mu g\ g^{-1}$ and 0.5 $\mu g\ g^{-1}$, respectively (5).

There are two official methods for determination of 3-nitro in feeds and premixes. One method involves extraction of feed with 2% K_2HPO_4, precipitation of proteins at isoelectric point followed by treatment with activated charcoal at pH 12. The resultant colored solution is analyzed by visible spectrophotometry at 410 nm (6). The second method, on the other hand, is not specific for 3-nitro, but determines the total arsenic. The method consists of extraction of samples with aqueous

ammonium carbonate solution followed by direct analysis on a graphite furnace atomic absorption spectrometry (AAS) for total arsenic (7). Recently, a high performance liquid chromatography (HPLC) method using ultraviolet detection at 243 nm without post-column reaction has appeared in the literature for roxarsone (3-nitro) in poultry feed (8).

There is no method to detect intact 3-nitro in tissues. Currently, the total residues of arsenic are determined by atomic absorption method following a wet digestion step (9). This paper describes a simple, rapid, reproducible and cost effective extraction technique involving microwave assisted process (MAP™) (10, 11), and detection by visible spectrometry at 410 nm for determination of 3-nitro in swine tissues (liver, kidney and muscle). The method was also applied to detect intact 3-nitro in incurred samples derived by feeding the compound for 28 days.

MATERIALS

Chemicals and reagents

3-Nitro (3-nitro-4-hydroxyphenylarsonic acid; roxarsone) was obtained from Sigma (St.Louis, MO.). Trifluoroacetic acid was HPLC/Spectro Grade, 99.5% pure from Chromatographic Specialties (Brockville, ON.). Glacial acetic acid (aldehyde free) was 'Baker Analyzed' reagent (J.T. Baker, Toronto, ON.). Isopropyl alcohol and ethanol were HPLC grade obtained from Caledon (Georgetown, ON.).

Instrumentation

The liquid chromatography system consisted of two model 302 solvent delivery pumps, a model 803C manometric module, and an auto-sampling injector model

231-401 with a 50 μL loop (all from Gilson). The detector was a Waters 486 Tunable Absorbance Detector set at 410 nm. The temperature of the column was kept at 30°C using a Waters Temperature Control Module. The column used was a PRP-1 (Hamilton), 10 μm, 250 x 4.1 mm, 75 Å and a PRP-1 guard column of 2 μm, 25 x 2.3 mm i.d. A Hewlett-Packard HP3394 integrator collected the chromatographic data.

For the extraction of tissue samples a Panasonic microwave oven model NE-7850C was used as well as a sonicator (Heat Systems) and an homogenizer (Brinkmann). Extracts were centrifuged in a refrigerated centrifuge (Mistral 3000*i* ,rotor head 43124-129, Johns Scientific Inc.)at -5°C. Extracts were evaporated to dryness in vacuo with a rotary evaporator Büchi, model RE121 (Brinkmann).

METHOD

Biological Samples

Weanling pigs were fed 0, 50 and 150 mg kg^{-1} of 3-nitro for 28 days. At the end of the experiment, animals were killed, the whole-liver, both kidneys and a portion of hind-quarters muscle were passed through a meat grinder, frozen and stored at -20°C until analyzed. Details on animal experimental procedure are reported elsewhere (4).

Standards and spiked samples

3-Nitro working standard solutions of 10, 20, 30, 40 and 60 μg mL^{-1} were prepared using mobile phase (water-isopropyl alcohol- trifluoroacetic acid 94:6:0.1 v/v/v) and were kept at room temperature.

One gram of previously frozen ground tissue was weighed into a 125 mL screw-capped specimen container.

Individual tissue samples were spiked with 100 μL of the appropriate 3-nitro working standard solutions to produce samples with tissue equivalencies of 1.0, 2.0, 3.0, 4.0 and 6.0 μg g^{-1}, respectively.

Extraction of 3-nitro from tissues

Spiked tissue samples were left to stand at room temperature for about 30 minutes prior to extraction by MAP™, homogenization or sonication. Twenty-five mL of absolute ethanol and 500 μL glacial acetic acid were added to each container and mixed gently. The samples were then homogenized or sonicated for 1.5 minutes or individually irradiated in a microwave oven for 9 seconds at maximum power but the content was not allowed to boil. The supernatant from the microwave extraction was immediately decanted into a 50 mL screw-capped polypropylene centrifuge tube. Samples from the three extraction techniques were then centrifuged at 1380 x g for 20 minutes at 0°C. The clear supernatant was decanted into a 250 mL round-bottom flask and evaporated to dryness under vacuum with a waterbath temperature of 69°C. Two mL mobile phase was added to dissolve all residue adhering to the glass and was transferred into a microfuge tube. The extract was centrifuged at 16000 x g for 8 minutes. The sample was transferred into a glass sample vial for HPLC analysis. An aliquot of 50 μL was injected onto the column. Standards of 3-nitro were subjected to the identical irradiation and extraction procedures prior to analyses. Incurred samples were also treated similarly to determine 3-nitro residues.

HPLC procedure

The mobile phase, water-isopropyl alcohol-trifluoroacetic acid (94:6:0.1 v/v/v), was filtered

through a 0.45 μm filter (Nylaflo®, Gelman Sciences), degassed under vacuum and delivered at a flow rate of 1.0 mL min^{-1}. Sodium hydroxide solution, 0.15 N, was provided at a flow rate of 1.0 mL min^{-1} by a second pump and mixed through a low pressure tee with the column eluate before detection at 410 nm. The purpose of the post-column reaction with sodium hydroxide was to generate a chromophore of 3-nitro to be visualized at 410 nm.

RESULTS AND DISCUSSION

Existing methods for the extraction of 3-nitro from feed involve laborious, lengthy and expensive clean-up steps before samples can be analyzed (6-9). We evaluated the classical extraction techniques of homogenization and sonication with the recently patented microwave-assisted process (MAP™) as a rapid, cost-effective and reproducible alternate method (10,11). Recoveries of spiked 3-nitro from liver, kidney and muscle using different extraction methods are listed in Table 1. Table 1 shows that recoveries of 3-nitro from spiked tissue samples were superior with the MAP™ technique than with the other two techniques.

Chromatograms of standard 3-nitro and extracts of spiked liver samples by MAP™, homogenization and sonication, shown in Figure 1, show that 3-nitro elutes in a reasonable time of under 10 minutes. The blank samples did not exhibit significant interferences in that region. The extracts from homogenization and sonication methods contained more impurities than that from MAP™ (Fig 1). The same extraction procedures were repeated for kidney and muscle tissues. Again, no interferences due to endogenous substances were found in either kidney or muscle extracts. Further studies were, then, carried out with MAP™ extraction technique.

TABLE 1

Recoveries of 3-nitro from spiked tissues by different extraction techniques[1]

Tissues	Techniques	%Recoveries
liver	homogenization	50.6 ± 6.4
	sonication	60.4 ± 7.3
	MAP™	82.4 ± 2.5
kidney	homogenization	37.6 ± 1.4
	sonication	42.7 ± 4.8
	MAP™	74.7 ± 1.8
muscle	homogenization	40.5 ± 7.8
	sonication	44.3 ± 4.5
	MAP™	80.8 ± 1.8

1) n=5, spiked at 4 $\mu g\ g^{-1}$.

Recovery studies at five different levels of fortification of 3-nitro in liver, kidneys and muscle were carried out using extraction by MAP™ and results are shown graphically in Figure 2. The average percentage recoveries from liver were 82.4 ± 2.5; muscle 80.8 ± 1.8; and 74.7 ± 1.8 for kidney. The calibration curves, representing mean peak areas versus concentration, show excellent linearity for concentrations from 0 to 6.0 $\mu g\ g^{-1}$. The correlation coefficients (r) were 0.9999, 0.9997, 0.9999 and 0.9998 for standard 3-nitro, liver, muscle and kidney, respectively.

The described method was successfully applied to swine liver, muscle tissue fortified with roxarsone in

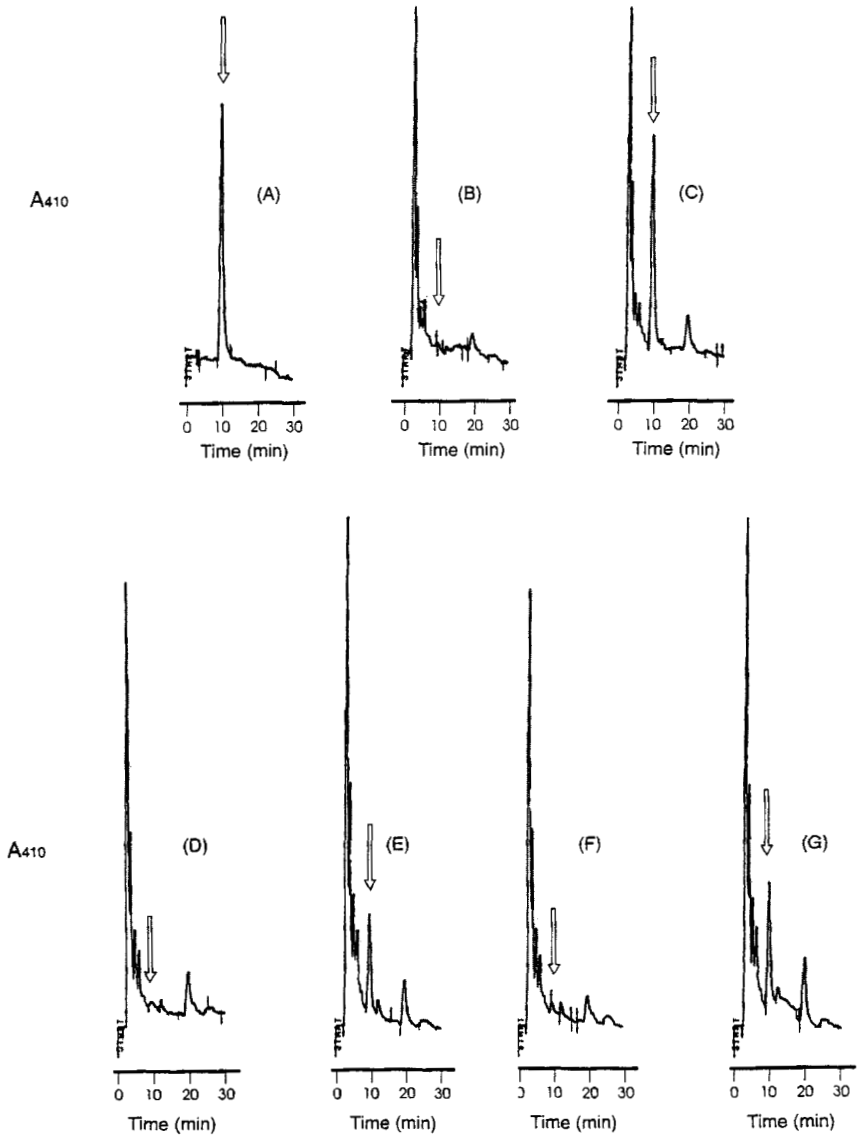

Figure 1. Chromatograms of (A) 3-nitro standard 100 ng, (B) blank liver MAP™ sample, (C) spiked liver MAP™ sample at a concentration of 4 $\mu g\ g^{-1}$, (D) homogenized blank liver sample, (E) spiked homogenized liver sample at a concentration of 4 $\mu g\ g^{-1}$, (F) blank sonicated liver sample and (G) spiked sonicated liver sample at a concentration of 4 $\mu g\ g^{-1}$. Arrow indicates 3-nitro.

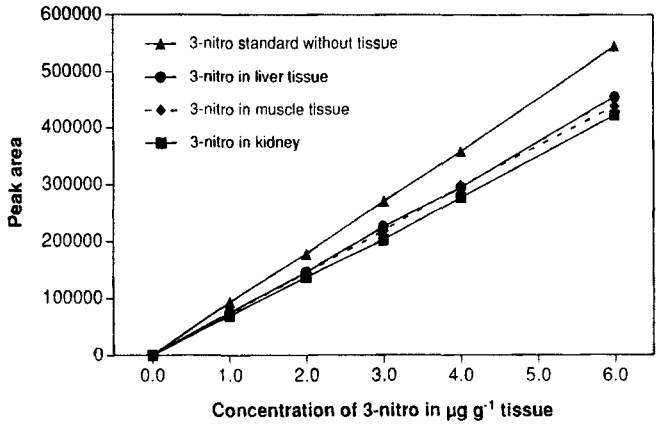

Figure 2. Recovery of 3-nitro in liver, muscle and kidney tissues spiked in the range of 1.0 to 6.0 $\mu g\ g^{-1}$.

the range of 1.0 to 6.0 $\mu g\ g^{-1}$ with excellent linearity and a limit of quantitation of 0.25 $\mu g\ g^{-1}$ (S/N >3:1). Consistent recoveries of roxarsone were obtained in the tissues (75-82%). The MAP™ method enabled us to process a large number of samples in a short time since no clean-up was required.

The tolerance for total arsenic residues in tissues is set at 0.5 $\mu g\ g^{-1}$ for muscle and kidney and 2.0 $\mu g\ g^{-1}$ for liver. These values are equivalent to about 1.75 $\mu g\ g^{-1}$ and 7.02 $\mu g\ g^{-1}$ of 3-nitro, respectively. The calibration curves in Figure 2 indicate the suitability of the method for detection of 3-nitro well below the lower established tolerance level.

Arsenic residues were determined by the atomic absorption technique in the incurred samples from feeding trials (4). Data showed that arsenic residues were the highest in liver, closely followed in kidney and considerably less in muscle. The arsenic residues in liver at 150 mg kg^{-1} supplementation level were 2.04 μg

g^{-1} for liver, 1.76 $\mu g\ g^{-1}$ for kidney and 0.14 $\mu g\ g^{-1}$ for muscle at 0 withdrawal period. The value reached 0.87 $\mu g\ g^{-1}$, 0.46 $\mu g\ g^{-1}$ and 0.1 $\mu g\ g^{-1}$ following the 5-d withdrawal period.

Liver samples containing the highest arsenic residues (2.04 $\mu g\ g^{-1}$) were subjected to the developed extraction method (MAP™) followed by analysis on HPLC. It was surprising to note that no 3-nitro was detected in any of the tissue samples of pigs fed the compound at 150 mg kg^{-1} for 28 days. Since 3-nitro was not detected in samples containing the highest arsenic residues, no further attempts were made to analyze the samples containing the lower amounts of arsenic.

The total absence of 3-nitro in incurred samples clearly suggest that the arsenic residues found in the sample are due to compounds other than 3-nitro. However, the nature of the arsenic moieties is not known. One can speculate that the compound has undergone metabolism to produce compounds including inorganic arsenicals which could not be detected by methods other than the atomic absorption method for total arsenic.

On the basis of data presented here, we see MAP™ as an alternate to classical extraction techniques including solvent extraction and supercritical fluid extraction for its versatility, simplicity, environmental safety and cost effectiveness. The technique has the potential for incorporation in routine analysis by regulatory agencies. The simplicity and cost-effectiveness features dictates further exploration with a variety of organic residues in various media including vegetables and fish.

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