Title

Evidence of non-extractable florfenicol residues: The development and validation of a confirmatory method for total florfenicol content in kidney by UPLC-MS/MS

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Abstract

The parent compound florfenicol (FF) is a broad spectrum antibacterial compound licensed in the UK for use in cattle, pigs and the aquaculture industry. The analysis of porcine tissues in this study demonstrates that significant amounts of solvent non-extractable FF related residues are present in incurred tissues (kidney and muscle) from treated animals. The results indicate that methods based on solvent extraction alone carry a high risk of reporting false negative results. The use of a strong acid hydrolysis step prior to solvent extraction of tissue samples is necessary for an accurate estimate of the total tissue florfenicol content. A robust and sensitive method for the determination of total florfenicol residue content in kidney samples by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has been developed and validated. This method covers the synthetic amphenicol drug florfenicol (FF) and its metabolites, measured as the marker residue florfenicol amine (FFA) as per Commission Regulation (EU) 37/2010. Non-extractable and intermediate metabolites are converted to the hydrolysis product FFA, and then partitioned into ethyl acetate. Extracts are solvent exchanged prior to a dispersive solid-phase extraction step, then analysed using an alkaline reverse phase gradient separation by UPLC-MS/MS. The method was validated around the maximum residue levels (MRLs) set out in commission regulation (EU) 37/2010 for bovine kidney in accordance with commission decision 2002/657/EC. The following method performance characteristics were assessed during a single laboratory validation study: selectivity, specificity, sensitivity, linearity, matrix effects, accuracy and precision (decision limit (CCα) and detection capability (CCβ) has been determined).

Keywords: florfenicol amine, incurred tissue, non-extractable, kidney, muscle, hydrolysis, liquid chromatography, mass spectrometry, UPLC-MS/MS.

Introduction

Florfenicol (FF) is a member of the amphenicol antibiotic drug family which includes both chloramphenicol (CAP) and thiamphenicol (TAP). It is a fluorinated analogue of CAP that is less likely to cause the blood dyscrasias occasionally found in humans following a dose-unrelated exposure to that drug. Both FF and TAP lack the p-nitrobenzene component of chloramphenicol, thought to be responsible for the idiosyncratic reaction that is seen in humans and which led to a prohibition on its use in food-producing animals.(Agency 1996)

As an important veterinary medicine, FF exhibits a broad antibacterial spectrum of activity, higher than TAP and with a higher bioavailability by comparison with the tetracycline and quinolone antibiotics in several species.(Park, Lim et al. 2006) It is effective against both gram positive and negative bacteria, exerting its action through the inhibition of protein synthesis by deactivation of bacterial ribosome sub-units. It is marketed under several different tradenames (including Nuflor, Resflor and Florocol) for the treatment of a number of bacterial infections in aquaculture, livestock and poultry production, including bovine respiratory disease (BRD) associated with *Pasteurella* or *Haemophilus* bacterial strains, for treatment of foot rot associated with *Fusobacterium* or *Bacteroides* strains and swine respiratory disease (SRD) associated with *Actinobacillus, Pasteurella* and *Bordatella infections.* In treated animals, FF is rapidly metabolised to a number of intermediate metabolites (mainly florfenicol alcohol, monochloroflorfenicol and florfenicol oxamic acid) which are then rapidly converted to florfenicol amine (FFA). Most of these are excreted via the urine, indicating that the kidneys are the major route of clearance.(Jianzhong Liu, Ki-Fai Fung et al. 2003)

FF has MRLs (in all food-producing species) listed in Table I of Commission Regulation (EU) No 37/2010.(Commission. 2010) The MRLs in bovine and porcine kidney are 300µg/kg and 500µg/kg, respectively, with the marker residue defined as ‘the sum of florfenicol and its metabolites measured as florfenicol amine’.(Agency 1996) It is not permitted for use in animals from which milk or eggs are produced for human consumption. In the UK, it is licensed for use in cattle, pigs and salmon, either by injection or via feeding-stuffs.

Several methods have been published for the analysis of FF alone or in combination with FFA. These include several multi-amphenicol methods(Luo, Chen et al. 2010, Alechaga, Moyano et al. 2012, Rezende, Filho et al. 2012, Zou, Zhao et al. 2013, Tao, Zhu et al. 2014, Pan, Wu et al. 2015) and several multi-class antibiotic methods.(Fedorova, Nebesky et al. 2014, Rezk, Riad et al. 2015, Schneider, Lehotay et al. 2015) However, these methods rely on the extraction of tissue samples with an organic solvent, followed by a solid-phase clean-up step, either in a column format or as a dispersive step. The assumption has therefore been made that FF related residues are extractable and mainly present as either FF or FFA. However, metabolism studies using radio-labelled FF have shown that non-extractable residues are predominant in tissues from numerous species (Schering-Plough 1996). To account for all FF related residues in tissue it is necessary to include a hydrolysis step prior to sample extraction to convert the parent drug and its metabolites to the single marker residue FFA.(Wrzesinski, Crouch et al. 2003)

Consequently, there are few screening or confirmatory methods in the literature that fulfil the MRL definition, and a lack of data on the effect of hydrolysis on incurred residues measured in tissue samples. Methods published by some authors include a strong acid hydrolysis step prior to sample extraction to account for all FF related tissue content. The first of these was in channel catfish using HPLC-UV(Wrzesinski, Crouch et al. 2003) and the second a confirmatory method in porcine muscle by HILIC LC-MS/MS.(Kong, Deng et al. 2014) Although the latter method met the requirements of commission decision 657/2002(Commission 2002) and provided data relating to the total FF content in incurred porcine muscle samples, there remains a lack of data relating to the extractable versus non-extractable FF related content in incurred tissues.

To the best of the author's knowledge, this is the first UPLC-MS/MS method presented for the analysis of total FF residue content in tissue samples. The data presented here also demonstrate that between 79 to 96% of FF residues in the incurred tissues studied were non-solvent extractable, which is of particular relevance for regulatory laboratories world-wide. The results unequivocally show that the use of a solvent extraction method alone cannot be used, and that a whole tissue hydrolysis procedure must be used to meet the residue marker definitions set in EU legislation. It is likely that there is significant under-

reporting of tissue samples which exceed the statutory florfenicol MRL (or equivalent) within Europe (and world-wide) as a result of the widespread use of methods lacking a whole tissue hydrolysis step.

Experimental

Reagents and Materials

Analytical grade reagents and HPLC grade solvents were used throughout. Methanol was obtained from Romil (Cambridge UK). Acetonitrile, sodium hydroxide (NaOH) pellets, ammonium hydroxide solution (35%), concentrated hydrochloric acid (HCl, 12.1N), di-potassium hydrogen orthophosphate (K_2HPO_4), Eppendorf tubes (1.5ml) and disposable borosilicate glass turbovap tubes (16 x 100mm) from Fischer scientific (Leicestershire, UK). Ethyl acetate was obtained from Sigma Aldrich (Dorset, UK). Grade 1 (18MΩ.cm) water was used throughout, obtained from an in house Milli-Q system (Millipore corp., Livingston, UK). Endcapped C18 sorbent material (Sepra $C_{18\text{-}E}$, 50µm) from Phenomenex (USA). Screw capped glass centrifuge tubes (50 ml) with Teflon lined caps were used for hydrolysis. Analytical standard powders for Florfenicol (purity >99%) and Florfenicol amine (purity >99.6%) were obtained from Sigma-Aldrich and Witega (Berlin, Germany) respectively.

Individual stock solutions of Florfenicol (FF) or Florfenicol amine (FFA) were prepared at 1mg/ml in methanol. Working standards were prepared for FF at 14.45µg/ml (equivalent to 10µg/ml of FFA, for use during spiked experiments), and FFA at 10 µg/ml by dilution of stock aliquots with methanol. Standards were stored in 25ml amber glass vials at -20ºC for up to 6 months. The working standard for FF was used to prepare method matched calibration curves (spiked pre-hydrolysis) and for fortification of recovery samples. A single level solvent standard (post hydrolysis and post extraction) was also prepared for FF and FFA from the working standards for identification of FF (to ensure complete conversion to FFA).

Preparation of additional reagent solutions

(a) 6N HCl – Add 50ml of concentrated HCl to 50ml of water and mix carefully. Prepare daily as required.

(b) 1M K_2HPO_4 – 17.42grams of di-potassium hydrogen orthophosphate was added to 100ml of water and mixed until dissolved. Prepared fresh daily.

(c) NaOH, 50% (w/w) – Add 50ml of water to a beaker immersed in an ice bath, then add 50 grams of NaOH pellets carefully, stirring until dissolved with a glass rod. Transfer to a 100ml volumetric cylinder and adjust to 100ml with water.

(d) 0.5% ammonia in methanol – Add 250µl of 35% ammonia solution to 50ml of methanol in a measuring cylinder and mix. Prepare fresh daily.

(e) HPLC mobile phase A – 0.05% v/v ammonia in water (pH >10.6) – Add 125µl of 35% ammonia solution to 250ml of UPLC grade water. Check final pH and adjust to above pH10.6 by adding additional ammonia solution in 25µl aliquots. Prepare fresh daily.

(f) HPLC mobile phase B – 100% acetonitrile

(g) Weak needle wash 20% methanol – add 50ml of methanol to 200ml of water and mix.

(h) Strong needle wash 90% methanol – mix 225ml of methanol with 25ml of water.

Instrumentation and conditions

UPLC separations were carried out using a Waters UPLC sample manager and binary solvent system. A reverse phase gradient separation was achieved on a Waters Acquity BEH C18 chromatographic column (50 x 2.1mm, 1.7µm) with an in line filter assembly (0.5µm porosity) (Waters, Milford, USA). The sample compartment was set at 10ºC, column temperature at 50ºC, and an injection volume of 5µl used. The mobile phases were (A) 0.5% ammonia in water and (B) acetonitrile, with a flow rate of 0.4ml/min. The flow was diverted to waste during sample injection cycles at 0.1-0.9 min and again from 1.85min until the end of an injection cycle. Linear gradient steps were used with initial conditions set at 100% A, decreasing to 50% A after 2min, 10%A at 2.5min, held for 0.7min, then returned to 100% A at 4min. A re-equilibration period of 2min was used. Total analysis time was 6min per sample.

A Waters Premier XE triple-quadrupole was used for mass spectrometric analysis, which was connected to the UPLC system via an electrospray ionisation (ESI) interface

source. FF was analysed in negative ESI, with FFA analysed in positive ESI mode. Fast polarity switching was not required as sufficient chromatographic separation was achieved. The following instrument conditions were used: source temperature 150ºC, desolvation gas 450ºC, cone gas flow 80 l/hr, desolvation gas flow 1000 l/hr, capillary voltages were +3kV in positive mode and -2.2kV in negative mode. Nitrogen was used for source, cone and desolvation gas, with argon as the collision gas. For both modes analyser settings were optimised for maximum transmission and set at unit mass resolution. Detection was performed in multiple reaction monitoring mode (MRM). Precursor and product ion transition experiments were optimised by infusion of individual standard solutions at 10µl/min using the integrated syringe pump into mobile phase via a zero dead volume T-piece. The MRM experiment is summarised in **Table 1**. The most intense MRM was used as the quantitative ion, with the remaining MRMs being used for ion ratio confirmation.

Samples

All control tissue samples were confirmed to be negative for FF related residues prior to use in validation studies. The method validation study was carried out using bovine kidney. In addition, incurred porcine kidney and muscle samples were obtained from pigs that had previously been fed florfenicol medicated feed as part of a withdrawal study. Samples were available from pigs at withdrawal periods of 0, 3, 6, 10, 12 and 14 days. These were analysed for extractable and non-extractable residues, and with the whole tissue hydrolysis procedure. All samples were minced using a laboratory blender and then frozen until needed at -20ºC. On the day of analysis samples were defrosted at room temperature and mixed thoroughly prior to weighing of aliquots.

Sample hydrolysis and extraction

For the whole tissue hydrolysis procedure, 2 grams of tissue sample was weighed into a 50ml screw cap hydrolysis tube. Selected blank samples for recovery evaluation or matrix standard calibrants were fortified at this stage with the FF working standard and allowed equilibrate for 10 minutes before proceeding.

Hydrochloric acid (6N, 4ml) was added to each tube, which was then vortexed for 30 seconds. All tubes were capped and incubated in a water bath for 2 hours at 100ºC. Tubes were vortexed intermittently to ensure complete digestion of the tissue sample, removed and allowed to cool for 30 minutes.

The pH of the hydrolysate in each sample tube was then adjusted to above pH 10 by adding 5ml of 1M di-potassium hydrogen orthophosphate solution followed by increasing amounts of 50% sodium hydroxide (2ml added initially to each tube, and then adjusted drop wise using a Pasteur pipette). To each tube, 5 ml ethyl acetate was then added. The tubes were capped and shaken for 30 seconds prior to centrifuging at 2000 rpm for 10 min. The ethyl acetate extract was transferred to a disposable clean 16 ml glass tube. The ethyl acetate extractions were repeated on a further two occasions and then combined for individual samples. All tubes were evaporated to dryness at 50 ± 2 ºC under nitrogen. To each tube, 1ml of 0.5% ammonia in methanol was added, vortex mixed for 1 minute and sonicated for 1 minute. To each tube 1ml of water was then added and vortex mixed for 1 minute.

Dispersive solid phase extraction (dSPE): Aliquots of 1ml were transferred to Eppendorf tubes containing 150mg of endcapped C18 sorbent, vortex mixed for 1 min and then ultra-centrifuged at 13000rpm for 5minutes at room temperature. Aliquots were then transferred to vials for LC-MS/MS analysis.

Method validation

The validation of the whole tissue hydrolysis method was carried out according to Commission Decision 2002/657/EC in bovine kidney tissue on each of three separate days. On each day, thirty aliquots of negative bovine kidney were weighed into separate 50ml screw cap hydrolysis tubes. Recovery samples were then spiked at 0.5, 1 or 1.5 times the MRL with seven replicates at each level as follows: 30µl, 60µl or 90µl of the 14.45µg/ml FF secondary standard (equivalent to 150, 300 or 450 µg kg^{-1} FFA equivalents) was added to 2g tissue. Three negative samples and six matrix calibrants were also prepared on each day. Matrix standards were prepared to cover a linear range from 0-1000 µg kg^{-1} (FFA equivalents) and

fortified prior to hydrolysis. All tubes were then subjected to the whole tissue hydrolysis procedure.

Incurred sample studies

For the incurred residue hydrolysis studies, matrix control samples were prepared as follows: Two negative controls, four recovery samples fortified at 0.5 x MRL and six matrix calibrant tubes in the range 0-1000 µg kg^{-1} were prepared for each analysis (as FFA equivalents). To investigate *solvent non-extractable* versus *solvent extractable* FF related residues in incurred samples the following experiment was designed. Incurred kidney or muscle samples and controls were weighed singly into screw cap hydrolysis tubes. To each tube 2 ml of water and 12 ml of acetonitrile was added. The samples were homogenised for 1 min, then centrifuged at 3500 rpm for 10 minutes at 4ºC. The extracts were decanted into separate turbovap tubes and the tissue pellets retained in the hydrolysis tube.

The extracts were evaporated to dryness under nitrogen at 50˚C and 2ml of 6N hydrochloric acid was then added, vortexed gently and sonicated for 5 minutes. The reconstituted extract was transferred to a 50ml hydrolysis tube. A further 2ml of 6N hydrochloric acid was added to each turbovap tube, vortexed and combined with the previous fraction. To the separate tissue pellets 4mls of 6N hydrochloric acid was added and each tube vortexed for 30 seconds. At this stage control sample tubes were spiked for recovery assessment and matrix standard calibrant preparation (tissue pellets and extract tubes). All tubes were capped and subjected to the sample hydrolysis/extraction method previously described, prior to analysis by LC-MS/MS.

In addition to the hydrolysis experiments described, the incurred kidney samples were analysed by the following summarised procedure to assess the levels of ‘free’ FF or FFA present in extracts when using a typical solvent extraction procedure alone. Tissue samples and control samples (2 grams) were weighed into 50ml centrifuge tubes. Samples for recovery estimation were fortified with both FF and FFA at 100 µg kg^{-1}. All samples were extracted by homogenisation with 2 ml of water and 12 ml of acetonitrile. The whole extract was then subjected to a dispersive clean-up with 0.5grams of C18 material. Extracts were transferred to turbovap tubes and calibration standards fortified at this stage to cover a linear

range from 0-100 µg kg^{-1} (both FF and FFA). Samples were evaporated and reconstituted to a final volume of 2ml with 50% methanol, then analysed by the LC-MS/MS procedure described.

Results and discussion

Optimisation of UPLC conditions

The chromatographic separation was optimised by assessing various mobile phase conditions on a Waters BEH C18 particle UPLC column. This hybrid particle has a stable pH range of 1-12 allowing different pH conditions to be assessed. FFA is a hydrophilic compound with a polar amino group which easily ionises at lower pH values. It was found that increasing the pH of the mobile phase improved retention and peak shape for FFA. This was expected as the amino group would not be fully ionised at higher pH values, resulting in a reduced hydrophilic nature. **Figure 1** demonstrates the separation of FFA and FF using the final mobile phase and gradient conditions developed. In the separation used, FFA eluted at four times the column void volume (t0) which exceeds the minimum acceptance criteria required by Commission Decision 2002/657/EC(Commission 2002) of twice the column void volume. A retention factor (k) of 2.7 for FFA was also calculated which is within the desirable range of 1-5 (k= retention time FFA-T0/T0). Excellent peak shape and retention time stability was achieved for both compounds during analyses (FF was monitored to ensure complete conversion to FFA during the hydrolysis step). No interfering peaks were observed close to the retention time of either analyte. The UPLC column was subjected to >3000 routine sample injections with no loss in chromatographic performance observed.

Optimisation of hydrolysis and extraction procedure

Initial optimisation of ethyl acetate extractions was carried out at pH10 and 12. FFA standards were prepared (in triplicate) in a 1M di-potassium hydrogen orthophosphate solution. FFA has a pKa value of 7.5, therefore for an efficient extraction of polar FFA drug residues from aqueous extracts into an immiscible organic solvent, a pH adjustment to >9.5 is required to ensure >95% of the drug is in its neutral state and amenable to extraction. A triplicate extraction with 5ml of ethyl acetate at either pH was sufficient to recover more than

80% of the added FFA content. A further 15% was recoverable through an additional extraction step when compared with standards prepared post extraction (all experiments were carried out in triplicate, data not shown). Adjustment to at least pH 10 was therefore used in the final hydrolysis method.

Several parameters were investigated for the hydrolysis procedure, including temperature, time, acid strength and the stability of FFA residues in strong acid. Initial trials used 1N or 6N HCl for the hydrolysis of FF solvent standards for 4 hours at 100ºC. The results demonstrated incomplete conversion of FF to FFA when using 1N HCl. The use of a strong acid hydrolysis (6N HCl) step was necessary to convert all FF related residues to the marker FFA, as described by other authors.(Wrzesinski, Crouch et al. 2003, Kong, Deng et al. 2014) In a further trial to optimise hydrolysis time and temperature, an incurred avian muscle sample was used. A FF solvent standard, tissue blank (for matrix standard), FF tissue recovery at 100 µg kg^{-1} and the incurred sample (mean measured content 95 µg kg^{-1}) were prepared at each of the following timepoints: 2, 4, 6 and 8 hours. These were subjected to hydrolysis at temperatures of 70ºC and 100ºC. Matrix standards were prepared by spiking both FF and FFA into the ethyl acetate extracts (post-extraction) prior to the drying and reconstitution step. The complete conversion of FF to FFA was achieved within 2 hours at 100ºC for the solvent standard, incurred tissue and recovery samples (data not shown). No remaining FF was evident beyond the initial sampling time of 2 hours, and there was no significant increase beyond this time-point in FFA concentrations for the incurred sample. In contrast the same experiment at 70ºC failed to achieve complete conversion in the same samples, with FF still measurable after 8 hours in the recovery and incurred tissue samples. It was also observed that some tissue material remained in the 70ºC hydrolysis tubes for several time points indicating incomplete tissue digestion at the lower temperature.

Optimisation of sample clean-up

The use of a dispersive solid phase extraction step (dSPE) was evaluated for sample clean-up to reduce sample type related matrix effects. These can have a significant impact on the robustness of a method due to enhancement or suppression of analyte ionisation in the electrospray ion source. Typically sorbents such as C18 or primary secondary amine (PSA),

alone or in combination are used for dSPE, depending on the matrix. PSA sorbent is effective in removing polar acidic compounds such as free fatty acids(Kong, Deng et al. 2014), however it is less effective at removing other non-polar materials.(Rezende, Filho et al. 2012) It is not uncommon for a sorbent used in this way to also remove a proportion of the target analyte(s) during any dSPE step. This is usually accepted as a trade off, providing the losses are concentration independent. In this study the use of endcapped and non-endcapped C18 material was evaluated by preparing mixed FF and FFA solvent standards at 500ng/ml in 50 to 70% methanol (with 0.5% ammonia). The results in **Table 2** show that the use of endcapped C18 material is necessary to prevent significant losses of FFA during the dSPE step. Most losses were attributed to residual acidic silanol groups on the non-endcapped C18 material interacting with FFA, as little difference was observed for FF. The inclusion of ammonia at 0.5% in the methanol component helped to reduce losses by blocking unwanted secondary ionic interactions, and as expected was more effective with the endcapped material. The percent of methanol used for the dSPE also has a significant impact if using a non-endcapped material. In the method presented the final extracts are prepared in 50% methanol with ammonia, as increasing the methanol component did not significantly improve recovery of FFA when using the endcapped sorbent. A lower methanol content may also reduce potential non-polar interferences.

Stability studies

FFA proved to be very stable in 6N HCl at 100ºC, the results in **Figure 2** demonstrate the stability of solvent standards (prepared by spiking with FFA prior to hydrolysis) for up to 8 hours. The stability of FFA in extracts was investigated by preparing matrix standards (in multiple tissue types) and analysing on the same day against a freshly prepared FFA solvent standard. The extracts were stored for one week at 4ºC, then reanalysed against a freshly prepared solvent standard. The results of the normalised data (against the solvent standard) demonstrated that FFA extracts were stable for at least one week in tissue extracts, regardless of the matrices evaluated. Other authors have already demonstrated the stability of FFA as solvent standards in a freezer for up to six months(Kong, Deng et al. 2014), or in

incurred tissue samples subjected to repeated freeze/thaw cycles.(Wrzesinski, Crouch et al. 2003)

Matrix effects

Kidney and muscle extracts for bovine and porcine tissues were prepared by the whole tissue hydrolysis procedure described (with and without dSPE). Matrix effects were then assessed by two different approaches against the developed UPLC method. Firstly, the prepared tissue extracts were injected and analysed (as MRM experiments) with post-column infusion of a mixed FF/FFA standard. This was carried out at several different levels (10ng/ml, 100ng/ml, 250ng/ml and 1000ng/ml in 50% methanol) and compared with a solvent blank injection. Secondly, matrix and solvent standards were prepared at 100ng/ml for FF/FFA from these extracts by reconstitution of the relevant matrix extract with known standard amounts or with methanol. These were injected without post-column infusion.

The results for the infusion experiments did not indicate a difference between matrices at any of the standard concentrations used. No significant baseline difference was observed around the retention time of FFA or FF. However, it was useful in providing evidence that the dSPE step did remove significant non-polar sample interferences (usually attributed to phospholipids or lipoproteins) as the non-dSPE traces indicated significant baseline depression at a much later retention time as shown in **Figure 3**. Such co-extractants have the potential to cause method robustness issues such as 'ghost peaks' during repeated injection cycles and can impact on chromatographic peak quality and column lifetime (due to a build up of sample material on the head of the column).

In contrast, the second approach which compared matrix and solvent standards (shown in **Table 3**), demonstrated that the inclusion of a dSPE step significantly reduced matrix related ion suppression effects for both FFA and FF, which could not be observed using the post-column infusion experiments. The matrix effect (ME) was calculated and expressed as a %ME as follows:

$$\%ME = \frac{(\text{matrix standard peak area} - \text{solvent standard peak area})}{\text{solvent std peak area}} \times 100$$

The results indicate that the matrix effect was still evident after clean-up, and that the use of matrix matched standards is required for analyses. The inclusion of PSA in combination with C18 or alone was not investigated as the performance characteristics of the method were found to be within acceptable limits (refer to validation results). In the whole tissue hydrolysis method described, recovery and matrix standard samples are fortified prior to the initial hydrolysis step. Hydrolysis efficiency, extraction losses and matrix effects are therefore accounted for in the procedure.

Incurred tissue studies and solvent non-extractable residues

The results for the solvent non-extractable (SNE) study in porcine kidney and muscle are presented in **Tables 4** and **5** (assessing hydrolysed tissue pellets and hydrolysed extracts separately). The results demonstrate that at zero days withdrawal, >80% of the measurable FF content in kidney and >65% in muscle tissues is 'solvent non-extractable', with a tendency for the proportion of SNE residues to increase with withdrawal time. This is in agreement with the data submitted for the original licence approval of Nuflor which also indicated significant non-extractable residues in tissue radioisotope studies.(Schering-Plough 1996)

The combined values for extracted and non-extracted FFA content demonstrate that the MRL of 500 µg kg^{-1} in porcine kidney was exceeded for up to 10 days after withdrawal. Pig muscle samples did not exceed the MRL of 300 µg kg^{-1} at any point. The results indicate that kidney tissues should be sampled for any residue surveillance programme to ensure compliance with EU Regulation 37/2010(Commission. 2010) and to ensure that appropriate withdrawal periods are being followed. The results of pharmacokinetic studies of FF have also indicated a major kidney clearance in cattle and pigs.(Jianzhong Liu, Ki-Fai Fung et al. 2003)

Of particular significance, the results indicate that methods of analysis, which rely on a solvent extraction step alone, will have a 100% false negative rate, even at a zero day withdrawal period. This is alarming considering the number of methods published intended to cover multiple amphenicol drugs in a single analysis, or indeed multi-residue analyses which include FF and occasionally FFA in the analytical suite. These procedures are validated using materials spiked with solvent standards around the MRL, which has lead analysts to

assume the method is fit for purpose due to sufficient precision and accuracy of fortification experiments. Consequently, there is a possibility of significant under-reporting of 'non-compliant' samples. The results presented here reinforce the need for the use of incurred materials during method development, in particular where non-extractable or bound residues need to be taken into account.

Further work was carried out to investigate the possibility of recommending a 'lower threshold screening value' for FF and FFA below the appropriate tissue MRL by using a solvent extraction method alone (without a hydrolysis step). In theory, a result could trigger the use of a whole tissue hydrolysis method if FF and FFA were above this 'threshold' level in tissue. This would only be possible if FF and FFA in tissue extracts was present at detectable levels as 'free' residues (not bound to low molecular weight co-extracted material), and if the proportions remained constant relative to the whole tissue content over the withdrawal period of interest (up to 10 days in the pig kidney samples). The results for the incurred porcine kidney extracts not subjected to hydrolysis are also presented in **Table 4**. The levels of 'free' FF or FFA measured were at trace levels in most samples, with no consistent ratio to the total measured hydrolysis content, demonstrating that this approach cannot be recommended.

Validation results

Selectivity, specificity, sensitivity and linearity

The selectivity of the method was verified by analysing negative tissue samples with each of the validation and incurred sample batches. Representative MRM chromatograms of blank, spiked and incurred tissue samples are shown in **Figure 4**. The absence of interfering peaks close to the retention time of either analyte in negative bovine or porcine tissue extracts demonstrated the selectivity of the analytical method. The retention time in spiked or incurred positive samples corresponded to calibration standards within the ±2.5% tolerance permitted for LC methods. **Figure 5** shows the linearity of FFA in hydrolysed extracted standards over the calibration range 50-1000 µg kg^{-1}. All calibration plots exceeded a correlation coefficient (R^2) of higher than 0.97 for each of the three validation days in bovine kidney (**Table 6**) and the incurred tissue studies (porcine kidney and muscle).

The specificity of the method was determined by comparison of the MRM ion ratios for FFA in validation spiked controls and incurred tissue samples against matrix standards. Two MRMs per analyte are required to fulfil the minimum identification criteria required by commission decision 2002/657/EC(Commission 2002), however a third MRM was included for FFA. Ion ratios were calculated as the ratio of each qualifier ion against the quantifier ion using Waters Targetlynx quantitation software. All validation controls and incurred results were within the acceptable limits as set in the legislation, and exceeded the 3 identification points required (5.5 total points were achieved as one precursor and three product ions were monitored). The ion ratio precision on different days and for different matrices was also calculated. The RSD value of the ratios on each day are lower than the permitted tolerances set by 2002/657/EC(Commission 2002) (refer to **Table 6**), indicating that the selected MRMs are sufficiently specific for the identification of FFA.

Accuracy and Precision

Validation was carried out according to decision 2002/657/EC(Commission 2002) by spiking seven replicates for each level to be assessed, at concentrations corresponding to 0.5, 1.0 and 1.5 times the MRL. The accuracy and precision of the method was evaluated by comparing the recoveries of FFA in spiked bovine kidney samples against the target spiked concentration, at different concentrations over three validation days. The data are presented in **Table 7**. Accuracy values from 92% to 108% at the MRL were obtained over three days, with a mean overall recovery (accuracy) of 100% for FFA over all concentration levels. Method precision was estimated at each validation level as follows: Firstly as intra-day repeatability, calculated as the relative standard deviation for repeatability (%RSD_r) obtained within a batch, and as within laboratory repeatability (%RDS_R), calculated as the relative standard deviation over the three validation days by a single analyst. Table 8 shows that the precision RSD_r values ranged from 6.6% to 11% and the RDS_R values from 2.9% at the 1.5xMRL level to 13.7% at the 0.5 MRL level. For substances with a permitted limit, the RDS_R values at half the MRL should be less than the predicted Horwitz equation target value of 23% for within laboratory reproducibility.(Commission 2002) The results demonstrate that method precision meets the legislative requirements for validation.

Decision limit (CCα) and Detection capability (CCβ)

The calculated CCα and CCβ values are shown in **Table 7**. The values were calculated according to decision 2002/657/EC(Commission 2002) by plotting the calculated concentration against the added concentration for each of the three days at the validated levels of 0.5, 1.0 or 1.5 times the MRL. For MRL substances, any sample with a confirmed concentration greater than the MRL with a statistical certainty of at least 95% (CCα) is non-compliant with community legislation. An α-error and β-error of 5% was applied based on the inter-day RSD data obtained at the MRL.

Conclusions

The results of the incurred tissue study presented here demonstrate that significant FF related residues remain in kidney and muscle samples from FF treated pigs that are solvent non-extractable (SNE). A confirmatory UPLC-MS/MS method has been validated in bovine kidney that can be used to quantify total FF related content in tissue samples. Tissue samples which are taken as part of a national residue control program must include a whole tissue hydrolysis procedure when analysing for FF related content. If a method for tissue residues, not incorporating a hydrolysis step is used, there is a significant potential for false negative results.

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Compound	*ESP Mode*	*Time (min)*	*Precursor*	*Product*	*Cone (V)*	*CE*
FFA	Positive	0.9 – 1.4	248	230	20	13
				130.2		21
				151		23
FF	Negative	1.4 – 1.9	356	336	25	10
				185		20

Table 1: Tandem mass spectrometry conditions

		FFA	FF
%Methanol	Endcapped C18	% Recoverable	% Recoverable
50	Yes	94	91
50	No	59	90
60	Yes	95	90
60	No	72	88
70	Yes	97	99
70	No	80	94

Table 2: Optimisation of the dSPE step. FFA and FF solvent standards prepared in increasing % methanol (containing 0.25% ammonia) at 500ng/ml. 1ml of standard subjected to dSPE with 150mg of C18 material. The data has been normalised against solvent standards not subjected to dSPE.

Matrix Standard	*% ME (without dSPE) FFA*	***% ME (with dSPE) FFA***	*% ME (without dSPE) FF*	***% ME (with dSPE) FF***
Porcine Kidney	-31.3%	-19.8%	-37.7%	-23.2%
Bovine Kidney	-39.6%	-17.8%	-36.0%	-23.2%
Porcine Muscle	-24.8%	-18.9%	-43.7%	-25.2%
Bovine Muscle	-19.1%	-8.6%	-27.4%	-17.6%

Table 3: Matrix effect (%) in different matrices with and without the inclusion of a dSPE clean-up step, for FFA and FF compared to solvent standard responses (peak area counts). Standards were prepared by reconstitution of known standard amounts with the relevant matrix extract or with 50% methanol (containing 0.25% ammonia). Negative values indicate the percentage ion suppression effect in the relevant tissue extract.

					Extract not subjected to hydrolysis procedure	
WITHDRAWAL DAY	**EXTRACTABLE (µg kg^{-1} FFA)**	**NON-EXTRACTABLE (µg kg^{-1} FFA)**	**COMBINED TOTAL (µg kg^{-1} FFA)**	**% NON-EXTRACTABLE**	**FFA (µg kg^{-1})**	**FF (µg kg^{-1})**
DAY 0	347.8	1292.1	**1639.9**	**78.8**	68.6	4
DAY 0	482.5	1971.9	**2454.4**	**80.3**	52.1	37.9
DAY 0	293.8	1215	**1508.8**	**80.5**	49.3	11.4
DAY 3	81.5	1062	**1143.5**	**92.9**	2.4	<2
DAY 3	97.2	771.7	**868.9**	**88.8**	3.9	<2
DAY 6	151	1081.8	**1232.8**	**87.8**	4.8	6.2
DAY 6	87.8	405.1	**492.9**	**82.2**	4.1	2.7
DAY 10	93.8	310.3	**404.1**	**76.8**	2	ND
DAY 10	75.7	471.3	**547**	**86.2**	<2	ND
DAY 10	50	396.7	**446.7**	**88.8**	<2	ND
DAY 10	39.4	373.7	**413.1**	**90.5**	ND	ND
DAY 12	30.6	260.8	**291.4**	**89.5**	<2	3
DAY 12	28.4	259.7	**288.1**	**90.1**	<2	<2
DAY 14	13.5	290.9	**304.4**	**95.6**	ND	<2
DAY 14	9.7	235.4	**245.1**	**96.0**	ND	ND

Table 4: Solvent extractable and solvent non-extractable study results (columns 2-5) from incurred porcine kidney samples. Samples were obtained from animals which were fed on Florfenicol medicated feed (at 10mg florfenicol per kg body weight per day for five consecutive days) and then subjected to withdrawal periods of 0 to 14 days (Note: the recommended withdrawal period for Nuflor is 14 days). The MRL listed in 37/2010 EC for porcine kidney is 500 µg kg^{-1}. In addition, tissue extracts were analysed using an alternate method (without a hydrolysis step) to determine the levels of FF or FFA present as unbound residues in extracts ('free' content; columns 6-7). Recovery of the method used was 81% for FF and 84% for FFA; recovery correction has not been applied.

WITHDRAWAL DAY	EXTRACTABLE (µg kg^{-1} FFA)	NON-EXTRACTABLE (µg kg^{-1} FFA)	COMBINED TOTAL (µg kg^{-1}) FFA	% NON-EXTRACTABLE
DAY 0	35.05	86.88	**121.93**	**71.3**
DAY 0	55.87	107.07	**162.94**	**65.7**
DAY 0	28.81	57.9	**86.71**	**66.8**
DAY 3	11.03	64.36	**75.39**	**85.4**
DAY 3	11.44	87.63	**99.07**	**88.5**
DAY 6	16.73	92.02	**108.75**	**84.6**
DAY 6	13.53	83.41	**96.94**	**86.0**
DAY 10	12.38	61.77	**74.15**	**83.3**
DAY 10	8.97	52.35	**61.32**	**85.4**
DAY 10	11.24	57.81	**69.05**	**83.7**
DAY 10	14.61	66.37	**80.98**	**82.0**
DAY 12	10.44	34.99	**45.43**	**77.0**
DAY 12	8.75	42.45	**51.2**	**82.9**
DAY 14	10.36	62.86	**73.22**	**85.9**

Table 5: Solvent extractable and solvent non-extractable study results from incurred porcine muscle samples obtained from animals which were fed Florfenicol medicated feed and then subjected to withdrawal periods of 0 to 14 days. The MRL listed in 37/2010 EC for porcine muscle is 300 µg kg^{-1}

Validation	Mean MRM Ratio 1 (n=35)	Mean MRM Ratio 2	Ratio 1 %RSD	Ratio 2 %RSD	Ratio 1 Permitted tolerance (%)	Ratio 2 Permitted tolerance (%)	R^2
DAY 1	0.26	0.12	3.33	2.52	25	30	0.999
DAY 2	0.26	0.12	4.34	3.34	25	30	0.989
DAY 3	0.25	0.12	4.89	2.68	25	30	0.973

Table 6: Ion ratio precision in Bovine kidney over 3 validation days (n=35 for each day). The coefficient of determination (R^2) on each validation day is also presented.

Table 7: Precision and accuracy results for each validation level in bovine kidney (n=7 on each of 3 days at each

Compound	*Concentration level (µg kg^{-1})*	*Accuracy (%)*	*Intra-day Precision (%RSDr)*	*Inter-day Precision (%RDSR)*	*MRL (µg kg^{-1})*	*CCα (µg kg^{-1})*	*CCβ (µg kg^{-1})*
FFA	150	103	11	13.7			
	300	102	6.6	8.7	300	364	428
	450	95	10	2.9			

level). Note: %RSDr, relative standard deviation for repeatability; %RSDR, relative standard deviation for within laboratory repeatability.

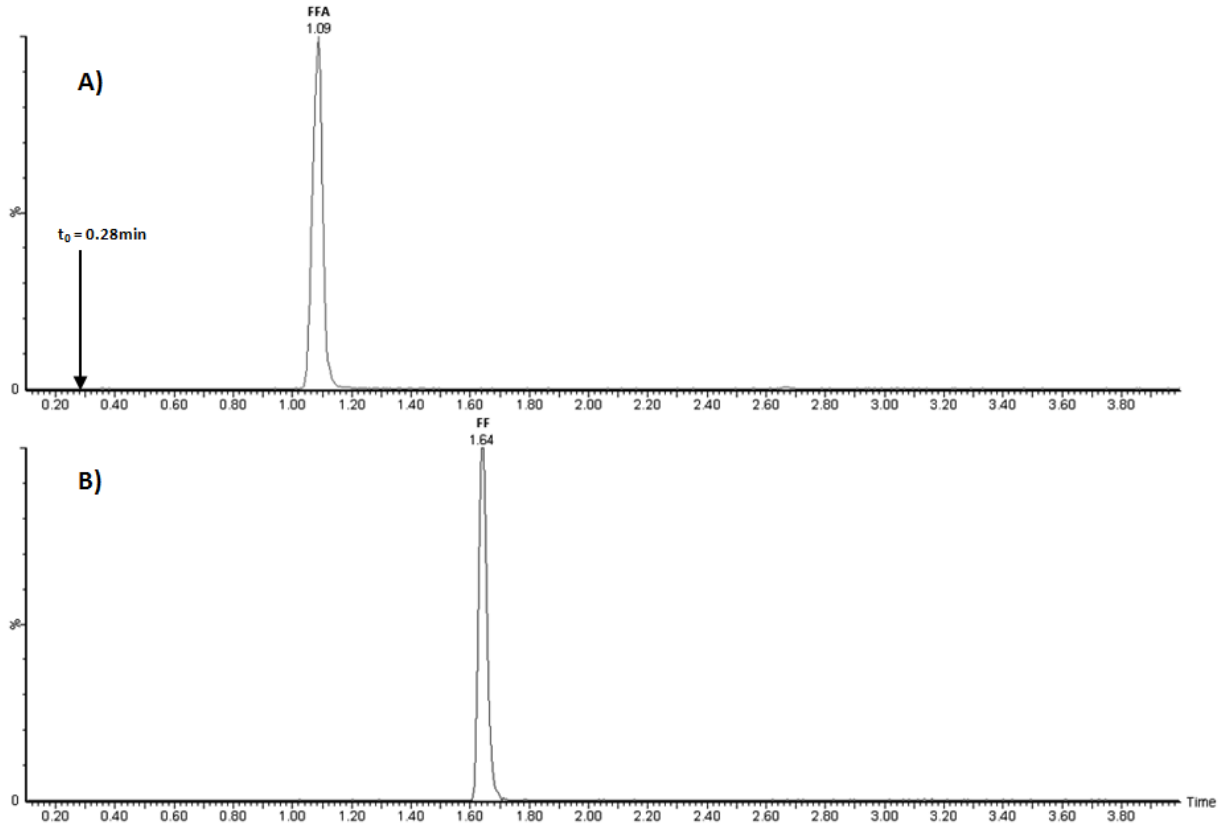


Figure 1: A mixed solvent standard injection at 100ng/ml (equivalent to 100 µg kg^{-1} in tissue) showing the separation of FF and FFA. The y-axis indicates % normalised relative intensity of total ion counts (TIC) for the MRM ion channels monitored. The column void volume is indicated at 0.28min. Trace A - TIC trace of FFA with a retention time of 1.09min; Trace B - TIC trace of FF with a retention time of 1.64min.

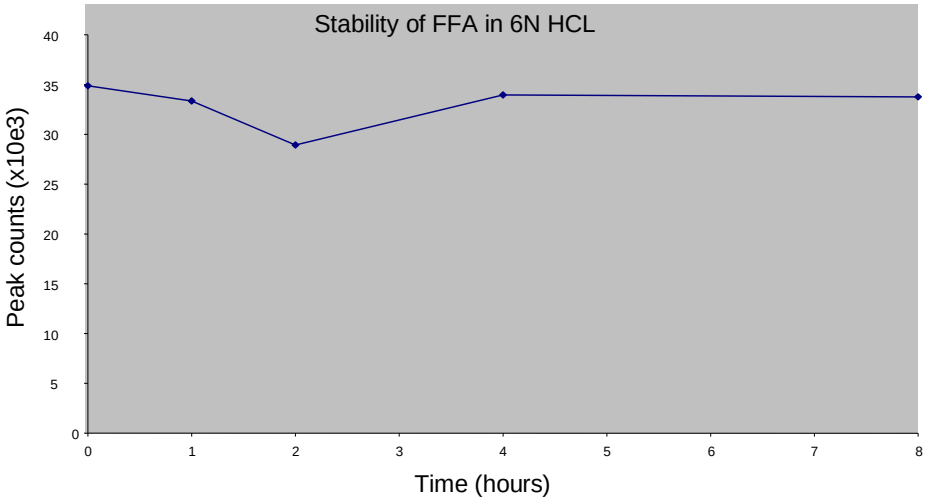


Figure 2: Stability of FFA in 6N HCl. FFA solvent standards were prepared at 100ng/ml and subjected to hydrolysis for up to eight hours. Peak area counts for the quantification MRM (248 > 230) are shown.

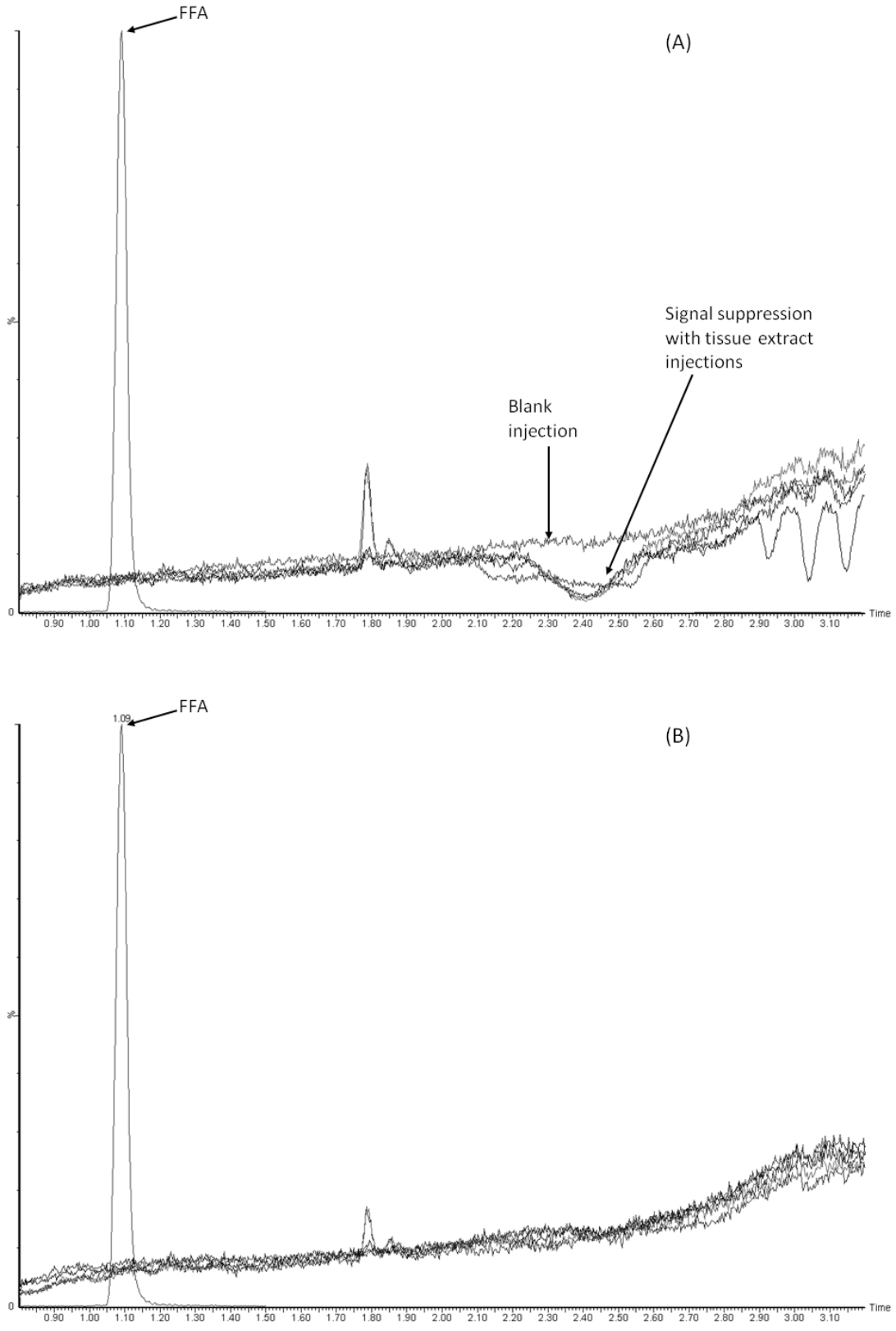


Figure 3: Post column infusion of FFA at 100ng/ml with injections on column of a solvent blank and negative extracts of porcine kidney or muscle, and bovine kidney or muscle. The y axis indicates the % relative baseline response for the quantification MRM channel 248>230 for FFA. **(A)** Without a dispersive C18 step **(B)** With dispersive C18 clean-up. An overlay of a 100ng/ml FFA standard injected on column (without post column infusion) is shown at a retention time of 1.09min.

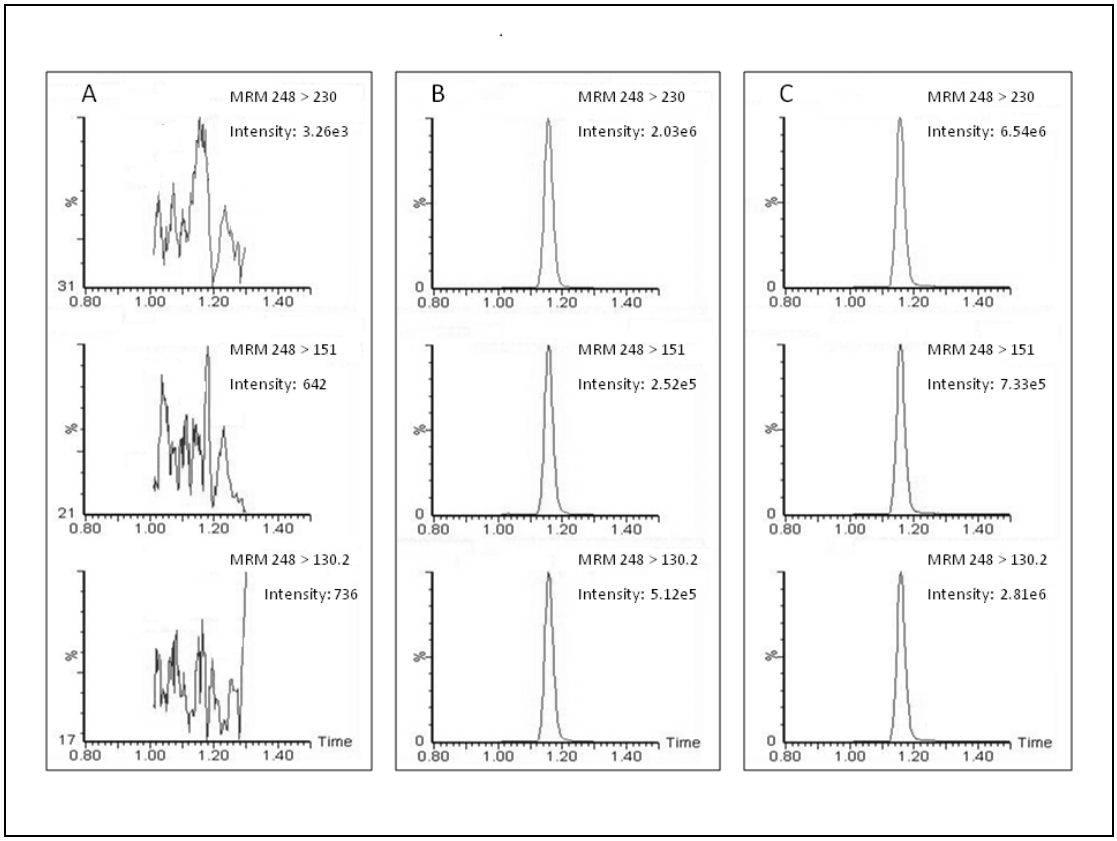
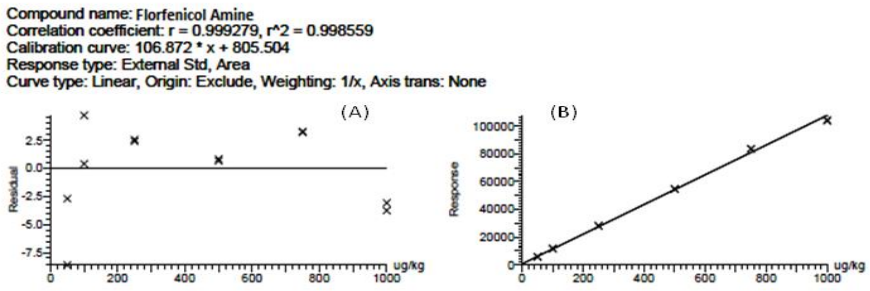


Figure 4: Representative MRM ion chromatograms for FFA in porcine kidney samples: **(A)** Negative tissue control, **(B)** Kidney sample fortified at 300 µg kg^{-1} FFA tissue equivalents and **(C)** incurred porcine kidney tissue sample at 1224 µg kg^{-1}. The y-axis has been normalised to the maximum MRM ion count intensity

Figure 5: Linearity and residual plots of florfenicol amine (FFA) in matrix standards prepared in bovine kidney. Matrix standards were spiked with florfenicol prior to the hydrolysis procedure (as FFA equivalents) to cover a concentration range of 50-1000 µg kg^{-1}



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