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Extraction and analysis of moxidectin in wombat plasma and faeces

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ABSTRACT

Sarcoptic mange in wombats results from a skin infestation by *Sarcoptes* mites and if untreated, results in a slow and painful death. Moxidectin is a pesticide used to treat internal and external parasites in cattle, but has shown to effectively treat other animals, including wombats. Two methods were developed to analyse wombat plasma, and methods were also developed to analyse faeces and fur. Moxidectin-D3 was used as an internal standard and behaved almost identically to moxidectin, resulting in recoveries of 95–105 % across the three matrices, even when matrix interferences caused signal suppression as high 20 %, or when moxidectin loss was high. This was presumably due to the high binding efficiency of plasma for MOX and MOX-D3. Moxidectin limits of detection were 0.01 ng/mL in plasma, 0.3 ng/g dry weight equivalent for faeces and 0.5 ng/g for fur. This study also developed a method to isolate plasma macromolecules, allowing the extraction of bound moxidectin for quantitative purposes, with an LoQ of 0.05 ng/mL. This method was subsequently used to determine that moxidectin was 97–99.4 % bound to lipoproteins in wombat plasma and 98–99 % bound in sheep, cow and horse plasma. The method reported for plasma was quick, cheap, and conducive to large sample batches, while providing high sensitivity. While faecal samples required additional cleanup steps to reduce the matrix effect, co-extracted matrix components such as undigested chlorophyll continued to result in ionisation suppression in the MS/MS. The methods reported here were used to monitor moxidectin in wombats treated with a single pour-on treatment, and confirmed that the moxidectin concentration in wombat plasma had decreased by more than 90 % by 28 days after application, while providing protection against sarcoptic mites over the majority of their life cycle. Clearance of moxidectin occurred via faecal elimination over the four week period and while moxidectin accumulated on fur due to application as a pour-on, concentrations declined rapidly by the four week period as fur fell out and was replaced by fresh fur that did not contain moxidectin.

1. Introduction

Wombats are quadruped herbivore marsupials that are endemic to Australia, and are subdivided into the common bare-nosed wombat (*Vombatus ursinus*), the northern hairy-nosed (*Lasiorhinus krefftii*), and the southern hairy-nosed wombats (*Lasiorhinus latifrons*). All three species are threatened by land clearing, predation, fire, wild dogs, drought, collisions with cars and diseases, such as sarcoptic mange [1], which is caused by the introduced parasitic mite, *Sarcoptes scabiei*. Sarcoptic mites burrow into the outer layer of epidermis (stratum corneum) after contacting mammals, resulting in scabies in humans or sarcoptic mange in animals [2]. Mange in wombats causes loss of fur, thickening of the skin, and skin crusting [3]. These symptoms result in excessive scratching, which causes skin abrasions that may lead to fly strike and skin infections. Thickening of the skin may also restrict animal mobility and reduce their ability to forage, thereby increasing the likelihood of starvation. These outcomes increase suffering and are ultimately fatal to infected animals, with suggestion that animals are only likely recover with human intervention to eliminate the mite infestation [3].

Treatment of mange usually relies on pour-ons or subcutaneous

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injection of anti-parasitic drugs that were developed for use on domestic or farm animals to control fleas, ticks and mites. Ivermectin (IVR) as Stromectol™ [4] and moxidectin (MOX) as Cydectin® [5] have both been used to treat wombat species by killing the mites responsible for mange. Application rates of 0.2 mg/kg MOX [5] and 0.2–0.3 mg/kg IVR [4,6] as subcutaneous injections, and 4 mg/kg MOX as a pour-on [7] have been reported as effective. However, a single oral dose of MOX was reportedly more active than two consecutive oral doses of ivermectin in pigs [8], which was attributed by the authors to a MOX residence time that was nine times longer than IVR, which better covered the various stages in the 30–60 day mite lifespan [9]. Approximately 90 % of IVR and MOX are excreted unmetabolised by animals in faeces [10], which may pose a risk to the dung beetles that degrade animal faeces. This may increase when high application and excretion rates are administered, and leaching into the soil profile from deposited faeces is prevented by strong binding of anti-parasitic drugs by faecal components occur. Consequently, the safe use of anti-parasitic drugs used for treating mange require suitable analytical methods to identify faecal concentrations for monitoring potential environmental contamination, as well as to confirm chemicals like MOX do not accumulate in the bloodstream of wombats being treated for mange.

Due to a relatively low application rate, MOX plasma and faecal concentrations are expected to be proportionally lower, requiring sample clean-up and concentration to improve quantification. MOX has high fat solubility and is thought to bind extensively to plasma proteins, particularly high-density lipoprotein (HDL) [11]. The use of protein precipitation has been reported to quantify MOX in plasma [5] which is a rapid and cheap approach, but compromises sensitivity, particularly if matrix suppression becomes significant during analysis. While liquid–liquid extraction has been reported [12], solid-phase extraction (SPE) is the most common approach for concentrating and purifying plasma [10,13–15] and faeces [10], which can improve the limit of quantitation (LoQ) by increasing the MOX concentration, while reducing matrix effects. The inclusion of a suitable internal standard is essential for analysis of complex biological matrices, but many studies ignore this aspect [10,15–17]. Other studies have used IVR [13], selamectin [5] and doramectin [12], but these chemicals have different structural features than MOX, and may have vastly different solubilities and matrix binding coefficients. Only one study appears to have used deuterated (^{2}H) MOX [18] and no studies have used ^{13}C analogues of MOX as internal standards. While heavy analogues are not suitable for analysis by LC-UV [19] or LC-fluorescence [10,12,13,16], isotope dilution is perfectly suited to LC-MS/MS analysis, which has become the most common analytical approach for moxidectin analysis [5,14,15,17]. The following work reports sensitive LC-MS/MS methods for the analysis of moxidectin in wombat plasma and faeces, compares them to some existing approaches, and demonstrates their application to a study on the use of a pour-on moxidectin to combat sarcoptic mange in wild wombats.

2. Materials and methods

2.1. Reagents and standards

IVM, MOX and MOX-D3 were manufactured by Toronto Research Chemicals (Mississauga, Canada) and purchased from Sapphire Bioscience (Sydney, Australia), and were used to prepare stick solutions used for constructing calibration curves, for matrix spiking and QC preparations. All organic solvents were purchased from ChemSupply Australia (Adelaide, Australia), sodium chloride/magnesium sulfate was purchased from Agilent (Melbourne, Australia), and ammonium acetate was purchased Sigma Aldrich (Sydney Australia).

2.2. Plasma binding of MOX

The extent of wombat plasma binding by MOX was determined due to its high lipophilicity and greater likelihood of being bound in the aqueous phase. Size exclusion centrifugation using 3 kDa filters was used to separate macromolecules from the aqueous phase. Plasma from sheep, cows and horses was also tested to determine whether plasma binding was species specific. Pooled plasma (500 µL) from at least four animals of each species was transferred to Eppendorf tubes (1.5 mL) in triplicate per species. Tubes were spiked with MOX solutions (20 µL) to provide a concentration range of 0.1–200 ng/mL for wombat plasma, and 2 ng/mL for sheep, cow and horse plasma. Solutions were vortexed for 30 s and transferred to Merck Amicon Ultra regenerated cellulose centrifuge filters (0.5 mL, 3 kDa) and centrifuged (14,000 × *g*, 25 min) according to manufacturer specifications. The recovered supernatants were spiked with MOX-D3 (500 ng/mL), mixed, the volume measured, and the solution analysed by LC-MS/MS. The filter unit was inverted in a centrifuge tube (10 mL) and centrifuged (2000 × *g*, 1 min) according to manufacturer specifications. After removing the filter, the protein in the centrifuge tube was spiked with MOX-D3 (500 ng/mL), and acetonitrile (2 × 3 mL) was added with 4:1 NaCl/$MgSO_4$ (0.2 g). After vortexing (1 min), the tube was centrifuged (3000 × *g*, 5 min) and the combined acetonitrile extracts were dried under nitrogen (30 °C). The residue was resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis by LC-MS/MS.

2.3. Quantitation of MOX

Due to the unavailability of a large enough volume of clean wombat plasma to allow method development, sheep plasma was used to develop and validate the various extraction methods. Optimised conditions were validated on pooled wombat plasma. We compared several reported extraction approaches (protein precipitation, LLE and SPE) for extracting MOX from plasma for analysis to two newly developed approaches (LLE with protein precipitation, and extraction from isolated plasma macromolecules) (Fig. 1).

2.3.1. Protein precipitation and solvent extraction of MOX

Protein precipitation was achieved using either methanol or acetonitrile. Sheep plasma (1 mL) was spiked with MOX (20 µL) to produce a final concentration of 1 ng/mL in plasma. After vortexing, an internal standard mix consisting of MOX-D3 (20 µL, 500 ng/mL) and IVR (20 µL, 1000 ng/mL) were added and then vortexed. Acetonitrile, acetonitrile containing 1 % formic acid, or methanol (1 mL) were added and vortexed for 2 min to precipitate proteins. Tubes were centrifuged (20,000 × *g*, 5 min) and solutions were analysed by LC-MS/MS. Alternatively, extracts were either placed in a vacuum oven overnight to dry and then resuspended, or $MgSO_4$/NaCl was added to the water/organic solvent solutions to separate the acetonitrile phase, which was recovered and evaporated under nitrogen (30 °C). All dry residues were resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis.

Direct liquid/liquid extraction (LLE) of plasma using hexane, chloroform, dichloromethane or ethyl acetate (2 × 4 mL) was performed, followed by evaporation at 30 °C under nitrogen. Residues were resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis. Solvent extraction was also performed on spiked plasma after protein precipitation with acetone (1.5 mL), followed by vortexing (5 min) and then centrifugation (3000 × *g*, 5 min). The supernatants were transferred to clean tubes and instrument grade water (1.5 mL) was added, followed by hexane (3 mL), and the solutions vortexed (5 min). After centrifugation (3000 × *g*, 5 min), the hexane layer was recovered to a glass vial, and the aqueous phase extracted a second time with hexane (3 mL). Combined hexane extracts were evaporated at 25 °C under nitrogen. Residues were resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis.

2.3.2. Solid phase extraction of plasma

Solid phase extraction (SPE) was performed using 2 stacked Strata-X 200 mg/3 mL cartridges and Sep-Pak C18 1000 mg/ 6 mL. Spiked plasma containing MOX-D3 (20 µL, 500 ng/mL) was diluted with formic

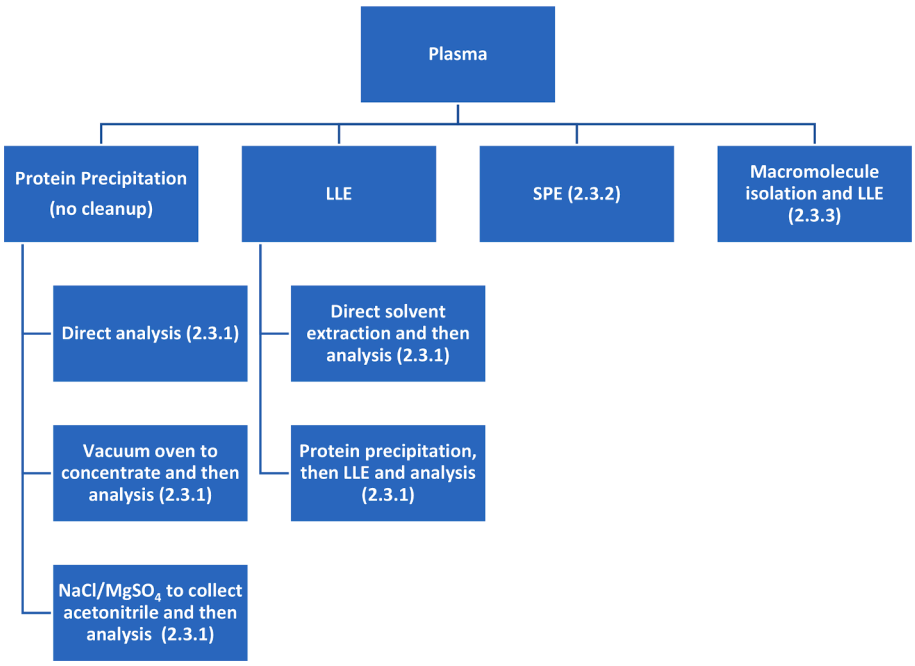

Fig. 1. Extraction approaches used quantify MOX in plasma.

acid in water (1 %, 2 mL) and applied to cartridges that had been pre-conditioned with two volumes of methanol and equilibrated with two volumes of instrument grade water. After application, cartridges were dried under vacuum for 15 min, and then eluted with methanol (5 mL). Dispersive SPE was also trialled by adding the diluted plasma to Agilent dSPE tubes, vortexed (5 min) and then centrifuged (3000 × *g*, 5 min). The supernatant was removed and the substrate was extracted in methanol (5 mL). SPE and dSPE extracts were evaporated at 30 °C under nitrogen and residues were resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis.

2.3.3. *Solvent extraction of plasma-bound MOX*

As MOX was found to be extensively bound by plasma, size exclusion centrifugation was used to separate macromolecules with bound MOX from the bulk plasma. MOX-spiked plasma (500 µL) was spiked with MOX-D3 (500 ng/mL) and transferred to Merck Amicon Ultra regenerated cellulose centrifuge filters (0.5 mL, 3 kDa or 10 kDa) and centrifuged (14,000 × *g*, 25 min). The filter unit was inverted in a centrifuge tube (10 mL) and centrifuged (2000 × *g*, 1 min). After removing the filter, acetonitrile or acetone (3 mL) was added. After vortexing (1 min), the tube was centrifuged (3000 × *g*, 5 min) and the extracts were dried under nitrogen (25 °C). The residue was resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis by LC-MS/MS.

2.4. *MOX extraction from faeces*

Clean faeces from at least 5 wombats was thawed, crumbled and then mixed. Subsamples (2 g) were weighed into centrifuge tubes and then spiked with MOX (20 µL) to provide final wet concentrations of 0.1–1000 ng/g. MOX-D3 (20 µL, 500 ng/mL) was added and allowed to stand for 15 min. Acetone (5 mL) was added, the tubes were vortexed for 2 min, and then sonicated (Thermoline UB-410) for 20 min at full power. After centrifuging (3000 × *g*, 5 min), the supernatant was decanted into a clean centrifuge tube containing formic acid (1.5 mL, 0.1 %) and hexane (4 mL), and vortexed for 3 min. After centrifuging (3000 × *g*, 5 min), the hexane layer was recovered and the aqueous layer was extracted a second time with hexane (4 mL). Combined extracts were reduced to 2–3 mL under nitrogen (25 °C). Florisil was activated over night at 500 °C and 1.5 g was added to glass columns, followed by anhydrous Na_2SO_4 (3 g). Hexane extracts were applied to the columns, and then washed with hexane (5 mL) and acetone in DCM (2 × 5 mL, 5 %). Columns were then eluted with acetone in DCM (2 × 5 mL, 20 %), followed by pure acetone (2 × 5 mL), or eluted with only pure acetone (2 × 5 mL). Extracts were evaporated under nitrogen (30 °C) and the residue was resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis by LC-MS/MS.

2.5. *MOX extraction from fur*

Fur (100 mg) was cut into lengths of a few mm into centrifuge tubes (10 mL) and spiked with MOX-D3 (20 µL, 500 ng/mL). After allowing to stand for 15 min to allow the solvent to evaporate, acetone (5 mL) was added and the tube sonicated for 20 min. After centrifugation (3000 × *g*, 5 min), the acetone was recovered, and hair extracted a second time with acetone (5 mL). Combined acetone extracts were evaporated under nitrogen (30 °C) and then resuspended in 5 mM ammonium acetate in acetonitrile (200 µL) for analysis by LC-MS/MS.

2.6. *LC-MS/MS conditions*

MOX was analysed using an Agilent Technologies 1200 Series LC with binary pump, degasser, column oven, autosampler, DAD (244 nm) and 6470 quadrupole tandem mass spectrometer. A Phenomenex Kinetex C8 (75 × 4.6 mm × 2.6 µm) column operated at 40 °C. The MS/MS used ESI+, and the ion transitions, retention times and parameters varied for each chemical and are shown in Table 1. Additional MS/MS

Table 1
Acquisition parameters for MS/MS.

MS/MS Parameter	IVR	MOX-1	MOX-2	MOX-D3
Ion transition (*m/z*)	893.5 → 552.8	640.5 → 528.3	640.5 → 498.3	643.4 → 531.4
Fragmentation voltage (V)	150	120	120	120
Collision energy	19	4	8	4
Cell Accelerator (V)	1	5	5	5
Dwell (ms)	20	100	20	20
RT (min)	8.06	8.28	8.28	8.27

parameters were gas temperature/flow (150 °C, 8 L/min), sheath gas temperature/flow (350 °C, 10 L/min), nebuliser (30 psi), capillary voltage (3500 V) and charging voltage (500 V). Mobile Phase A was aqueous ammonium acetate (0.05 % m/v) and Phase B was acetonitrile/water (95 %:5% v/v) containing ammonium acetate (0.05 % m/v). The gradient held Phase B at 20 % for 0.5 min, then ramped to 90 % by 4.5 min, and held until 9 min at a flow rate of 0.6 mL/min. The injection volume was 20 µL and the calibration range was 0.2–250 ng/mL, but was tested up to 1000 ng/mL.

2.7. Validation of optimised MOX extraction from plasma, faeces and fur

The optimised protein precipitation/LLE method was first validated using pooled, clean sheep plasma (1 mL) was spiked with MOX (20 µL) in a centrifuge tube (10 mL) to give final concentrations of 0.01, 0.05, 0.1, 0.5, 2, 10, 50, 100 and 200 ng/mL in plasma in triplicate. Pooled, clean wombat plasma was also used to provide MOX concentrations of 0.05, 0.1 1 and 2 ng/mL. After mixing the spiked plasma, MOX-D3 (20 µL, 500 ng/mL) was added and mixed. Acetone (1.5 mL) was added and vortexed (2 min), and the tube centrifuged (3000 × *g*, 5 min). The supernatant was recovered to a new tube and aqueous formic acid (1.5 mL, 0.1 % v/v) was added. The solution extracted twice with hexane (2 × 3 mL) using vortexing (2 min), and the organic phase was separated using centrifugation (3000 × *g*, 5 min). Combined hexane extracts were dried under nitrogen (25 °C) and the residue resuspended in ammonium acetate in acetonitrile (200 µL, 5 mM) for analysis by LC-MS/MS. Matrix suppression was determined by extracting blank pooled plasma and spiking the dried residue prior to resuspension in 5 mM ammonium acetate in acetonitrile, and comparing it to the pure resuspension buffer.

Fur and faeces were extracted as previously described and validated over the 0.1–1000 ng/g in faeces and 10–500 ng/g in fur. Florisil columns used for clean-up of faecal extracts were eluted using pure acetone. Matrix suppression in faeces was performed by extracting approximately 20 g of blank faeces (the equivalent of 10 faeces samples) according to the methodology in Section 2.4, and resuspending the extracted matrix in resuspending solvent (2 mL). Sub-samples (200 µL) were spiked with MOX and MOX-D3, and compared to the same spikes in pure resuspending solvent for matrix suppression of both MOX and MOX-D3. Additionally, moisture in faeces was determined to allow MOX dry weight calculation by drying samples (10–15 g) for 48 h at 30 °C under vacuum with a gentle stream of nitrogen to assist with removal of water vapour.

2.8. MOX application and sample collection from wild wombats

Wombats were treated once with the current maximum allowable dose approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA PER90094) by pouring Cydectin® (100 mL, 5 g/L moxidectin) along their backline using a pole and scoop. Wombats were sedated and blood was collected in EDTA and lithium heparin tubes on days 1, 7, 14, 21 and 28 after treatment with moxidectin, with a control sample collected 5–14 days prior to Cydectin® application. As wild animals were used in the study, faecal samples were collected opportunistically off the ground after animals were observed and ownership could be confirmed, bagged and frozen (−20 °C) for analysis. Fur was sampled before and after Cydectin® using hair clippers to shave off a 2 cm by 2 cm square of fur from the shoulder, flank or rump of the wombat. Plasma, faeces and fur were analysed using optimised extraction conditions described in the previous section. Additionally, a selection of plasma samples were analysed using the size exclusion centrifugation method to isolate macromolecules containing bound MOX residues to validate this method. Quality control samples were included in each batch of incurred samples. This consisted of spiking blank plasma to concentrations of 0.1 and 2 ng/mL in plasma and 100 and 500 ng/g in faeces, all in duplicate and running duplicates randomly in the LC-MS/MS sample order.

3. Results and discussion

3.1. LC-MS/MS conditions

Initial attempts to identify the M + H ion using ESI+ resulted in a weak signal and scans showed the formation of Na and K adducts, which have been previously identified for moxidectin [15,20] and ivermectin [21] and seemed to be provoked in the current study by the inclusion of formic acid in the mobile phase. The resulting calibration was consistent but resulted in an upwardly-inflecting curve that could not be overcome, even when using MOX-D3. The replacement of formic acid with ammonium acetate increased the strength of the M + H ion signal and seemed to almost entirely eliminate the M + Na and M + K ions, and signal strength was doubled again by using widest resolution on Q1 and unit resolution on Q3. The use of a standard C18 column resulted in an unnecessarily long MOX retention time but this was shortened by the use of a C8 column instead. MOX and MOX-D3 peaks were very sharp and this was presumably due to their high hydrophobicity and low water solubility (Fig. 2). The use of a wider bore C8 column (4.6 mm) allowed a larger injection volume (20 µL) without band broadening or tailing due to MOX adsorbing on the front of the column after injection into the highly aqueous mobile phase. An attempt to use a 3 mm ID C8 column resulted in a shorter retention time, but peak height decreased and peak width increased by approximately 10 % compared to the 4.6 mm ID column, presumably due to the relatively large injection volume causing MOX to diffuse further into the column prior to adsorbing. MOX calibration was linear over the 0.2–250 ng/mL range, and a quantification range up to 1000 ng/mL.

3.2. MOX plasma protein binding

The binding of MOX by plasma proteins was investigated in wombat, horse, sheep and cows due to its potential to interfere in analysis of macrocyclic lactones, such as AVR [22]. MOX binding appeared to occur almost immediately after spiking plasma as no equilibration period was required prior to centrifugal filtration. The plasma binding of MOX for the four animal species was not significantly different. The mean MOX binding ranged from 97.9 to 98.7 % for the four animals at a 2 ng/mL plasma concentration and was as high as 99.4 % for wombats at 0.5 ng/mL (Table 2), confirming that MOX is almost entirely bound by plasma macromolecules. Another study in which goat plasma macromolecules were fractionated showed that plasma containing MOX at 10 ng/mL showed more than 98 % of MOX was bound to the lipoprotein fraction, with approximately 90 % of that component bound specifically by HDL and approximately 10 % by low density lipoprotein (LDL) [11]. As HDL tends to be quite large (>175 kDa), the use of filters with a larger pore size may be adequate to isolate HDL-bound MOX from other plasma components.

3.3. MOX extraction from plasma

3.3.1. Solvent extraction of plasma

Both MOX-D3 and IVR were trialled simultaneously as internal standards. Previous studies have reported other avermectins, such as selamectin [5], abamectin [21], ivermectin [13] and doramectin [12], or have simply used no internal standard at all [15–17,23–25] to account for variation in extraction efficiency. Our study found that MOX recovery when using IVR as an internal standard ranged from 35 to 190 % during the experimental variations of solvent types and volumes, even for replicates for the same extraction design. Conversely, MOX recoveries of 95–105 % were obtained when using MOX-D3 as an internal standard, even when the absolute MOX recovery was as low as 20 % in some of the poorer extraction approaches trialled. The difference in behaviour between MOX-D3 and IVR is likely due to the difference in lipophilicity of IVR and MOX, where MOX and MOX-D3 should behave in almost identical fashions. Other studies have likewise shown the

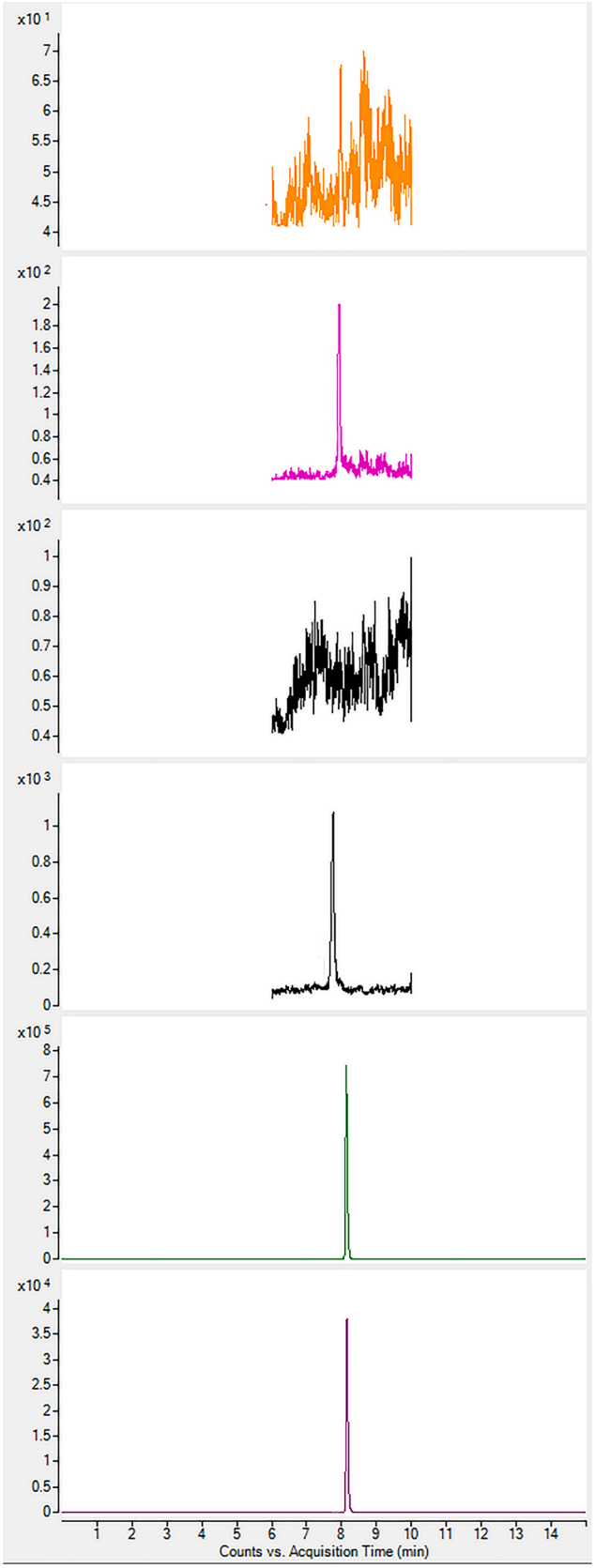

Fig. 2. Primary ion transition for blank plasma (A), 0.01 ng/mL MOX in plasma (B), blank faeces (C), 1 ng/g MOX in faeces (D), 100 ng/mL MOX-D3 (E), 5 ng/mL MOX (F) and 100 ng/mL IVR (G).

Table 2
Percentage of MOX bound to plasma components > 3 kDa (Mean ± StDev, n = 3).

Animal	MOX plasma concentration (ng/mL)	% Bound MOX
Wombat	0.1	98.9 ± 1.7
Wombat	0.5	99.4 ± 0.3
Wombat	2	98.5 ± 1.1
Wombat	20	97.0 ± 1.8
Wombat	40	97.5 ± 2.1
Wombat	100	99.5 ± 0.1
Wombat	200	99.1 ± 0.5
Sheep	2	97.9 ± 0.2
Cow	2	98.3 ± 1.5
Horse	2	98.7 ± 1.3

disparity between MOX recovery and the recoveries of internal standards such as IVR [13] and abamectin [14]. As a result of these inconsistent results, as well as the poorer sensitivity of IVR relative to MOX-D3, the inclusion of IVR in studies was discontinued and MOX-D3 was used as internal standard for all subsequent work.

Protein precipitation (or dilute and shoot) is the simplest, cheapest and most rapid approach for determining MOX in plasma, but it only results in the removal of proteins, and may still suffer from analyte suppression during analysis. Acetone, acetonitrile and methanol are commonly used to precipitate plasma proteins, but only the latter two are directly compatible with reverse phase HPLC. Acetonitrile and methanol were trialled in the current study, but both showed marked differences in their performance (Fig. 3A). While the use of MOX-D3 provided approximately 100 % recoveries, absolute MOX recoveries for methanol and acetonitrile were 21 % and 75 %, respectively, but the latter was improved to 82 % when 1 % formic acid was added. This may be a pH or salting effect which improved denaturation of the HDL and LDL components that were binding MOX in plasma. The poor performance of methanol may due to its higher polarity than acetonitrile, which may result in less desorption of the lipophilic MOX from HDL.

As the dilution of the plasma with solvent during this process resulted in poorer LoQs compared to processes that include a preconcentration step, attempts were made to remove water to isolate acetonitrile phase for evaporation and concentration (Fig. 3B). Acetone, acetonitrile, methanol and acetonitrile with 1 % formic acid were used to precipitate proteins and the resulting supernatant was placed in a vacuum oven to evaporate all solvents. Absolute MOX recoveries did not exceed 10 % for any treatment, while normalised MOX-D3 gave recoveries of 97–104 %. Evaporation controls containing only water and acetonitrile resulted in absolute and normalised recoveries of 57 and 95 %, respectively, indicating that evaporation under vacuum played a considerable role in MOX loss. The use of drying agents to remove water to allow evaporation of organic solvents under nitrogen was also trialled. Spiked plasma was treated with acetonitrile in 1:1, 1:2, 1:3 and 1:5 ratios to precipitate protein and the water/acetonitrile phase was then treated with $MgSO_4$/NaCl to remove water and separate the acetonitrile. Results showed that absolute MOX recovery increased with the addition of acetonitrile ratio, but recovery was still less than 50 % at a ratio of 1:5 (Fig. 3C). The exact binding mechanism of MOX to HDL and LDL is not known but difficulty extracting MOX may indicate strong adsorption to surface apoproteins, or diffusion through the phospholipid surface into the hydrophobic core of HDL particles [26], thereby restricting MOX extraction. Greater acetonitrile to plasma ratios may improve disruption of HDL components and improve MOX release or it may reduce the polarity of the aqueous phase enough to increase MOX solubility, but increases cost and time of sample analysis. Of all these approaches, dilute and shoot is the simplest, quickest and cheapest but comes at the expense of the MOX LoQ by a factor of approximately five, which may be the reason for limited use of this approach in the literature [5,24].

Direct liquid/liquid extraction (LLE) of plasma does not appear to have been reported for MOX. Low or non-polar solvents were compared

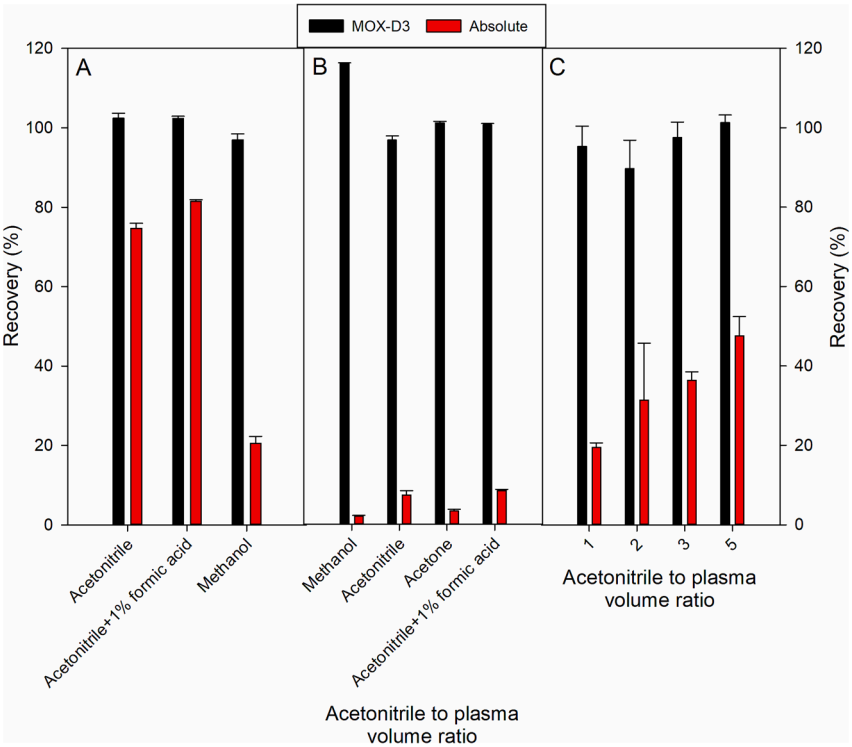

Fig. 3. MOX recoveries from plasma by protein precipitation (A), vacuum evaporation after protein precipitation (B) and varying acetonitrile:plasma ratio with the use of drying agents after protein precipitation (C).

for direct MOX extraction without protein precipitation (Fig. 4A). While hexane provided the best recovery with 34 %, all solvents performed poorly because they failed to desorb MOX from LDL and HDL, and suggesting either extremely strong sorption to the HDL surface, or that MOX may diffuse into the hydrophobic core of HDL particles. Protein precipitation with acetone followed by LLE with hexane or ethyl acetate showed a better recovery for hexane (Fig. 4B). Ethyl acetate extraction resulted in only 41 % MOX recovery, while the first hexane extraction alone accounted for more than 70 % of the MOX recovered of around 90 %. Hexane is also an appealing extraction solvent as it has a lower boiling point and required a lower temperature for evaporation to dryness, reducing the likelihood of MOX loss. Increasing the water or hexane volumes during the extraction resulted in poorer absolute MOX recovery. Whether this is an indication that subtle changes in solution polarity change the strength of the interaction between MOX and HDL, or whether MOX diffusion out of HDL is reduced, cannot be determined from these results. The addition of formic acid to the LLE stage and using hexane lowered the absolute MOX recovery slightly, but dramatically reduced the variability between the replicates for both absolute and MOX-D3, making it the preferred option. Increasing the volume of acetone to further improve absolute MOX recovery was counterproductive, and whether this was due to a change in the polarity of the aqueous solution, the formation of micelles or whether it simply reduced the efficiency of the hexane extraction step cannot be determine from these data. The single volume addition of acetone to precipitate plasma proteins was the most effective approach for extracting MOX, and may indicate a rupture of HDL particles which allowed MOX to be extracted, or a greater solvating power which allowed MOX to desorb from the surface of HDL.

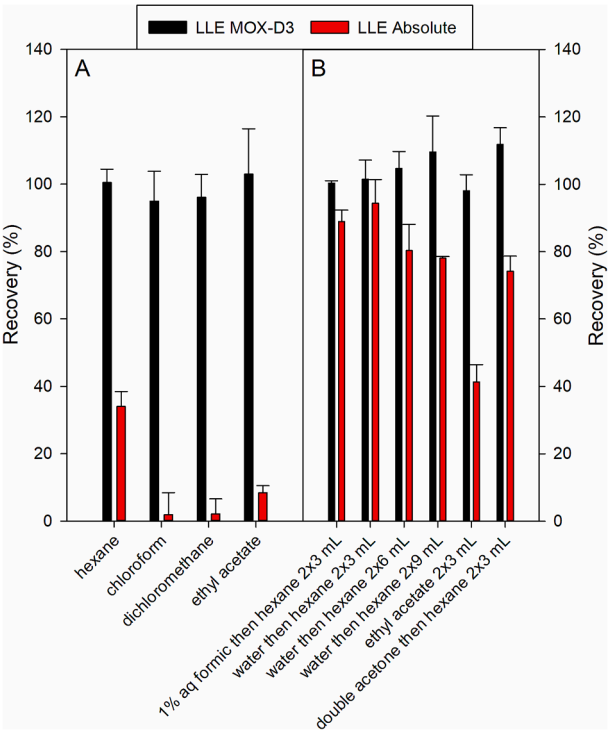

Fig. 4. MOX plasma recoveries using direct LLE with no protein precipitation (A), and protein precipitation with acetone followed by LLE (B).

3.3.2. SPE, dSPE and plasma protein extraction

We compared Strata-X, C18 and dispersive SPE (dSPE). SPE absolute recoveries from the initial cartridge using Strata-X and C18 were approximately 15 % and 20 % respectively, and another 10 % was recovered from each of the second cartridges, whereas dSPE resulted in less than 2 % (Fig. 5A). Double stacking SPE cartridges showed a failure to trap the highly hydrophobic MOX and may indicate that the inclusion of 50 % methanol was sufficient to interfere with MOX binding by SPE cartridges, or that MOX may bind to the hydrophobic tail of lipids from plasma to assist their transit through SPE cartridges. SPE using C18 is the

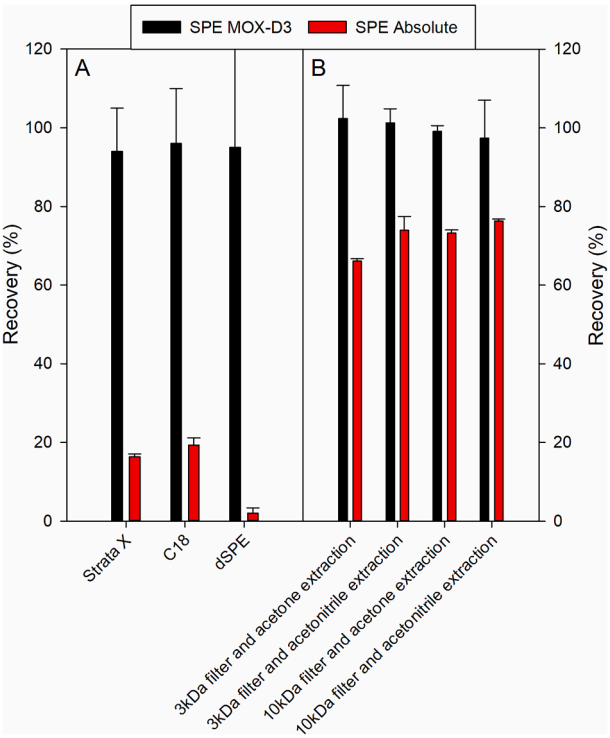

Fig. 5. MOX recovery using SPE and dSPE (A) and 3 kDa/10 kDa protein filters with acetone or acetonitrile as extracting solvent (B).

most widely reported extraction and cleanup approach for the analysis of MOX in plasma [12–15,17,21,23,25], with absolute recoveries of 60 % [14] and 94 % [13] from human plasma. The conundrum of these two approaches is the conflicting issue of requiring considerable solvent power to either disrupt HDL or desorb MOX from LDL/HDL initially, while requiring low solvent power when attempting to adsorb MOX on the SPE substrate. Work in the previous section of this study suggested that methanol performed poorly at releasing MOX for quantitation (Fig. 3A), and is likely to perform the same way when used at only 50 % concentration. Attempts in this study could not replicate other efficiencies reported [13,14], even when using larger capacity 1 g C18 cartridges, and may be an indication that a larger sample size was an issue, however other studies used automated SPE systems, which may have provided more consistent control over the process, compared to the manual approach used in the current study. The use of acetone to desorb MOX, followed by its evaporation prior to SPE to reduce its solvation power may be an avenue of further investigation.

Our initial work on plasma binding indicated that plasma lipoproteins acted as a natural SPE substrate by rapidly and efficiently binding MOX. Filtration of plasma macromolecules, including LDL and HDL containing bound MOX, from the majority of smaller molecules, salts and water in plasma as a quantitative approach for MOX pharmacokinetic studies has not been reported to date. Both 3 kDa and 10 kDa filters were tested for MOX recovery using wither acetone or acetonitrile for desorbing MOX from the isolated protein. As expected, 3 kDa filters took slightly longer to filter the plasma and were more impacted by the choice of extraction solvent, whereas the choice of solvent for MOX extraction was not significant for 10 kDa filters (Fig. 5B). Due to the large size of LDL and HDL which are primarily responsible for almost all MOX binding in plasma [11], it is likely that a filter with a larger pore size could be used to hasten the filtration process further, while still retaining LDL and HDL for MOX extraction. Extraction of macromolecules resulted in clear final extracts that were free from salt and many organic molecules that could suppress ionisation and make the MS spray chamber dirty, leading to column or MS/MS blockages. While LLE after protein precipitation results in greater absolute recoveries, extraction of isolated lipoproteins also offers the advantage resolving the lipoprotein binding of MOX while also allowing quantification. The use of MOX-D3 ensured that consistent extraction recoveries.

3.3.3. *Validation of LLE and lipoprotein isolation using wombat plasma*

Spiked sheep plasma (n = 4) and wombat plasma (n = 3) were used to validate the method for solvent extraction after protein precipitation. Fewer concentrations were validated for wombat plasma due to the limited supply of clean plasma, so concentrations were selected in the approximate range of the study using wild wombats. Absolute recoveries in both animals for 0.1–10 ng/mL ranged from 91 to 93 % while MOX-D3 normalised results were 95–103 % over the same range (Fig. 6A and B). Once the MOX sheep plasma concentration exceeded 10 ng/mL, absolute recovery started to drop significantly ($P < 0.05$) as the extraction became less efficient, and fell to 48 % at 200 ng/mL. However, MOX-D3 normalised recoveries were still 96–101 %, reinforcing the effectiveness of MOX-D3 as an internal standard. A similar trend for the extreme ends of the recovery range have been reported previously [13]. Matrix suppression was investigated by extracting clean plasma, evaporating to dryness, and then resuspending. The solutions were spiked with MOX and MOX-D3 and comparing them to spikes in pure resuspending solvent (Fig. 6C). The matrix spikes were significantly lower than pure solvent spikes across the 0.05–2 ng/mL range, indicating the co-extraction of suppressing species in the hexane extracts. This conclusion was supported visually by the presence of a pale yellow pigment in the extract after pre-concentration, and may have been bilirubin due to its low water solubility. MOX-D3 normalised results were 96–104 % across this concentration range, indicating that the matrix suppression impacted MOX and MOX-D3 equally, and therefore would not impede on the ability of the method to provide consistent and accurate results.

While the dilute and shoot approach is the quickest, simplest and cheapest approach to sample analysis, it comes at the cost of sensitivity through sample dilution and possible matrix suppression, with an LoQ reported at 1 ng/mL [5]. The nine-fold dilution reported by the authors undoubtedly reduced their LoQ, but this dilution also affected the matrix and may have also offset matrix suppression in samples. SPE can overcome both of these issues but the current work was not as successful as other studies, which have reported LoQs of 0.2 ng/mL [13], 0.1 ng/mL [14,23] and 0.05 ng/mL [25]. LLE after protein precipitation for MOX analysis appears to have only been reported once [12] with a LoQ of 0.22 ng/mL while using fluorescence detection, and may in fact provide greater sensitivity with MS/MS detection instead. The LoQ from the current study using LLE after protein precipitation was 0.01 ng/mL, which is an improvement over the 0.05 ng/mL reported in one study [23] but the value will be offset by the smaller volume of plasma used in another study compared to that used in the current study [14]. Within batch variabilities were 10.7 % and 2.50 % for absolute MOX and MOX-D3 normalised results, respectively, while the between batch variabilities were 15.8 % and 3.9 % for absolute MOX and MOX-D3 normalised results, respectively.

3.4. *MOX extraction from faeces and fur*

Basic solvent extraction of faeces using methanol and acetonitrile was initially trialled for MOX recovery from faeces, but the approach was discarded quickly due to poor recoveries arising from their inability to extract or desorb MOX from particulates in the faeces. The success of acetone and hexane extraction of plasma was used to identify a more suitable extraction procedure. Sonication proved valuable to MOX extraction by approximately doubling recoveries over non-sonicated samples. Up to 30 % of applied MOX was recovered from faeces using acetone extraction and LLE into hexane while using florisil to remove the matrix (Fig. 7A). Analysis of the hexane and 5 % acetone in DCM fractions showed no MOX present, indicating their suitability for washing

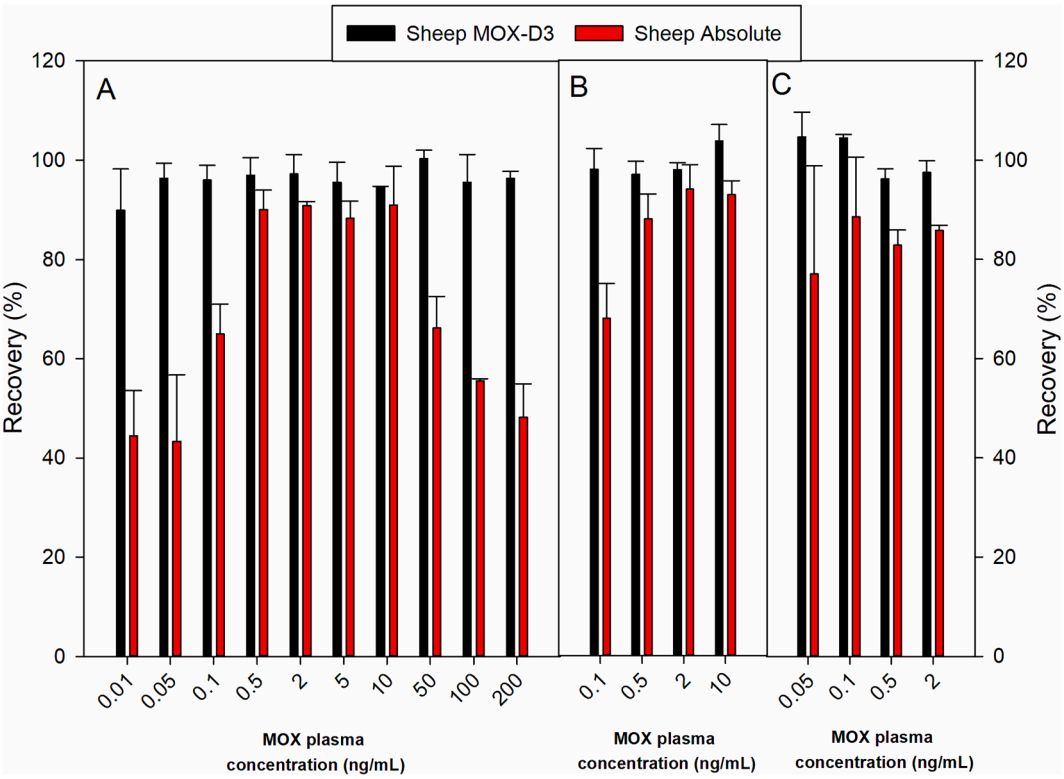

Fig. 6. MOX recoveries using protein precipitation LLE for sheep plasma (A), wombat plasma (B), and recoveries for extracted blank matrix spiked with MOX to determine MS/MS matrix suppression (C).

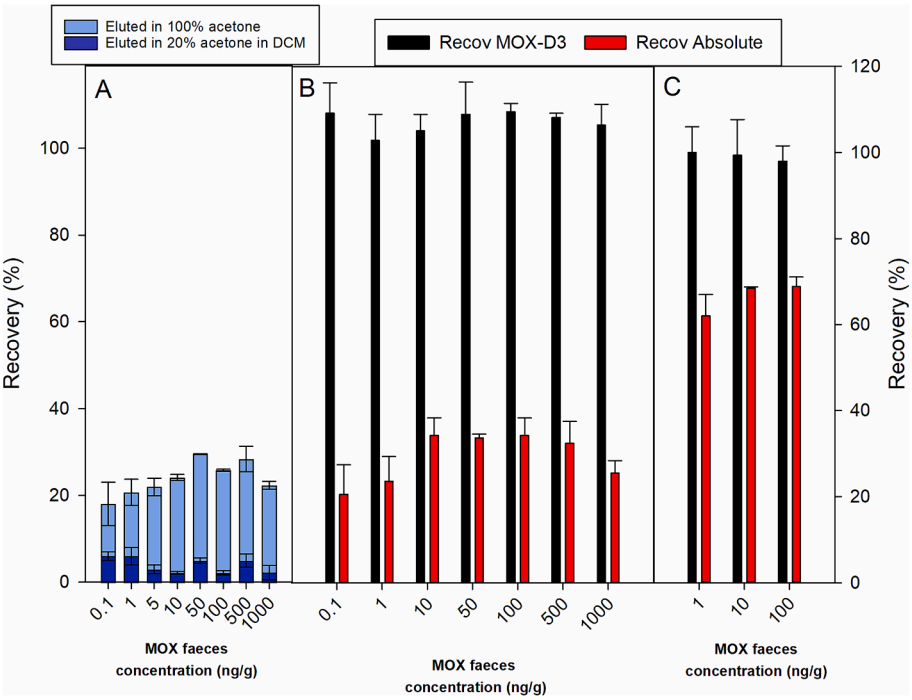

Fig. 7. Absolute MOX recoveries from faeces using 20 % acetone in DCM followed by 100 % acetone (A), elution using only 100 % acetone (B), and matrix suppression of blank extracted matrix that was spiked with MOX and MOX-D3 (C).

the column after application of the faeces extract in hexane, provided that the hexane extract volume was reduced to approximately 2 mL. If the 2 × 4 mL hexane sample extracts were applied to the column directly, MOX did not appear to be completely retained by the column and was detected in small amounts in the 5 % acetone in DCM washes, which were useful at eluting a yellow pigmented chemical, presumed to be carotene from undigested grass. Likewise, the use of activated florisil seemed essential at retaining MOX because florisil taken directly from the bottle resulted in broadening of MOX band on the cleanup column, with some elution during the washing phases. The main interfering

species in the MOX extraction was chlorophyll and variation of the acetone concentration in DCM used as the eluting solvent could not separate the two components. MOX elution using 20 % acetone in DCM occurred to only a small extent and still resulted in coelution with chlorophyll, while the subsequent 100 % acetone elution contained the majority of the MOX, but was also effective at eluting chlorophyll. As a result of these issues, it was considered best to only used 100 % acetone to elute MOX (Fig. 7B), and this approach resulted in absolute recoveries of 20–35 % MOX. As with plasma, normalisation with MOX-D3 resulted in extremely consistent MOX recoveries, which ranged from 102 to 108 % across the concentration range tested. Matrix suppression by chlorophyll was investigated by extracting clean faeces, and then spiking the resuspended extract with MOX and MOX-D3, and comparing them to pure resuspending solvent. Recoveries across the three concentrations tested were not significantly different from each other and indicated that chlorophyll and other interfering chemicals suppressed the signal by approximately 30 %, regardless of MOX concentration (Fig. 7C). This may be due directly to ionisation suppression in the spray chamber, or that increased viscosity of the extract impacted the injection process in the autosampler, relative to pure solvent, but this was not resolved in the current study. As with plasma samples, normalisation of results with MOX-D3 demonstrated the signal reduction was consistent for both MOX and MOX-D3. While it would be more ideal if MOX and chlorophyll eluted separately to improve signal strength, the positive aspect of these circumstances is that chlorophyll served as a visual cue to indicate when MOX was likely moving on the florisil column, ensuring that any potential MOX elution in the washing steps was counteracted.

The difficulty in extracting MOX from faeces may be due to extensive binding by particulates, which has been reported to be more than 90 % bound MOX in sheep [27]. Partitioning into the hydrophobic core of undigested food particulate material may restrict the ability to extract MOX, and may also be a contributing factor to MOX being excreted by animals in faeces largely unmetabolised [28]. LoQs of 5 ng/g [29] and 0.5 ng/g [10] in lamb and horse faeces, respectively, have been reported but it is unclear if these are on a wet or dry weight basis. The LoQ in the current study was 0.1 ng/g in wet faeces, which equated to an LoQ of 0.3 ng/g dry weight equivalent. Within and between batch variabilities were 4.6 % and 7.1 %, respectively for MOX-D3 normalised results. As Australia is the driest populated continent on earth, popular belief is that wombats, like many native Australian animals, have faeces with very low water contents compared to many other terrestrial animals. Faeces used in the current study had water contents ranging from 47 to 85 %, which can impact heavily on the consistency of a method. However, MOX-D3 performed extremely well as an internal standard and accounted for the anomalies arising from the inconsistency between faecal samples, such as water and chlorophyll contents.

As MOX was applied as pour-on in the study involving wild wombats, fur concentrations were always likely to be very high for the duration of the sampling period for the study, making method sensitivity a lower priority. Extraction of MOX from fur was comparatively more straight forward than plasma, and even more straightforward than for faeces, due to the lack of water in fur, the high analyte concentrations, and the low matrix interferences resulting from the extraction process. Using a sample size of 100 mg of fur to ensure a representative sample, the LoQ for the current method was 0.5 ng/g. The same LoQ was reported for MOX in cattle hair [30] using a 12 h incubation in acetonitrile using 200 mg hair, compared the 20 min sonication and 100 mg wombat fur sample in the current study. Neither method required further cleanup of the extracts, and there was no significant different between the spikes applied to extracted blank fur and spikes in pure resuspending solvent.

3.5. Naturally incurred samples

Incurred plasma samples were analysed using the optimised and validated method that used solvent extraction after protein precipitation. Selected incurred samples were also extracted using the lipoprotein isolation method. Results for a single animal from a larger study are shown in Fig. 8A. The plasma MOX concentration peaked at one week and rapidly declined over the next three weeks, and persistence may be connected to a high lipid content of plasma [31], which prevented metabolism due to extensive MOX binding. The faecal concentration peaked after day 4 at more than 12,000 ng/g and 4,500 ng/g in dry and wet faeces, respectively, indicating that dermal application of MOX results in rapid clearance from wombats, and MOX undergoes limited metabolism [28], as has been reported for other animals [10,29]. Cydectin® was applied to the skin in the current study and as wombats skin tends to be quite thick skin compared to other animals [32], absorption into the blood stream is a slow process that take more time and results in lower plasma concentrations. For example, subcutaneous injection of MOX to wombats in another study resulted in an average peak concentration of 99 ng/mL after 14 h [5]. The peak plasma concentration in the current study may have occurred earlier than 7 days, but due to sampling interval limitations associated with wild animals, a more accurate value could not be determined. MOX does not appear to have been measured in wombat faeces previously, but has been detected in horse faeces up to 75 days after oral administration, with concentrations up to approximately 2500 ng/g, but the study is not clear whether this was on a wet or dry mass basis [10]. While MOX concentrations in wombat faeces were five times greater than for horses, its leeching into soil is likely to be limited due to a high K_{OC}. MOX has a reported half-life of about 2 months [33], and combined with low animal numbers and

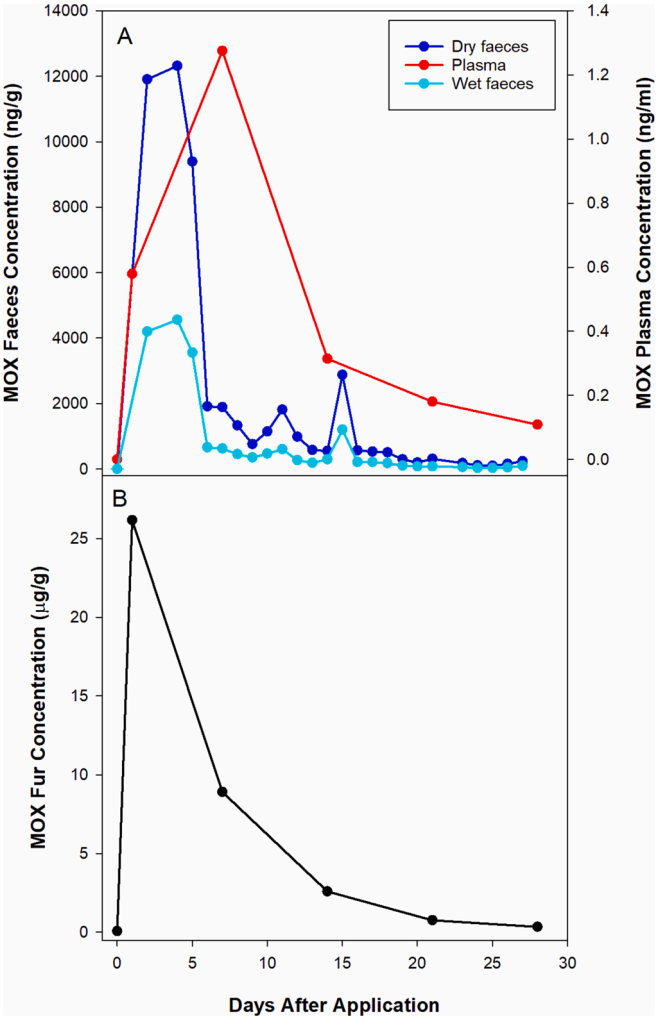

Fig. 8. MOX concentration in plasma, and wet and dry faeces for one animal (A) and fur (B).

population densities, is unlikely to pose a large risk to native dung beetle populations. Additionally, MOX is a methoxime derivative of nemadectin, which is produced by the soil bacterium (*Streptomyces*) (Awardi). As nemadectin was first discovered in Australian soils, adaptation of soil microbes to the degradation of MOX may occur more rapidly than otherwise expected. The MOX concentration in fur peaked immediately after application at approximately 26 μg/g to the (Fig. 8B) and dissipated over the next three weeks. This is likely due to MOX-laden fur falling out and being replaced with fresh fur that contained little or no MOX, resulting in a dilution of the matrix. Hair from cattle was reported to contain 0.444 μg/g MOX [30], but this was after a subcutaneous injection rather than as a pour-on, as in the current study. QC results for MOX for batches were within 5 % of the known concentrations when using MOX-D3 to normalise MOX, indicating the robustness of the faecal extraction method.

Analysis of selected samples by LLE after protein precipitation as well as by the lipoprotein isolation method were performed to validate the latter method (Fig. 9). The gradient of the line indicates a slight bias towards the lipoprotein isolation method, which is consistent with MOX-D3 normalised mean recoveries for spiked plasma of 99.2 ± 3.1 % and 102.3 ± 4.5 % for LLE and the lipoprotein isolation method, respectively. Differences between the two methods were more apparent at lower concentrations, but this variation would also be expected between replicates at low concentration when extracting plasma using the most methods. Correlation between the two methods was good, but the R^2 increased to more than 0.99 when the two outliers were removed. Absolute recovery of MOX via the lipoprotein isolation method was slightly poorer than by LLE and results in a slightly poorer LoQ, but normalisation with MOX-D3 removed the variation in the results and provided consistent and reliable concentrations. Within and between batch variabilities for the lipoprotein isolation method were 5.9 % and 7.8 %, respectively, and the LoQ was 0.05 ng/mL, compared to the LoQ of 0.01 ng/mL achieved using LLE after protein precipitation. However, it should be noted the former method only used 0.5 mL compared to 1 mL in the latter. QC plasma samples run in each batch of extractions using spiked plasma showed normalised recoveries of 6 %, indicating that both methods were suitable and reliable for the analysis of MOX.

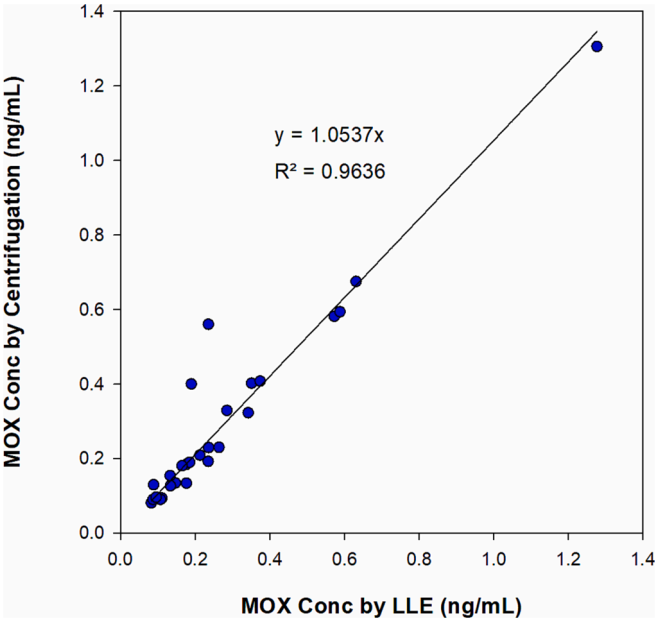

Fig. 9. Correlation of naturally incurred wombat plasma samples analysed using LLE after protein precipitation, and the lipoprotein isolation method, both using MOX-D3 normalisation.

4. Conclusions

Suitable methods of analysis are essential for confirming the fate of pesticides applied to animals. Due to its hydrophobic nature, establishing LC-MS/MS conditions for the quantitation of MOX was relatively straightforward. However, MOX hydrophobicity was a double-edged sword when it came to extracting MOX from plasma and faeces, because it is the likely reason for its recalcitrant nature. The work in this study showed that MOX was bound rapidly by plasma and faecal components, and may in fact partition into the hydrophobic core of HDL in plasma, and to the core of undigested food particulates in faeces. This extensive binding of MOX possibly explains why MOX is eliminated largely unmetabolised, and also raises questions about its activity towards sarcoptic mites in plasma if it is almost entirely bound and potentially sequestered by hydrophobic plasma components. We compared MOX-D3 and IVR as internal standards and quickly established the poor performance of IVR, and exceptional performance of MOX-D3 as internal standards. Even when the absolute MOX recovery was poor during method development for plasma and faeces, the behaviour of MOX-D3 mirrored that of MOX, resulting in internal standard-moderated results that approximated 100 % MOX recovery. While further work is needed to better understand the causes of lower MOX/MOX-D3 recoveries to allow improved methodology to be established, the current methodology was more than adequate to demonstrate that MOX applied topically to wombats is absorbed slowly into the blood stream, never reaches high concentrations as seen when using subcutaneous injection, and is eliminated from the body mostly unmetabolised in faeces. These observations suggest MOX is suitable for use on wombats provided it is effective at controlling *Sarcoptes* mites.

Author statement

Gregory Doran – Method development, validation, formal data processing and analysis, writing and editing original draft.

Hayley Stannard – Sample collection, project conceptualisation, project management, funding acquisition, manuscript reviewing and editing.

Marie Wynan – Sample collection and management, project conceptualisation, manuscript reviewing and editing.

Ray Wynan – Sample collection and management, manuscript reviewing and editing.

Amanda Cox – Sample collection and management, project conceptualisation, manuscript reviewing and editing.

Howard Ralphe – Sample collection and management.

Animal ethics

The project was approved by the Charles Sturt University ACEC, approval number: A21410.

CRediT authorship contribution statement

Gregory S. Doran: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Marie Wynan:** Conceptualization, Resources, Writing – review & editing, Investigation. **Ray Wynan:** Resources, Writing – review & editing, Investigation. **Amanda Cox:** Conceptualization, Resources, Writing – review & editing. **Howard Ralph:** Resources. **Hayley J. Stannard:** Conceptualization, Funding acquisition, Resources, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Hayley Stannard reports financial support was provided by Wombat Protection Society of Australia. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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