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Determination of buparvaquone residues in bovine tissues and milk by HPLC/MS/MS

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Abstract

Buparvaquone (BPQ) is an antiprotozoal drug, used to treat *Theileria* infections in cattle. It is not registered in Australia, knowledge about its tissue residue profile in cattle is based on obsolete technology and there is no modern tissue residue analytical method available. As a precursor to a tissue residue depletion study, this project developed a procedure for the determination of BPQ residues in edible beef tissues and milk by reverse-phase high performance liquid chromatography (HPLC), coupled with tandem mass spectrometry (MS). BPQ residues are extracted from muscle, fat, liver, kidney and milk samples using acetonitrile/acetone. Quantitation is done via matrix-matched external standards of BPQ with an ACQUITY UPLC System (Waters) with a Xevo TQ-S MassLynx 4.1 XP Workstation, using a BEH C18 1.7 µm 50 x 2.1 mm (Waters) column. The parameters used for validation of the method were linearity, system precision, accuracy and precision. A Limit of Quantitation (LOQ) of 0.01 mg/kg for bovine tissues and 0.005 mg/kg for milk, and a Limit of Detection (LOD) of 0.005 mg/kg for bovine tissues and 0.002 mg/kg for milk were achieved.

Executive summary

Theileria orientalis is a blood-borne protozoon which infects cattle and has been known to occur in Australia (mainly in Queensland) for more than a century. The infection was known as Benign Theileriosis and seldom caused clinical signs in infected animals. For approximately the past decade, cattle on the eastern seaboard of Australia have become ill, showing signs of anaemia, icterus, depression, weakness, abortion and death. They have been diagnosed as being infected with *T orientalis*, of which there is now known to be at least three subtypes in Australia, one of which is consistently found in clinical cases. This disease has been given the name Bovine Anaemia caused by *T orientalis* and has since been found to occur in cattle in Victoria and Western Australia.

There is no remedy registered for treating this disease in Australia. Buparvaquone (BPQ) has been registered elsewhere in the world and was found to be effective against induced infections with two isolates in splenectomised calves. Veterinarians can apply to the Australian Pesticides and Veterinary Medicines Authority (APVMA) for Consent to Import the remedy for treating patients under their care, but they will then bear the responsibility for any residues found in edible tissues from such animals. There is currently no information on which veterinarians can base a recommendation of a suitable with-holding period (WHP) to avoid violative residues in BPQ treated cattle slaughtered for human consumption. Prior to conducting a tissue residue depletion study on which to base WHP recommendations, an analytical method for BPQ residues in edible tissue had to be developed, because the method used previously was not readily available and was deemed to have used obsolete technology.

The principle of the method is that BPQ residues are extracted from bovine tissues with acetonitrile/acetone (80:20). An aliquot of the extract is removed and cleaned up by solid phase extraction (SPE) and BPQ residues determined by reverse-phase high performance liquid chromatography (HPLC), coupled with tandem mass spectrometry (MS) (HPLC/MS/MS) (multiple reaction monitoring – MRM). BPQ residues are extracted from milk by vortexing the sample with acetonitrile/acetone.NaCl, $MgSO_4$ and other selected salts are added to the extract to create a partition between the acetonitrile/acetone and water. An aliquot of the acetonitrile/acetone layer is taken and diluted and BPQ residues determined by HPLC/MS/MS (MRM).

A series of matrix matched external reference standard solutions of BPQ is prepared: a *stock standard* solution containing 1,000 mg/L in acetone, three *intermediate standards* containing 0.1, 1 and 10 mg/L in acetone, and *quantitation standards* in untreated control matrices of muscle, fat, liver, kidney (spiked to concentrations ranging from 0.2 to 0.0025 mg/kg) and milk (0.1 to 0.002 mg/kg) for producing calibration curves.

Sample preparation consists of weighing 5g of the tissue into a 250mL flask, (adding reference standards to control matrices for spiking of quantitation standards,) adding 50mL 80:20 acetonitrile/acetone, homogenising, mixing and centrifuging prior to SPE clean-up.

LC/MS/MS analysis was done on an ACQUITY UPLC System (Waters) with a Xevo TQ-S MassLynx 4.1 XP Workstation in a BEH C18 1.7 µm 50 x 2.1 mm (Waters) column. Quantification and identification were made by comparison with matrix matched external standards using comparative peak areas. A linear regression

equation was generated for analyte calibration standards with 1/x weighting using the concentration of the analyte (X-axis) versus the analyte peak area (Y-axis), excluding the origin. Concentrations of analyte in the final extracts were determined by substituting the peak area responses into the linear regression equation.

Quality assurance for this method consists of recoveries (untreated control samples fortified with the BPQ). Recoveries are run concurrently with every batch of test samples. Average recoveries must be between 70% and 110% with relative standard deviations (RSD) $\leq$ 20% acceptable. The method of analysis was validated using the following parameters: linearity, system precision, accuracy and precision.

It is foreseen that this method can be used for determining BPQ residues in the tissues of treated cattle, e.g. for determining the tissue residue depletion pattern for the setting of with-holding periods (WHP) for treated cattle destined for slaughter for human consumption. This will benefit veterinarians wishing to treat cattle infected with *T orientalis* and recommending an appropriate WHP, as well as the owners of those cattle, who may otherwise suffer the loss of untreated cattle, or of having to with-hold treated cattle from entry into the human food chain.

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1 Background

Theileria orientalis is a blood-borne protozoon which infects cattle and has been known to occur in Australia (mainly in Queensland) for more than a century. The infection was known as Benign Theileriosis and seldom caused clinical signs in infected animals. For approximately the past decade, cattle on the eastern seaboard of Australia have become ill, showing signs of anaemia, icterus, depression, weakness, abortion and death[1]. They have been diagnosed as being infected with *T orientalis*, of which there is now known to be at least three subtypes in Australia, one of which is consistently found in clinical cases[2]. This disease has been given the name Bovine Anaemia caused by *T orientalis* and has since been found to occur in cattle in Victoria and Western Australia.

There is no remedy registered for treating this disease in Australia. Buparvaquone (BPQ) has been registered elsewhere in the world for the treatment of East Coast Fever and Tropical Theileriosis in cattle and was found to be effective in Australia against induced infections with two isolates of *T orientalis* in splenectomised calves[3]. Veterinarians can apply to the Australian Pesticides and Veterinary Medicines Authority (APVMA) for Consent to Import the remedy for treating patients under their care, but they will then bear the responsibility for any residues found in edible tissues from such animals. There is currently no information on which veterinarians can base a recommendation of a suitable with-holding period (WHP) to avoid violative residues in BPQ treated cattle slaughtered for human consumption. Prior to conducting a tissue residue depletion study on which to base WHP recommendations, an analytical method for BPQ residues in edible tissue had to be developed, because the method used previously was not readily available and was deemed to have used obsolete technology.

When this project was first completed it was decided to keep the report confidential because of the intellectual property it contained. That decision has since been rescinded and the method made available for the benefit of the beef industry.

2 Project objective

The objective of the project was to develop and validate a method of analysis for the determination of residues of buparvaquone (BPQ) in animal tissues and milk, with a view to conduct residue depletion studies.

3 Methodology

3.1 Principle

BPQ residues are extracted from tissues with acetonitrile/acetone and cleaned up by solid-phase extraction (SPE). Residues are determined by reverse-phase high performance liquid chromatography (HPLC), coupled with tandem mass spectrometry (MS) (HPLC/MS/MS) (multiple reaction monitoring – MRM). Quantitation is via matrix matched external standard solutions.

[1] Izzo MM et al. (2010) *Aust vet J* 88: 45 - 51
[2] Kamau J et al. (2011) *Parasites & Vectors* 2011, 4:22
[3] MLA project B.AHE.0048.

BPQ residues are extracted from milk by vortexing the sample with acetonitrile/acetone. Selected salts are added to the mixture to create a partition between the acetonitrile/acetone and water. An aliquot of the acetonitrilie/acetone layer is taken and diluted. Residue determination and quantitation are as for tissue residues.

3.2 Reagents, standards, reagent solutions

3.2.1 Reagents

Acetonitrile (ACN), Nanograde, Labscan or equivalent.
Acetone, Nanograde, Labscan or equivalent.
Methanol, Nanograde, Labscan or equivalent.
Acetic acid, Chem Supply or equivalent.
Formic acid, AR grade, Sigma-Aldrich or equivalent.
Ammonium hydroxide, AR grade, Sigma-Aldrich or equivalent.
Potassium phosphate monobasic (KH2PO4), Chem Supply or equivalent.
Sodium chloride, AR grade, sieved, Univar or equivalent.
Magnesium sulfate anhydrous extra pure, Scharlau or equivalent.
Sodium Hydrogencitrate sesquihydrate, AR Grade, Scharlau or equivalent.
Tri-Sodium Citrate Dihydrate, AR Grade, Chemsupply or equivalent.

3.2.2 Standards

Buparvaquone analytical standard (Vet Pharma 99.68% or equivalent).

3.2.3 Reagent solutions

Acetonitrile/acetone (80:20)
0.05 Potassium phosphate monobasic.
1% (v/v) acetic acid in water
1% (v/v) acetic acid in acetonitrile/water (5:95)
1% (v/v) acetic acid in methanol/water (10:90)
2% acetic acid in acetonitrile
0.01% (v/v) formic acid and 0.05% (v/v) ammonium hydroxide in water.

3.2.4 Reference standards

Stock standard (~1,000 mg/L)
Intermediate standards: 10 mg/L:, 1 mg/L, 0.1 mg/L
Quantitation standards: Prepared by diluting intermediate standards with control matrix prepared from untreated control samples. At least six quantitation standards are prepared for each batch of sample analysis.

3.3 Apparatus

Calibrated analytical Balance (± 0.0001 g), Mettler AE240 or equivalent.
Calibrated top pan balance, Sartorius B 3100S ± 0.01 g, Mettler PM2000 ± 0.01 g or equivalent.
Ultrasonicator.
Vortex Mixer.
Tissumizer, Heidolph Diax 900, AA47.
Various calibrated autopipettes 200-1000 µL, 20-200 µL or equivalent.
10 mL and 50 mL centrifuge tubes.
250 mL plastic bottles.
5 mL Single use syringe (Talus) or equivalent.

Vacuum manifold.
Oasis HLB 6cc (0.2 g) Extraction Cartridge (Waters) or equivalent.
Centrifuge (KI scientific) or equivalent.
Sample filter, RC20 0.2 µm and RC 0.45 µm (Sartorius) or equivalent.
Solvent delivery system, Waters ACQUITY UPLC Core System or equivalent.
Waters Xevo TQ-S mass spectrometer detector or equivalent.
Standard MassLynx 4.1 XP workstation or equivalent.
ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 x 50 mm column or equivalent.

3.4 Procedure

3.4.1 Sample preparation

a. Liver, kidney, muscle and fat

- Weigh 5.0 $\pm$ 0.1 g of tissue into a 250 mL bottle.
- Fortify the recovery assays.
- Add 50 mL acetonitrile/acetone 80:20
- Tissumize liver, kidney & muscle for ca. 1 min; sonicate fat for 10 min.
- Place all samples on a shaker for 30 min.
- Remove from the shaker and centrifuge for 5 min at 2,000 rpm.
- Remove from the centrifuge, take a 10 mL aliquot from the supernatant and filter with an RC 0.45 µm syringe filter into a 50 mL centrifuge tube.
- Place samples in the freezer (~ -20°C) for one hour.
- Remove from the freezer and centrifuge for 5 min at 3,000 rpm.
- Remove from the centrifuge, allow samples to come to room temperature.
- Take a 4 mL aliquot of the supernatant and transfer to a 50 mL centrifuge tube.
- Add 6 mL of 0.05 M potassium phosphate monobasic, vortex to mix the sample and proceed to 2.4.2.

b. Milk

- Weigh 10.0 $\pm$ 0.5 g of milk into a 50 mL centrifuge tube.
- Fortify the recovery assays.
- Add 10 mL acetonitrile/acetone 80:20
- Vortex for ca. 1 min.
- Centrifuge the samples for 5 min at 3,000 rpm.
- Remove from the centrifuge and decant the supernatant into a a 50 mL centrifuge tube.
- Add 1g NaCl, 0.5g Na-hydrogen-citrate-sesquihydrate, 1g tri-sodium citrate dehydrate, 4g magnesium sulphate.
- Cap and vortex immediately for ca. 30 seconds.
- Centrifuge the samples for 5 min at 3,000 rpm.
- Remove from the centrifuge, take a 0.2 mL aliquot from the supernatant and transfer into a 10 mL centrifuge tube.
- Add 0.8 mL 2% acetic acid in acetonitrile.
- Vortex to mix the sample and filter with an RC 0.2 µm syringe filter into LCMS vials.
- Place vials in the refrigerator if LCMSMS is not to be done immediately.

3.4.2 HLB SPE cleanup

- Condition HLB SPE cartridge with 6 mL methanol, followed by 6 mL 1% acetic acid in water.
- Quantitatively transfer sample extract onto preconditioned SPE column.

- Wash the column with 6 mL 1% acetic acid in methanol/water, followed by 6 mL 1% acetic acid inacetonitrile/water. Discard eluents.
- Elute the sample with 5 mL 2% acetic acid in acetonitrile into 10 mL plastic centrifuge tube.
- Filter the samples with an RC 0.2 µm syringe filter into LCMS vials.
- Place vials in the refrigerator if LCMSMS is not to be done immediately.

3.4.3 LC/MS/MS Conditions

Instrument: ACQUITY UPLC System (Waters) with Xevo TQ-S MassLynx 4.1 XP Workstation
Column: BEH C18 1.7 µm 50 x 2.1 mm (Waters)
Oven Temperature: 40ºC
Mobile phase: A: 0.01% (v/v) Formic acid and 0.05% (v/v) Ammonium hydroxide in Milli-Q water
B: Methanol

Gradient:

Time, min	A(%)	B(%)
0	50	50
0.5	50	50
2	0	100
4	50	50

Run Time: 5 minutes
Flow rate: 0.4 mL/minute
Detector: Mode – electrospray negative polarity
Ions – Buparvaquone (Quantitation ion: 325.1/186, Confirmatory ion: 325.1/297.1)
Injection volume: 5 µL
Under the above conditions Buparvaquone will typically elute at approximately 1.7 minutes.

See Appendix for typical chromatograms.

3.5 Quantitation

Quantification and identification are made by comparison with matrix matched external standards using comparative peak areas. Concentration of standards must bracket the concentration of analytes found in test samples.

3.6 Calculations and reporting

A linear regression equation is generated for analyte calibration standards with 1/x weighting using the concentration of the analyte (X-axis) versus the analyte peak area (Y-axis), excluding the origin. Concentrations of analyte in the final extracts were determined by substituting the peak area responses into the linear regression equation as shown below:

$$Y = aX + b$$

With: Y = Analyte area, X = Analyte concentration in final solution, a = Slope, b = Intercept.
Thus: X (as µg/kg) = (Analyte area - b) / a

For the calculation of residues in the sample:

$$R\ (mg/kg) = X \times 0.001$$

Where : R = Residue found in the tissue as mg/kg, X = Final concentration of analyte in extract in µg/kg.
(Note: 0.001: unit conversion factor)

3.7 Quality assurance

The quality assurance for this method consists of recoveries (untreated control samples fortified with the BPQ). Recoveries are run concurrently with every batch of test samples. Average recoveries must be between 70% and 110% with RSD $\leq$ 20% acceptable. If recoveries are outside these limits consult Senior Project Chemist.

3.8 Validation

The parameters used for validation of this method of analysis were linearity, system precision, and recovery accuracy and precision. Detailed results of this validation are shown in the Appendix.

4 Results and discussion

Typical Limit of Detection (LOD) = 0.005 mg/kg for bovine tissues; 0.002 mg/kg for milk
Typical Limit of Quantitation (LOQ) = 0.01 mg/kg for bovine tissues; 0.005 mg/kg for milk

A set of at least five quantitation standards over the range of 0.002 mg/kg to 0.2 mg/kg gave linear responses for the area with a correlation (R^2) of at least 0.995.

A matrix matched quantitation standard at the LOQ injected 10 times to evaluate system precision yielded the following results:

Tissue	Peak area average	Standard deviation	RSD (%)
Liver	251	17.8	7.1
Kidney	152	9.0	5.9
Muscle	251	24.7	9.8
Fat	319	24.4	7.7

5 Success in achieving objectives

The objective of the project was to develop and validate a method of analysis for the determination of residues of buparvaquone (BPQ) in animal tissues and milk, with a view to conduct residue depletion studies. This was achieved successfully.

6 Impact on meat and livestock industry – Now and in five years time

This analytical method will enable the performance of a BPQ tissue residue depletion study for treated cattle. The results from such a depletion study will allow attending veterinarians to make an informed recommendation on a suitable with-holding period for cattle they wish to treat with BPQ, whilst the product is not registered in Australia. The method will also enable the analysis of residues in the tissues of treated cattle, should it be necessary to monitor the use of this remedy.

The availability of a drug with which to treat clinical cases of *T orientalis* infection will limit losses due to this emerging disease and have a positive animal welfare outcome.

7 Conclusions and recommendations

This method can be used for determining residues of BPQ in the tissues of treated cattle, specifically for performing a residue depletion study for the setting of a with-holding period.

It is recommended that this analytical method be used to promote the use of BPQ for the treatment of clinical cases of *T orientalis* infection in Australian cattle.

8 Bibliography

Izzo MM et al. (2010) Haemolytic anaemia in cattle in NSW associated with *Theileria* infections. *Aust vet J* 88: 45 - 51

Kamau J et al. (2011) Emergence of new types of *Theileria orientalis* in Australian cattle and possible cause of theileriosis outbreaks. *Parasites & Vectors* 2011, 4:22

MLA project B.AHE.0048.

Kinabo LD, Bogan JA (1988) Parvaquone and buparvaquone HPLC analysis and comparative pharmacokinetics in cattle. Acta Trop 45 (1): 87-94.

9 Appendix

Agrisearch Analytical Pty Ltd report, method number AATM-R170

1. Title

Determination of Buparvaquone Residues in Bovine Tissues and Milk by HPLC/MS/MS.

2. Scope

This analytical method describes the procedure for the determination of buparvaquone residues in bovine tissues and milk by reverse-phase High Performance Liquid Chromatography (HPLC) coupled with tandem mass spectrometric determination (LC-MS/MS). The method was validated over the concentration range of 0.002- 0.2mg/kg with a validated lower limit of quantitation of 0.01mg/kg in bovine tissues and 0.005mg/kg in milk. The method may by applicable to other animal tissues but has not been validated as yet.

3. Safety

Latex gloves, safety glasses and lab coat should be worn at all times.

4. Referenced documents

WI-33	Basic Operation of Waters ACQUITY Xevo TQ-S System (LC/MS/MS) and Routine Maintenance
SOP-01	Receipt, Storage, Sub-sampling and Disposal of Test samples
SOP-08	Quantitative Data - Analytical Standards
SOP-12	Test Results - Monitoring Quality
SOP-15	Estimation of the Uncertainty of Measurement
SOP-18	Validation of Regulatory Test Methods
WI-13 S	ub-sampling and Blending

5. Principle

Buparvaquone residues are extracted from bovine tissues with acetonitrile/acetone (80:20). An aliquot of the extract is removed and cleaned up by solid phase extraction (SPE). Buparvaquone residues are determined by HPLC/MS/MS (MRM). Quantitation is via matrix matched external standards of buparvaquone.

Residues of buparvaquone are extracted from milk by vortexing the sample with acetonitrile/acetone. NaCl and MgSO4 and other selected salts are added to the extract which creates a partition between the acetonitrile/acetone and water. An aliquot of the acetonitrile/acetone layer is taken and diluted. Buparvaquone residues are determined by HPLC/MS/MS (MRM). Quantitation is via matrix matched external standards of buparvaquone.

6. Reagents, standards, prepared solutions and standards

Other appropriate weights, volumes and dilutions may be used for standard and solution preparations instead of the typical preparations specified below.

6.1 Reagents

- Acetonitrile (ACN), Nanograde, Labscan or equivalent
- Acetone, Nanograde, Labscan or equivalent
- Methanol, Nanograde, Labscan or equivalent
- Acetic acid, Chem Supply or equivalent
- Formic acid, AR grade, Sigma-Aldrich or equivalent
- Ammonium hydroxide, AR grade, Sigma-Aldrich or equivalent
- Potassium phosphate monobasic (KH2PO4), Chem Supply or equivalent
- Sodium chloride, AR grade, sieved, Univar or equivalent
- Magnesium sulfate anhydrous extra pure, Scharlau or equivalent
- Sodium Hydrogencitrate sesquihydrate, AR Grade, Scharlau or equivalent
- Tri-Sodium Citrate Dihydrate, AR Grade, Chemsupply or equivalent

6.2 Standards

- Buparvaquone analytical standard (Vet Pharma, 99.68% or equivalent)

6.3 Reagent solutions

6.3.1 Acetonitrile/acetone (80:20)

Measure 800 mL of acetonitrile and 200 mL of acetone using a measuring cylinder and transfer to an appropriate container. Cap the container and invert several times to mix the contents.

6.3.2 0.05 M Potassium phosphate monobasic

Weigh 6.8g of potassium phosphate into an appropriate container and dissolve in one litre of Milli-Q water.

6.3.3 1% (v/v) acetic acid in water

Measure 500mL of Mill-Q water using a measuring cylinder and transfer to an appropriate container. Add 5mL of acetic acid. Cap the container and invert several times to mix the contents.

6.3.4 1% (v/v) acetic acid in acetonitrile/water (5:95)

Measure 475mL of Mill-Q water, 25mL of acetonitrile using a measuring cylinder and transfer to an appropriate container. Add 5mL of acetic acid. Cap the container and invert several times to mix the contents.

6.3.5 1% (v/v) acetic acid in methanol/water (10:90)

Measure 450mL of Milli-Q water, 50mL of methanol using a measuring cylinder and transfer to an appropriate container. Add 5mL of acetic acid. Cap the container and invert several times to mix the contents.

6.3.6 2% (v/v) acetic acid in acetonitrile

Measure 200mL of acetonitrile using a measuring cylinder and transfer to an appropriate container. Add 4mL of acetic acid. Cap the container and invert several times to mix the contents.

6.3.7 0.01% (v/v) formic acid and 0.05% (v/v) ammonium hydroxide in water

Measure 400mL of Milli-Q water using a measuring cylinder and transfer to a glass bottle. Add 0.2mL of ammonium hydroxide and 0.04mL of formic acid using autopipettes. Sonicate for 5 minutes.

6.4 Reference standards

6.4.1 Stock standards

Stock Standard (~1000mg/L): Prepare the stock standard solution of Buparvaquone by accurately weighing 50mg of the neat buparvaquone standard into a 50mL volumetric flask. Dissolve with acetone and dilute to the mark with approximately 50mL of acetone. (See SOP-08).

6.4.2 Intermediate standards

- **10mg/L standard**: Take a 0.5 mL aliquot of the stock standard solution (see section 6.4.1) and transfer to a 50 mL volumetric flask. It is filled up to the mark with acetonitrile to make a 10mg/L standard. Stopper the flask and invert several times to mix the solution. Transfer the solution into a labelled glass bottle. This standard solution should be stored in the fridge (ca. 4˚C) and will be stable for at least 1 month.
- **1mg/L standard** (freshly prepared on the day of analysis): Take a 0.1mL aliquot of the 10mg/L intermediate standard and dilute with 0.9mL of acetonitrile.
- **0.1mg/L standard** (freshly prepared on the day of analysis): Take a 0.1mL aliquot of the 1mg/L intermediate standard and dilute with 0.9mL of acetonitrile.

6.4.3 Quantitation standards

Quantitation standards are prepared by diluting the intermediate standards (see section 6.4.2) with control matrix prepared from untreateded control samples. At least six quantitation standards are prepared for each batch of sample analyses. Each sample is spiked with the appropriate amount buparvaquone standard at the beginning of sample preparation. The quantitation standards are therefore exposed to the same process as the samples. The following tables contain instructions for the preparation of matrix matched standards in tissues and milk.

Spiking levels on 5g tissues

Original Std. conc. (mg/L)	Aliquot taken (mL)	Concentration in Matrix (mg/kg)
0.1	0.125	0.0025
0.1	0.25	0.005
1	0.05	0.01
1	0.125	0.025
1	0.25	0.05
10	0.05	0.1
10	0.1	0.2

Spiking levels on 10g milk

Original Std. conc. (mg/L)	Aliquot taken (mL)	Final Spiked Level in Tissues (mg/kg)
1	0.02	0.002
1	0.05	0.005
1	0.1	0.01
10	0.025	0.025
10	0.05	0.05
10	0.1	0.1

In any specific run only the standards required to cover the range of residues found in the samples need to be run (minimum 4).

7. Apparatus

7.1 Calibrated analytical Balance (± 0.0001 g), Mettler AE240 or equivalent
7.2 Calibrated top pan balance, Sartorius B 3100S ± 0.01 g, Mettler PM2000 ± 0.01 g or equivalent.
7.3 Ultrasonicator
7.4 Vortex Mixer
7.5 Tissumizer, Heidolph Diax 900, AA47
7.6 Various calibrated autopipettes 200-1000 µL, 20-200 µL or equivalent
7.7 10 mL and 50 mL centrifuge tubes
7.8 250 mL plastic bottles
7.9 5 mL Single use syringe (Talus) or equivalent
7.10 Vacuum manifold
7.11 Oasis HLB 6cc (0.2 g) Extraction Cartidge (Waters) or equivalent
7.12 Centrifuge (KI scientific) or equivalent
7.13 Sample filter, RC20 0.2 µm and RC 0.45 µm (Sartorius) or equivalent
7.14 Solvent delivery system, Waters ACQUITY UPLC Core System or equivalent
7.15 Waters Xevo TQ-S mass spectrometer detector or equivalent
7.16 Standard MassLynx 4.1 XP workstation or equivalent
7.17 ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 x 50 mm column or equivalent

8. Sample preparation

Upon receipt, samples are either sub-sampled immediately or samples must be transferred to the freezer room at or below -15°C. The supplied sample must be thawed slightly and diced into small pieces with a sharp knife. If further homogenisation is required then mince the sample cryogenically with dry ice in a Bamix mincer. Make the sample as homogenous as possible so that the analytical portion is representative of the supplied sample. Samples are stored in the freezer at –15 ^{o}C, until the day before the analysis. They are then moved to the refrigerator at $_{5}^{o}$C to defrost overnight (Refer to WI-13).

9. Procedure

9.1 Sample preparation

9.1.1 Liver, kidney, muscle and fat samples

- Weigh 5.0 ± 0.1g of the tissue into a 250mL bottle.
- Fortify the recovery assays at this point.
- Add 50mL of acetonitrile/acetone (80:20) (see section 6.3.1).
- For liver, kidney and muscle: tissumize for approximately one minute.
- For fat: sonicate for 10 minutes.
- Place all samples on a shaker for 30 minutes.
- Remove from the shaker and centrifuge for 5 minutes at 2000 rpm.
- Remove from the centrifuge and take a 10mL aliquot of the supernatant and filter with an RC 0.45µm syringe filter into a 50mL centrifuge tube.
- Place samples in the freezer (approximately -20˚C) for one hour.
- Remove from the freezer and centrifuge for 5 minutes at 3000 rpm.
- Remove from the centrifuge and allow samples to come to room temperature.
- Take a 4mL aliquot of the supernatant and transfer to 50mL centrifuge tube.
- Add 6mL of 0.05 M potassium phosphate monobasic (see section 6.3.2) and vortex to mix the sample. Proceed to 9.1.1.1.

9.1.1.1 HLB SPE Cleanup

- Condition the HLB SPE (see 7.11) cartridge with 6mL of methanol followed by 6mL of 1% acetic acid in water (see section 6.3.3).
- Quantitatively transfer the sample extract onto the preconditioned SPE column.
- Wash the column with 6mL of 1% acetic acid in methanol/water (see section 6.3.5) followed by 6 mL of 1% acetic acid in acetonitrile/water (see section 6.3.4). Discard eluents.
- Elute the sample with 5 mL of 2% acetic acid in acetonitrile (see section 6.3.6) into 10mL plastic centrifuge tube
- Filter the samples with an RC 0.2 µm syringe filter into LCMS vials.
- Place vial in the refrigerator if the LCMSMS is not going to take place immediately.

9.1.2 Milk samples

- Weigh 10.0 ± 0.5 g of milk into a 50 mL centrifuge tube.
- Spike the recoveries at this point.
- Add 10 mL of acetonitrile/acetone (80:20) (see section 6.3.1).
- Vortex for approximately one minute.
- Centrifuge the samples for 5 minutes at 3000 rpm.
- Remove from the centrifuge and decant the supernatant into a 50 mL centrifuge tube.
- Add the following:
    - 1 g sodium chloride
    - 0.5 g sodium hydrogen citrate sesquishydrate
    - 1 g tri-sodium citrate dehydrate
    - 4 g magnesium sulfate
- Cap and vortex immediately for approximately 30 seconds.
- Centrifuge the samples for 5 minutes at 3000 rpm.
- Remove from the centrifuge and take a 0.2 mL aliquot of the supernatant and transfer into a 10 mL centrifuge tube.
- Add 0.8 mL of 2% acetic acid in acetonitrile (see section 6.3.6).
- Vortex to mix the sample and filter with an RC 0.2 µm syringe filter into LCMS vials.
- Place vial in the refrigerator if the LCMSMS is not going to take place immediately.

9.2 LC/MS/MS Analysis

9.2.1 Typical LC/MS/MS Conditions

Instrument:	ACQUITY UPLC System (Waters) with Xevo TQ-S MassLynx 4.1 XP Workstation		
Column:	BEH C18 1.7 µm 50 x 2.1 mm (Waters)		
Oven Temperature:	40ºC		
Mobile phase:	A: 0.01% (v/v) Formic acid and 0.05% (v/v) Ammonium hydroxide in Milli-Q water B: Methanol		
Gradient:	Time, min	A(%)	B(%)
	0	50	50
	0.5	50	50
	2	0	100
	4	50	50
Run Time:	5 minutes		
Flow rate:	0.4 mL/minute		
Detector:	Mode – electrospray negative polarity Ions – Buparvaquone (Quantitation ion: 325.1/186, Confirmatory ion: 325.1/297.1)		
Injection volume:	5 µL		

Under the above conditions Buparvaquone will typically elute at approximately 1.7 minutes.

NOTE: **Appendix B** shows typical chromatograms.

10. Quantitation

Quantification and identification are made by comparison with matrix matched external standards using comparative peak areas. Concentration of standards must bracket the concentration of analytes found in test samples.

11. Calculations and reporting

In general, a linear regression equation is generated for analyte calibration standards with 1/x weighting using the concentration of the analyte (X-axis) versus the analyte peak area (Y-axis), excluding the origin. Concentrations of analyte in the final extracts were determined by substituting the peak area responses into the linear regression equation as shown below:

Y = aX + b With:
Y: Analyte area
X: Analyte concentration in final solution

a: Slope
b: Intercept

Thus: X (as µg/kg) = (Analyte area - b) / a
For the calculation of residues in the sample:
R (mg/kg) = X x 0.001
Where:
R: Residue found in the tissue as mg/kg.
X: Final concentration of analyte in extract in µg/kg.

(Note: 0.001: unit conversion factor)

Typical Limit of Detection (LOD)	= 0.005 mg/kg for bovine tissues; 0.002 mg/kg for milk
Typical Limit of Quantitation (LOQ)	= 0.01 mg/kg for bovine tissues; 0.005 mg/kg for milk

12. Quality assurance:

The quality assurance for this method consists of recoveries (untreated control samples fortified with the buparvaquone). Recoveries are run concurrently with every batch of test samples. Average recoveries must be between 70% and 110% with RSD ≤ 20% are acceptable. If recoveries are outside these limits consult Senior Project Chemist.

13. Validation

This method of analysis has been validated using the following parameters: linearity, system precision, accuracy and precision. Results of this validation are shown below.

13.1 Linearity

A set of at least five quantitation standards over the range of 0.002 mg/kg to 0.2 mg/kg gave linear responses for the area with a correlation (R^2) of at least 0.995.

NOTE: Appendix A shows typical Calibration Curves.

13.2 System Precision

A matrix matched quantitation standard at the LOQ was injected 10 times to evaluate system precision:

13.2.1 10 µg/kg Buparvaquone in Liver Matrix System Precision:

Injection Number	Retention time (mins)	Peak Area
1	1.80	255.0
2	1.80	212.3
3	1.80	244.0
4	1.80	254.8
5	1.80	260.8
6	1.80	276.9
7	1.80	267.7
8	1.80	256.8
9	1.80	237.0
10	1.80	246.7
Average	1.80	251
Standard Deviation	0	17.8
RSD (%)	0	7.1

13.2.2 10 µg/kg Buparvaquone in Kidney Matrix System Precision:

Injection Number	Retention time (mins)	Peak Area
1	1.65	155.8
2	1.65	158.6
3	1.65	143.9
4	1.65	161.8
5	1.65	156.2
6	1.65	160.4
7	1.65	137.2
8	1.65	138.9
9	1.65	152.3
10	1.65	158.0
Average	1.65	152
Standard Deviation	0	9.0
RSD (%)	0	5.9

13.2.3 10 µg/kg Buparvaquone in Muscle Matrix System Precision:

Injection Number	Retention time (mins)	Peak Area
1	1.71	292.2
2	1.71	290.4
3	1.71	239.1
4	1.71	244.3
5	1.71	217.9
6	1.71	226.5
7	1.71	264.7
8	1.71	250.4
9	1.71	238.8
10	1.71	250.7
Average	1.71	251
Standard Deviation	0	24.7
RSD (%)	0	9.8

13.2.4 10 µg/kg Buparvaquone in Fat Matrix System Precision:

Injection Number	Retention time (mins)	Peak Area
1	1.74	337.9
2	1.74	359.9
3	1.74	356.3
4	1.74	297.4
5	1.74	311.9
6	1.74	308.2
7	1.74	315.0
8	1.74	307.4
9	1.74	288.3
10	1.74	304.2
Average	1.74	319
Standard Deviation	0	24.4
RSD (%)	0	7.7

13.2.5 5 µg/kg Buparvaquone in Milk Matrix System Precision:

Injection Number	Retention time (mins)	Peak Area
1	1.81	67.5
2	1.81	77.8
3	1.81	85.4
4	1.81	64.6
5	1.81	85.7
6	1.81	68.0
7	1.81	68.4
8	1.81	66.9
9	1.81	61.1
10	1.8	74.5
Average	1.81	72
Standard Deviation	0.003	8.5
RSD (%)	0.17	12

13.3 Accuracy & Precision (Recovery)

A method validation study was conducted to determine the accuracy and precision of the method for the determination of buparvaquone in bovine tissues and milk.

The overall recovery results for buparvaquone in bovine tissues and milk are summarized below and listed individually in Appendix C Tables 1-5.

Compound	Substrate	Fortification (mg/kg)	Replicates (n)	Mean Recovery (%)	RSD (%)
Buparvaquone	Liver	0.01	6	99	8.1
Buparvaquone	Liver	0.1	6	103	4.7
Buparvaquone	Kidney	0.01	6	94	6.5
Buparvaquone	Kidney	0.1	6	94	4.4
Buparvaquone	Muscle	0.01	6	94	7.8
Buparvaquone	Muscle	0.1	6	95	3.7
Buparvaquone	Fat	0.01	6	96	6.8
Buparvaquone	Fat	0.1	6	94	4.4
Buparvaquone	Milk	0.005	6	95	10
Buparvaquone	Milk	0.05	6	108	3.6

13.4 Uncertainty

From the above recovery results, the uncertainty of the method was calculated (see file AATM-S-170-UWS.xls):

Concentration (mg/kg)	Substrate	Analyte	Uncertainty (mg/kg)
0.01	Liver	Buparvaquone	0.003
0.1	Liver	Buparvaquone	0.022
0.01	Kidney	Buparvaquone	0.002
0.1	Kidney	Buparvaquone	0.023
0.01	Muscle	Buparvaquone	0.003
0.1	Muscle	Buparvaquone	0.022
0.01	Fat	Buparvaquone	0.002
0.1	Fat	Buparvaquone	0.023
0.005	Milk	Buparvaquone	0.001
0.05	Milk	Buparvaquone	0.011

14. References:

“Parvaquone and buparvaquone HPLC analysis and comparative pharmacokinetics in cattle”, Kinabo LD, Bogan JA, Acta Trop, 1988 Mar 45 (1): 87-94.

APPENDIX A

Typical Calibration Curves

Figure 1 - Buparvaquone in Liver Matrix Calibration Curve

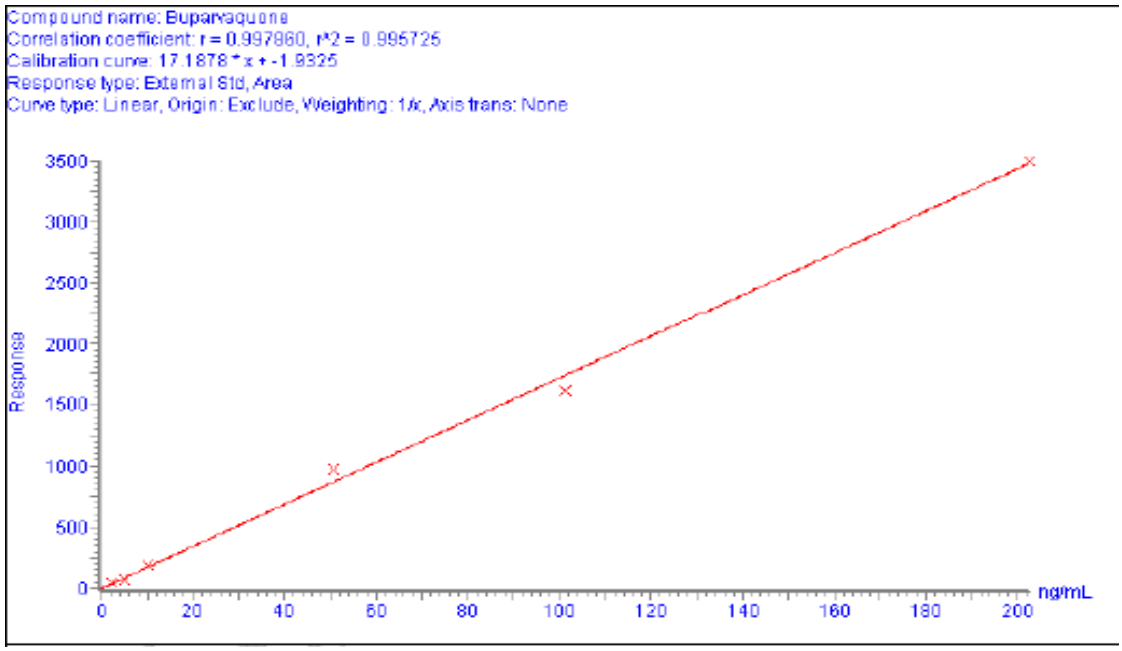


Figure 2 - Buparvaquone in Kidney Matrix Calibration Curve

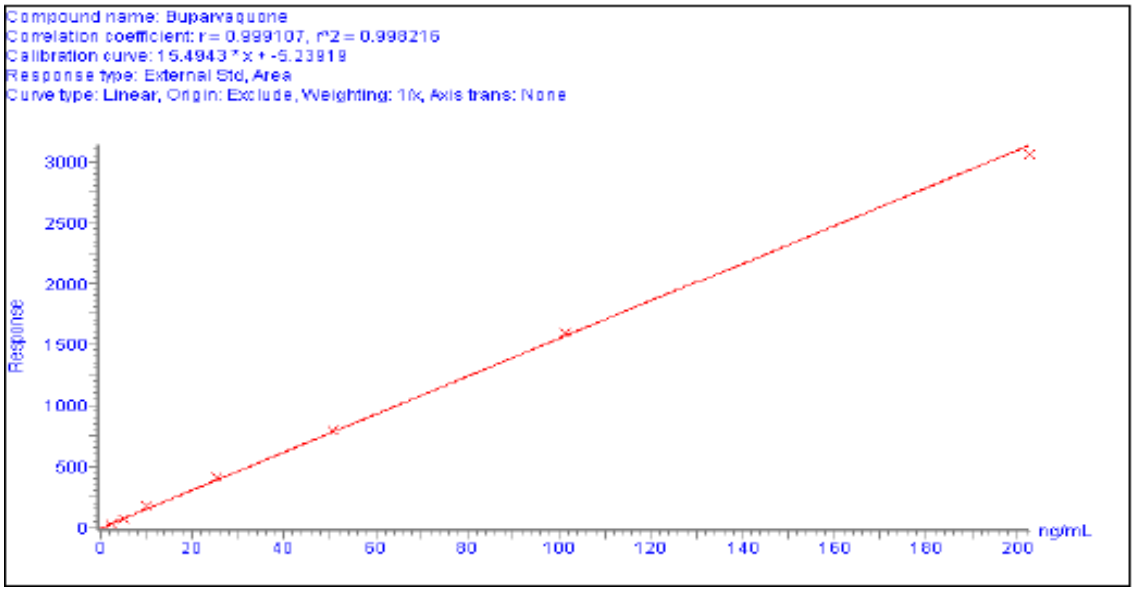


Figure 3 - Buparvaquone in Muscle Matrix Calibration Curve

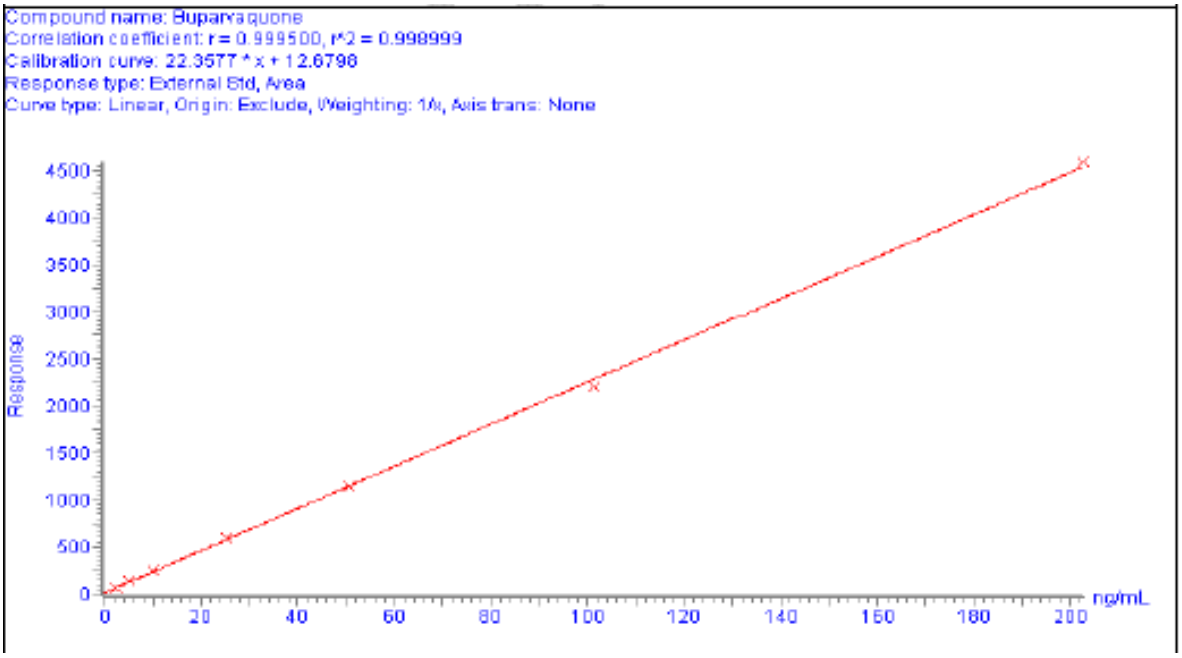


Figure 4 - Buparvaquone in Fat Matrix Calibration Curve

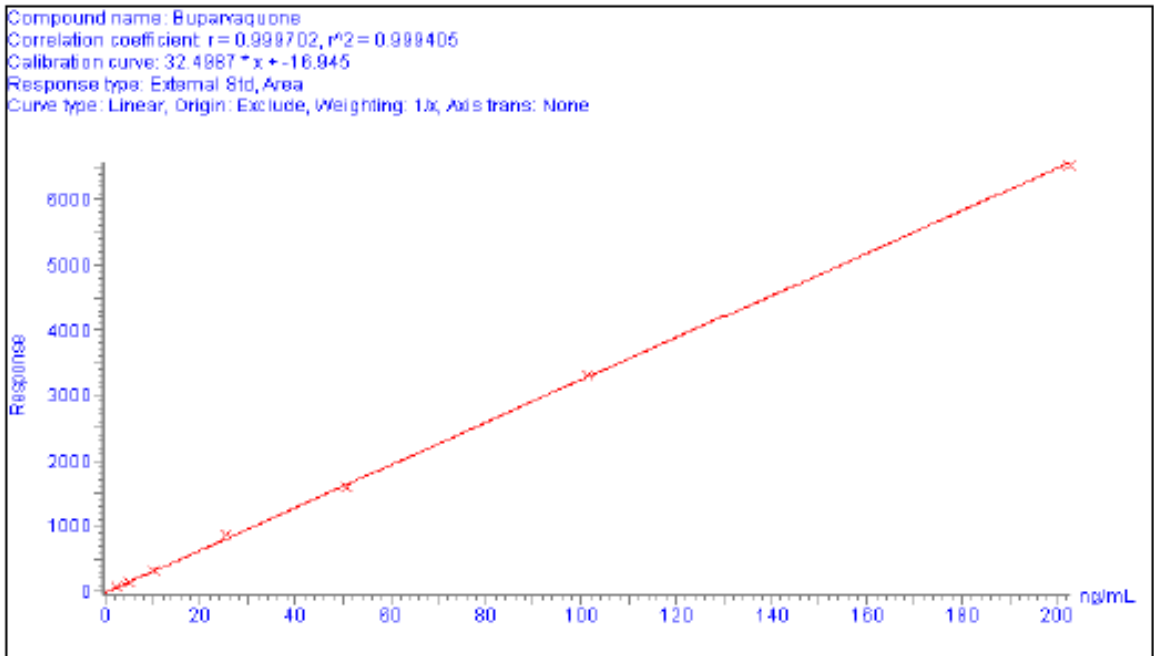
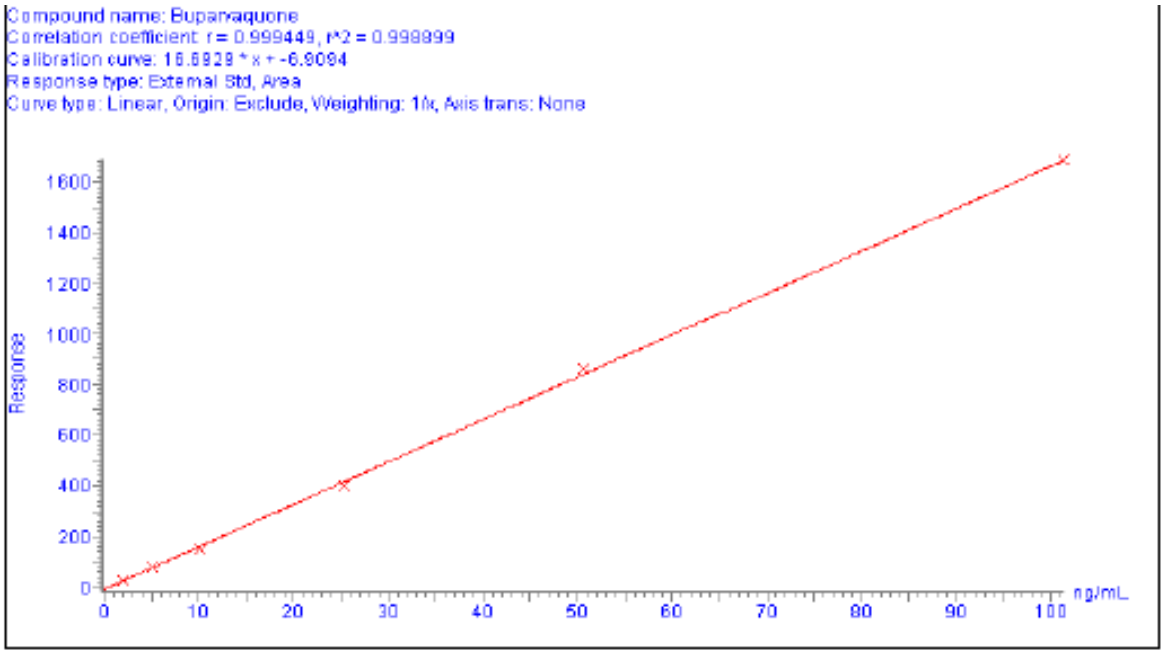


Figure 5 - Buparvaquone in Milk Matrix Calibration Curve



APPENDIX B

Sample Chromatograms

Figure 6 – Typical Chromatograms of Buparvaquone in Bovine Liver

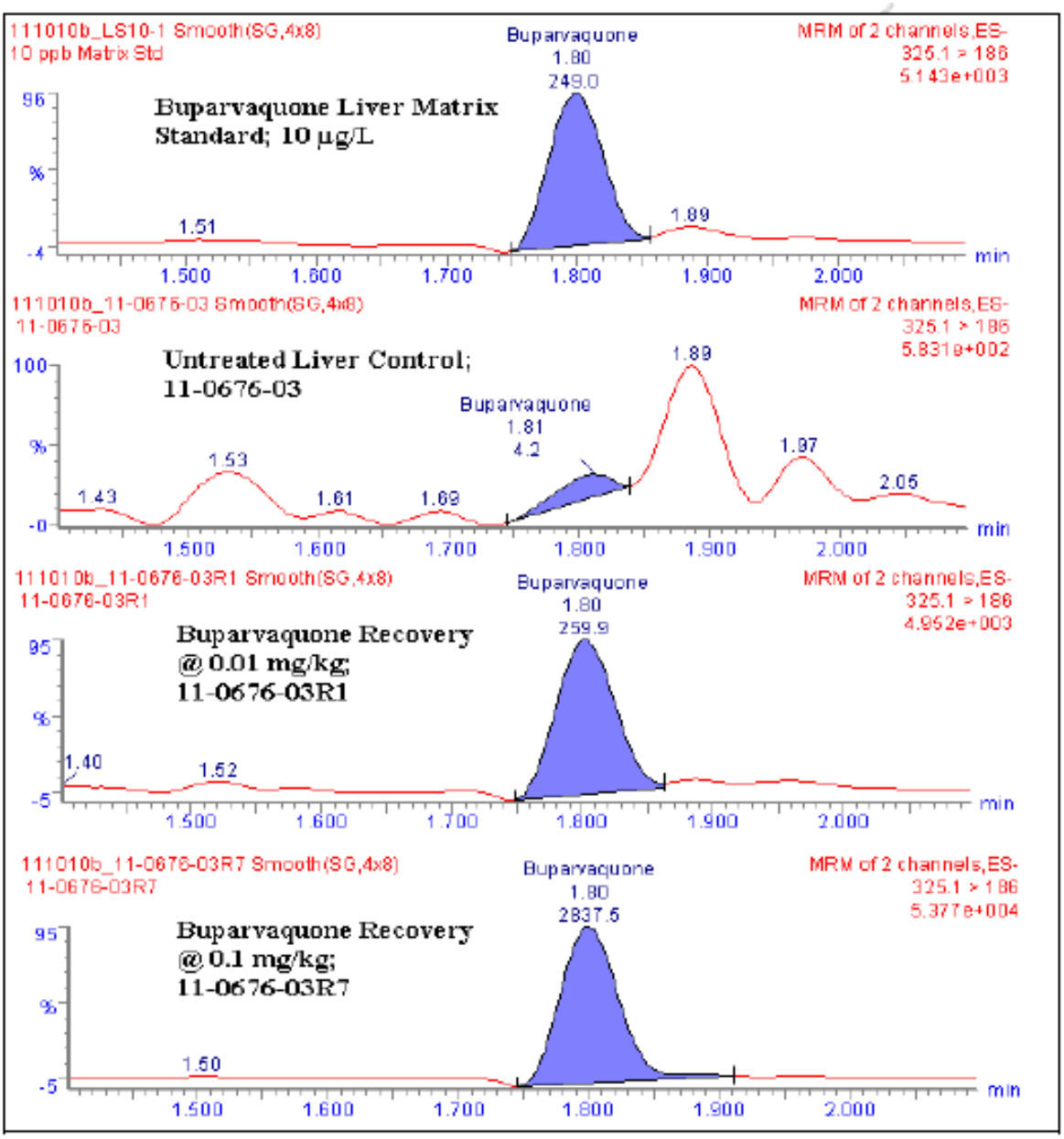


Figure 7 – Typical Chromatograms of Buparvaquone in Bovine Kidney

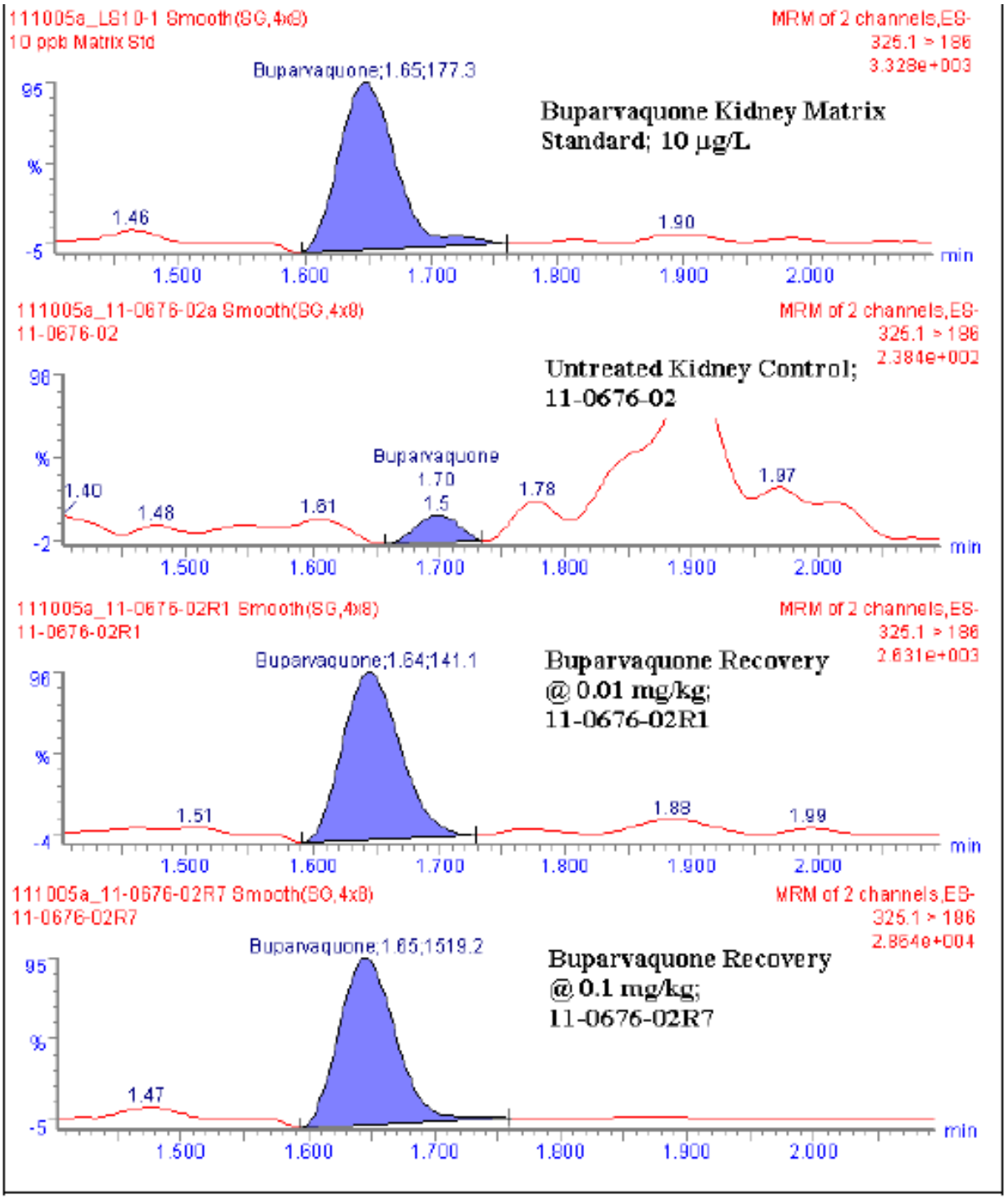


Figure 8 – Typical Chromatograms of Buparvaquone in Bovine Muscle

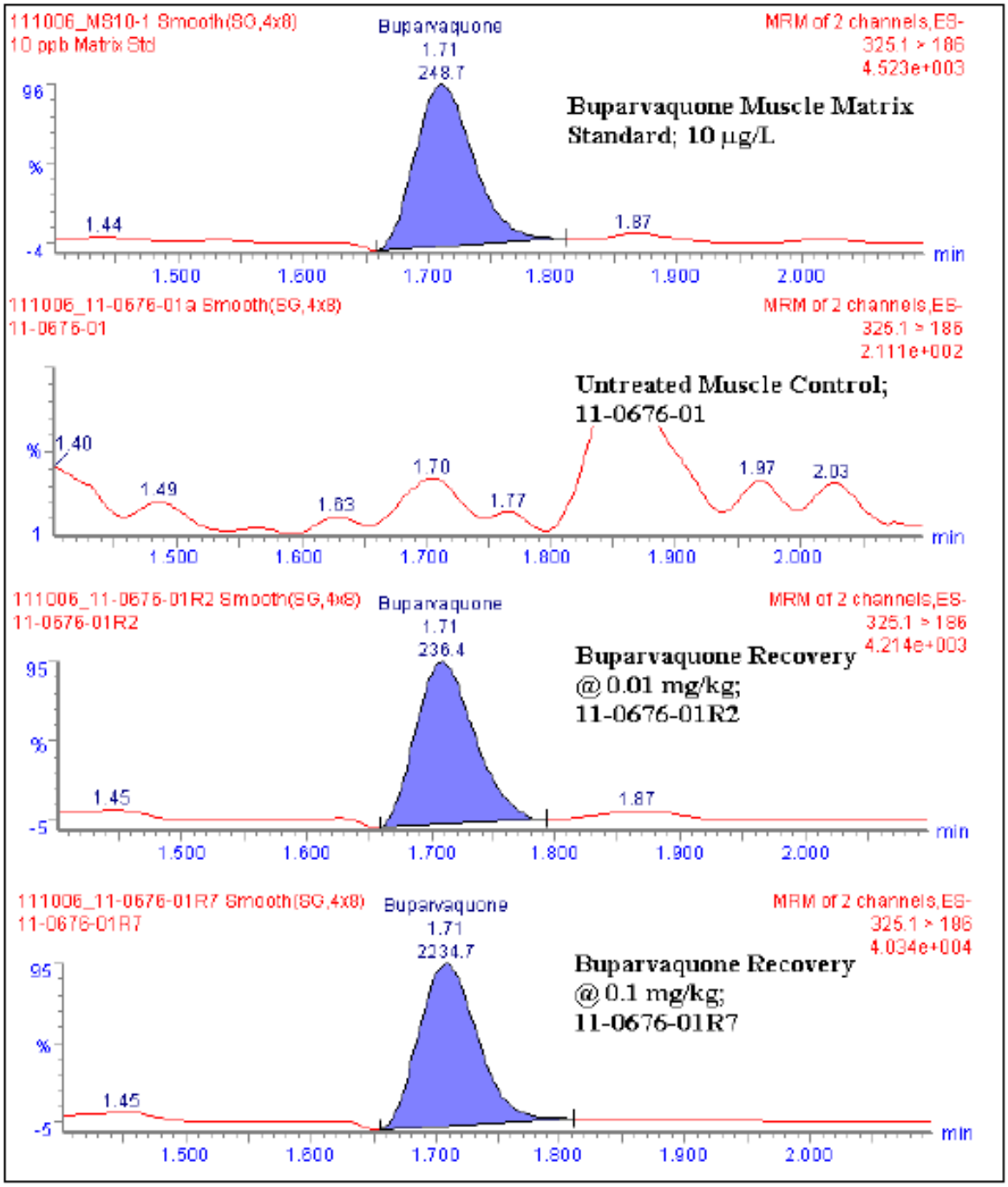


Figure 9 – Typical Chromatograms of Buparvaquone in Bovine Fat

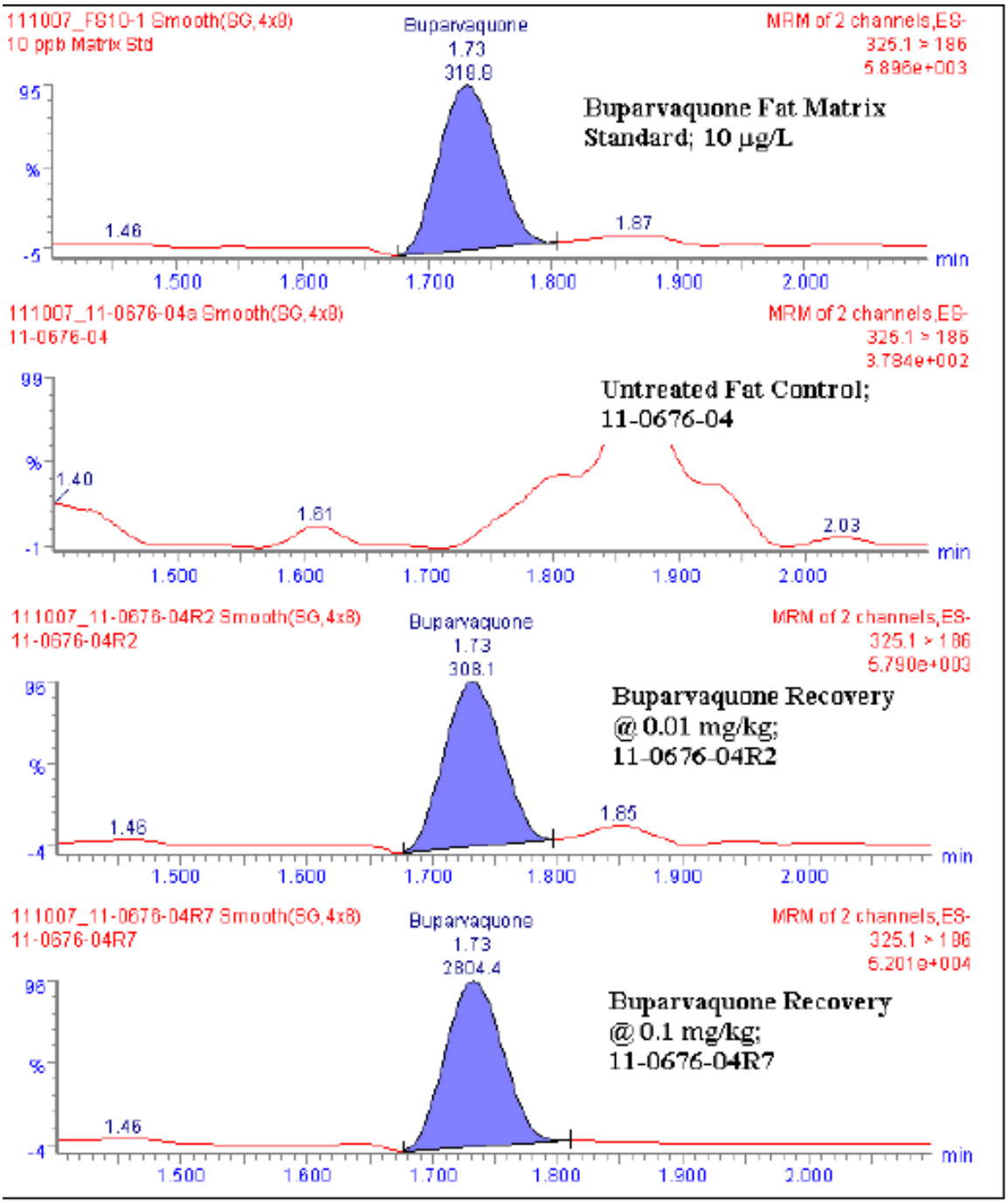
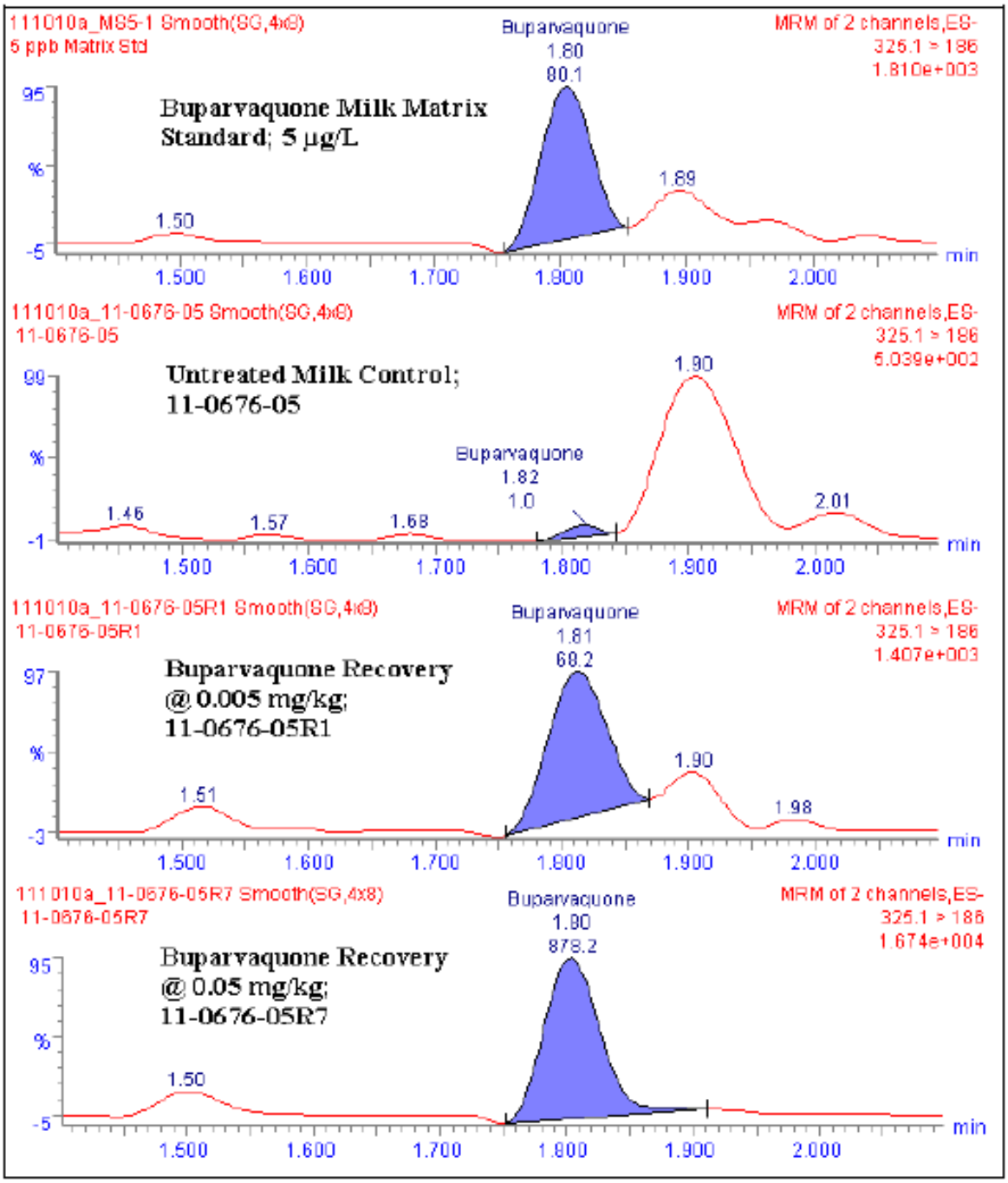


Figure 10 – Typical Chromatograms of Buparvaquone in Bovine Milk



APPENDIX C

Table 1 Recovery of Buparvaquone from Fortified Bovine Liver

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-03R1	Liver	0.01013	<LOD	0.01020	101
11-0676-03R2	Liver	0.01013	<LOD	0.00881	87
11-0676-03R3	Liver	0.01013	<LOD	0.01093	108
11-0676-03R4	Liver	0.01013	<LOD	0.00937	92
11-0676-03R5	Liver	0.01013	<LOD	0.01076	106
11-0676-03R11	Liver	0.01013	<LOD	0.01004	99
			Average	0.010	99
			SD	0.001	8.0
			RSD	8.089	8.1

Buparvaquone

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-03R7	Liver	0.1013	<LOD	0.1049	103
11-0676-03R8	Liver	0.1013	<LOD	0.1071	106
11-0676-03R9	Liver	0.1013	<LOD	0.1104	109
11-0676-03R12	Liver	0.1013	<LOD	0.0959	95
11-0676-03R13	Liver	0.1013	<LOD	0.1026	101
11-0676-03R14	Liver	0.1013	<LOD	0.1029	102
			Average	0.104	103
			SD	0.005	4.8
			RSD	4.703	4.7

Table 2 Recovery of Buparvaquone from Fortified Bovine Kidney

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-02R1	Kidney	0.01013	<LOD	0.00945	93
11-0676-02R2	Kidney	0.01013	<LOD	0.00992	98
11-0676-02R3	Kidney	0.01013	<LOD	0.01011	100
11-0676-02R4	Kidney	0.01013	<LOD	0.01022	101
11-0676-02R5	Kidney	0.01013	<LOD	0.00868	86
11-0676-02R6	Kidney	0.01013	<LOD	0.00901	89
			Average	0.010	94
			SD	0.001	6.2
			RSD	6.519	6.5

Buparvaquone

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-02R7	Kidney	0.01013	<LOD	0.0984	97
11-0676-02R8	Kidney	0.01013	<LOD	0.0947	93
11-0676-02R9	Kidney	0.01013	<LOD	0.1015	100
11-0676-02R10	Kidney	0.01013	<LOD	0.0953	94
11-0676-02R11	Kidney	0.01013	<LOD	0.0916	90
11-0676-02R12	Kidney	0.01013	<LOD	0.0903	89
			Average	0.095	94
			SD	0.004	4.1
			RSD	4.379	4.4

Table 3 Recovery of Buparvaquone from Fortified Bovine Muscle

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-01R1	Muscle	0.01013	<LOD	0.00888	88
11-0676-01R2	Muscle	0.01013	<LOD	0.01001	99
11-0676-01R3	Muscle	0.01013	<LOD	0.00979	97
11-0676-01R4	Muscle	0.01013	<LOD	0.00855	84
11-0676-01R5	Muscle	0.01013	<LOD	0.00955	94
11-0676-01R6	Muscle	0.01013	<LOD	0.01058	104
			Average	0.010	94
			SD	0.001	7.4
			RSD	7.808	7.8

Buparvaquone

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-01R7	Muscle	0.01013	<LOD	0.0994	98
11-0676-01R8	Muscle	0.01013	<LOD	0.0981	97
11-0676-01R9	Muscle	0.01013	<LOD	0.0916	90
11-0676-01R10	Muscle	0.01013	<LOD	0.0917	90
11-0676-01R11	Muscle	0.01013	<LOD	0.0984	97
11-0676-01R12	Muscle	0.01013	<LOD	0.0978	97
			Average	0.096	95
			SD	0.004	3.5
			RSD	3.693	3.7

Table 4 Recovery of Buparvaquone from Fortified Bovine Fat

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-04R1	Fat	0.01013	<LOD	0.00870	86
11-0676-04R2	Fat	0.01013	<LOD	0.01000	99
11-0676-04R3	Fat	0.01013	<LOD	0.01046	103
11-0676-04R4	Fat	0.01013	<LOD	0.00925	91
11-0676-04R5	Fat	0.01013	<LOD	0.01012	100
11-0676-04R6	Fat	0.01013	<LOD	0.01010	100
			Average	0.010	96
			SD	0.001	6.5
			RSD	6.762	6.8

Buparvaquone

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-04R7	Fat	0.01013	<LOD	0.0970	96
11-0676-04R8	Fat	0.01013	<LOD	0.0907	89
11-0676-04R9	Fat	0.01013	<LOD	0.0935	92
11-0676-04R10	Fat	0.01013	<LOD	0.1028	101
11-0676-04R11	Fat	0.01013	<LOD	0.0964	95
11-0676-04R12	Fat	0.01013	<LOD	0.0934	92
			Average	0.096	94
			SD	0.004	4.1
			RSD	4.371	4.4

Table 5 Recovery of Buparvaquone from Fortified Bovine Milk

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-05R1	Milk	0.005066	<LOD	0.004497	89
11-0676-05R2	Milk	0.005066	<LOD	0.005783	114
11-0676-05R3	Milk	0.005066	<LOD	0.004408	87
11-0676-05R4	Milk	0.005066	<LOD	0.004700	93
11-0676-05R5	Milk	0.005066	<LOD	0.004589	91
11-0676-05R6	Milk	0.005066	<LOD	0.004920	97
			Average	0.00482	95
			SD	0.00051	10.0
			RSD	10.5	10.5

Buparvaquone

Laboratory No.	Substrate	Spiking Level (µg/kg)	Buparvaquone in Control (µg/kg)	Buparvaquone Found Level (µg/kg)	% Recovery
11-0676-05R7	Milk	0.005066	<LOD	0.05302	105
11-0676-05R8	Milk	0.005066	<LOD	0.05500	109
11-0676-05R9	Milk	0.005066	<LOD	0.05556	110
11-0676-05R10	Milk	0.005066	<LOD	0.05748	113
11-0676-05R11	Milk	0.005066	<LOD	0.05366	106
11-0676-05R12	Milk	0.005066	<LOD	0.05209	103
			Average	0.0545	108
			SD	0.00195	3.8
			RSD	3.6	3.6