Title

MRM^3-based UHPLC-MS/MS method for quantitation of total florfenicol residue content in milk and withdrawal study profile of milk from treated cows.

Authors: Dermot V. Faulkner[a*], Margaret L. Cantley[a], David G. Kennedy, Christopher T. Elliott [b], Steven R. H. Crooks[a]

[a]Agri-Food and Biosciences Institute, Veterinary Sciences Division, Belfast, N. Ireland, BT4 3SD

[b]Queens University Belfast, Institute for Global Food Security, Belfast, N. Ireland, BT9 5DL

*Corresponding author. Email: dermot.faulkner@afbini.gov.uk

Abstract

Florfenicol is a broad spectrum antibacterial, licensed globally for treatment of animal and aquaculture diseases. In the EU, Canada and US it is not permitted for use in animals producing milk or eggs. There are no published methods for analysis of total florfenicol content in milk/milk products as these lack a hydrolysis step, failing to meet the marker residue definition. A method for determining total florfenicol content in milk that meets this definition is reported for the first time. Use of a UHPLC-MS/MS multiple reaction monitoring-cubed method improved the selective detection and quantitation of lower levels of florfenicol amine in milk compared to MRM only. Single laboratory validation data and withdrawal profile in bovine milk are presented. A withdrawal period of over 50 days is indicated in case of off-label use. Requirement for hydrolysis is demonstrated.

Keywords: Florfenicol amine; Milk; Hydrolysis; Withdrawal study; LC-MS/MS-MRM3; Validation

Abbreviations

AR, analytical reagent
CAP, chloramphenicol
CCα, Decision limit
CCβ, detection capability
dSPE, dispersive solid phase extraction
EMA, European Medicines Agency
FF, florfenicol
FFA, florfenicol amine
FWHH, Full Width at Half-Height
IM, intramuscular
LOQ, limit of quantitation
ME, matrix effect
MRL, maximum residue limit
MRM^3, multiple reaction monitoring (cubed)
MS/MS, tandem mass spectrometry
PT, proficiency test
RSD_r, relative standard deviation for repeatability
RSD_R, within laboratory reproducibility
TAP, thiamphenicol
TIS, turboionspray
VMPs, veterinary medicinal products
UHPLC, ultra-high performance liquid chromatography

1. Introduction

Florfenicol (FF) is an important veterinary medicine belonging to the amphenicol drug class, which includes chloramphenicol (CAP) and thiamphenicol (TAP). FF is a fluorinated analogue of TAP, both these synthetic drugs possessing a *p*-methyl sulfonyl group substitution in place of the *p*-nitro component of CAP, which resulted in the latter drug being banned for use in food production due to safety concerns over inducement of aplastic anaemia (Agency 1996; Thompson et al. 2020). Concerning the safety of consumers through exposure to FF from veterinary use, the European Medicines Agency (EMA) concluded that FF was highly unlikely to cause blood dyscrasias in consumers (such as aplastic anaemia). The EMA report (Agency 1996) highlights that the human epidemiological data could not be used to demonstrate FF safety in humans as it is not used in human medicine, and while lacking a p-nitro group, it does not unequivocally prove that it does not have the potential to cause similar effects.

FF is marketed under several different tradenames (including Nuflor, Resflor and Florocol), with a higher bioavailability than tetracycline or quinolone antibiotics in several species, via intramuscular (IM) or oral route. It has a broad antibacterial spectrum, being effective against both gram-positive and -negative bacteria. It exerts its action through the inhibition of protein synthesis by deactivating bacterial ribosome subunits (Varma et al. 1986). It is licensed globally for the treatment of bacterial infections in aquaculture, livestock or poultry production, including respiratory or foot rot infections in livestock, enteric colibacillosis in broilers and enteric septicaemia in fish.

In cattle, after IM injection, the fraction absorbed was 75% (Agency 1996). In treated animals, FF is rapidly metabolised to several 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 et al. 2003).

Recent Community legislation has strengthened the requirement for on-farm follow-up investigations following the detection of residues. In addition to a long-standing requirement to investigate all non-compliant findings of both licensed and unlicensed substances; the new legislation requires the follow up of concentrations of licensed veterinary medicinal products below the maximum residue limit (MRL) that have not been declared on the Food Chain Information certificates that accompany animals to the slaughter house (Commission 2019a). Commission Regulation (EU) No

37/2010 (Commission 2010) lists MRLs for FF in liver, kidney, muscle and fat (± skin) for all food producing species. The marker residue definition for FF is defined as 'the sum of florfenicol and its metabolites measured as florfenicol amine' (Commission 2010). It is listed as not permitted for use in animals from which milk or eggs are produced for human consumption and, as a consequence no MRL is set in these matrices. Sensitive analytical methods with low detection limits are therefore required for milk or egg analyses, as any confirmed detection is non-compliant with EU legislation.

A significant body of published work for FF analysis in food samples is based on the use of solvent extraction methods which lack a hydrolysis step. These do not meet the marker residue definition, failing to account for intermediate or bound metabolites, by analysing for the extractable fraction of FF alone, or in combination with FFA (Guidi et al. 2017; Pan et al. 2015; Fedeniuk et al. 2015; Tao et al. 2014; Rezende, Filho, and Rocha 2012; Alechaga, Moyano, and Galceran 2012; Schneider, Lehotay, and Lightfield 2015). Metabolism studies using radio-labelled FF have shown that non-extractable residues are predominant in edible tissues from numerous species (Schering-Plough 1996; Horsberg, Martinsen, and Varma 1994). Published work by these authors (Faulkner et al. 2016; Thompson et al. 2017) also demonstrated significant amounts of non-extractable residues in incurred tissues taken from FF treated pigs and the requirement for whole sample hydrolysis. These findings were subsequently supported by other authors (Imran et al. 2017; Saito-Shida et al. 2019). Physico-chemical methods for FF analysis in tissue, which lack a hydrolysis step do not meet the marker residue definition, and will lead to false negative or false compliant results due to an underestimation of the total residue content. To meet the residue definition, a hydrolysis step is required to release tissue bound residues and convert these, the parent drug and its metabolites to the single marker residue FFA (**supplementary Figure 1**). The work described here was undertaken to assess if hydrolysis is important in the analysis of milk samples, and develop a confirmatory method that fulfils the residue definition.

For milk, no published data is available on the residue profile in incurred samples and no indication of the impact of hydrolysis on the total measured FF content in incurred samples. Published methods on the analysis of FF residues in milk do not include a hydrolysis step and only account for the parent compound alone or in combination with FFA (Ruiz B et al. 2010; Rezende, Filho, and Rocha 2012; Power et al. 2014; Liu, Lin, and Fuh 2016). As no hydrolysis step is used, these published methods do not fulfil the marker residue definition. Consequently there is no reliable withdrawal study

data available for milk. Although FF medications are not licensed for use in milk production, veterinary practitioners may prescribe extra-label use of veterinary medicinal products (VMPs), according to the provisions of Commission Regulation 2019/6, Article 113 (Commission 2019b). Veterinary practitioners who prescribe off label use of FF to animals under their care are required to give guidance on an appropriate withdrawal time to limit residues entering the milk supply chain. This requires appropriate withdrawal studies, with data generated using appropriate methods of analysis.

This work was required as current physico-chemical procedures published for analysis of FF in milk do not fulfil the residue definition and there is a lack of published data in milk from treated cows which accounts for the contribution of all intermediate metabolites to total FF content in milk. To the best of the author's knowledge, there are no confirmatory methods in the literature that fulfil the marker residue definition in milk, and no data on the effect that hydrolysis (or lack of) has on the FF-related content in milk samples obtained from treated cows.

A validated method for the analysis of FF and its metabolites in milk (through conversion to FFA, after hydrolysis) which meets the marker residue definition listed in Commission Regulation (EU) No 37/2010 (Commission 2010), is presented for the first time. This quantitative method, based on UHPLC-MS-MS analysis with MRM^3 acquisition, permits the sensitive and specific confirmation of total FF residue content in milk. The MRM^3 workflow improved the selective detection and quantitation of lower levels of FFA in milk compared to MRM only. Results of the single laboratory validation study are presented. Data are also presented for the first time on the withdrawal profile in milk from three treated British Friesian dairy cattle and the contribution of intermediate metabolites to total FF content. It is demonstrated through analysis of incurred milk samples that physico-chemical methods for FF analysis in milk should include a hydrolysis procedure to avoid the risk of obtaining false negative results.

2. Material and Methods

2.1 Chemicals and materials

All reagents were AR grade or better. Methanol (HPLC grade) was purchased from Romil (Cambridge UK) and Ethyl acetate from Sigma Aldrich (Dorset, UK). Acetonitrile, sodium hydroxide, ammonium hydroxide, concentrated hydrochloric acid, di-potassium hydrogen orthophosphate were purchased from Fischer Scientific (Leicestershire, UK). Water (LC-MS grade) water was used throughout, obtained from an in house Milli-Q system (Millipore Corp., Livingston, UK). End-capped C18 material was

obtained from Phenomenex (USA). Eppendorf tubes (1.5mL) and disposable borosilicate glass turbovap tubes (16 x 100mm) were obtained from Fischer Scientific (Leicestershire, UK).

Analytical standard powders for FF (purity >99%) and FFA (purity >99.6%) were obtained from Sigma-Aldrich and Witega (Berlin, Germany) respectively. Individual stock solutions of FF or FFA were prepared at 1mg/ml in methanol. Working standards were prepared by serial dilution of stock solutions with methanol.

2.2 Instrumentation and conditions: UHPLC-MRM^3 analysis

UHPLC separations were carried out using a Schimadzu Nexera UHPLC system. A reverse phase gradient separation was achieved on a Waters Acquity BEH C18 chromatographic column (50 x 2.1mm, 1.7µm) with in line filter assembly (0.2µm 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^{-1}. The flow was diverted to waste during sample injection cycles from 0.1-1.6 min and from 2.6 min until the end of an injection cycle. Linear gradient steps were used with initial conditions set at 100% A, held for 0.2min, decreasing to 50% A at 2.2min, 10%A at 2.4min, held for 0.7min, then returned to 100% A at 3.5min. A re-equilibration period of 2.5min was used. Total analysis time was 6min per sample.

A Sciex Linear QTrap 6500 system was used for mass spectrometric analysis, which was connected to the UHPLC system via a TurboIonspray® (TIS) interface source. FFA was analysed in positive polarity and FF in negative polarity (switching not required as sufficient chromatographic separation was achieved). The following instrument conditions were used: desolvation temp 425ºC, curtain gas flow 35 l/hr, gas 1 at 30 l/hr, gas 2 at 45 l/hr, ionspray voltages were +5.5kV in positive mode and -4.5kV in negative mode. Zero grade air was used for all source gases. Nitrogen was used as collision gas and set at 12l/hr (high) for all experiments. Analyser settings were optimised for maximum transmission. Unit mass resolution was used for MS-MS experiments with the ion trap (Q3) operated at a scan rate of 1000 Da/sec to achieve a resolution of <0.3Da Full Width at Half-Height (FWHH), for the second stage fragmentation process. Detection was performed in multiple reaction monitoring mode, acquiring both MS-MS and MS/MS/MS (MRM^3) experimental data simultaneously (MS/MS data was acquired for comparison against MRM^3 data). Transition experiments were optimised by infusion of individual standard solutions prepared in mobile phase (1:1 v/v) at 10µL min^{-1} using the integrated

184 syringe pump. The final MS experiment is summarised in **Table 1**. For FFA the most intense MRM^3
185 transition was used as the quantitative ion, with the remaining MRM^3 transition being used for ion ratio
186 confirmation.
187
188 ## 2.3 Sample extraction
189 ### *2.3.1 Sample materials*

190 Negative full fat raw milk samples were obtained from cows not receiving any treatment to provide
191 negative controls for the method validation and incurred study analyses. Control milk samples were
192 confirmed as negative for FF related residues prior to use. Incurred raw milk samples were available
193 as part of a withdrawal study being conducted in Friesian milking cows which had received the
194 recommended dose of FF (further described at section 2.5 – incurred sample studies). Incurred
195 samples were analysed using the developed hydrolysis procedure, and with an alternative methodology
196 lacking a hydrolysis step. On the day of analysis samples were defrosted at room temperature and
197 mixed thoroughly prior to weighing of aliquots.
198
199 ### *2.3.2 Sample hydrolysis, extraction and clean-up*

200 For the whole milk hydrolysis procedure, milk samples (2g) were weighed into 50mL screw cap
201 hydrolysis tubes. Negative milk samples for recovery evaluation or matrix standard calibrants were
202 fortified at this stage with a FF working standard (72.25ng mL^{-1}) and allowed to equilibrate for 10min
203 before proceeding.
204 Hydrochloric acid (6N, 4mL) was added to each tube, and the tubes then capped and vortex
205 mixed for 30s. All tubes were incubated in a water bath for 2h at 100ºC, with vortex mixing every 30min
206 prior to removal and cooling for 30min. The pH of hydrolysates was then adjusted to above pH 10 by
207 addition of 5mL of 1M di-potassium hydrogen orthophosphate solution, followed by increasing amounts
208 of 50% sodium hydroxide. Ethyl acetate (5mL) was added to each sample and the tubes re-capped
209 before inverting for 30s, followed by centrifugation at 2600 x g for 10min. Ethyl acetate layers were
210 transferred to separate disposable 16mL glass tubes. Ethyl acetate extractions were repeated on a
211 further two occasions and combined for each individual sample. All tubes were evaporated to dryness
212 at 50 ± 2ºC under nitrogen. To each tube, 1mL of water was added, vortex mixed (1min) and sonicated

(1min). Extracts were transferred to individual Eppendorf tubes and ultra-centrifuged at 17900 x g for 5min.

A dispersive solid phase extraction (dSPE) procedure was used for further extract clean-up. Firstly, 0.5mL of 0.5% ammonia in methanol was added to individual labelled Eppendorf tubes containing 75mg of end-capped C18 sorbent. A 0.5mL aliquot of the extract aqueous layer was transferred to its corresponding Eppendorf tube (avoiding any surface oil). Tubes were vortex mixed for 1min, then ultra-centrifuged for 5min at 17900 x g (room temperature). Aliquots were then transferred to vials for analysis.

2.3.3 *Incurred sample analysis with hydrolysis*

Hydrolysis studies followed the method previously described with recovery and matrix calibrants prepared at a level appropriate to expected contents along with each batch of incurred milks (as FFA equivalents, fortified prior to hydrolysis with FF only) as follows: two negative controls, four recovery samples fortified between 0.5-5 (low level) or 20-100µg kg^{-1} (high level) FFA equivalents, and six matrix calibrant tubes at FFA equivalent concentrations of 0.25, 0.5, 1.25, 2.5, 3.75 and 5µg kg^{-1} (linear range 0.25-5µg kg^{-1} FFA equivalents). For samples containing higher levels, calibrants were prepared at 5, 10, 25, 50, 75 and 100µg kg^{-1} (linear range 5-100µg kg^{-1} FFA equivalents).

2.3.4 *Incurred sample analysis without hydrolysis*

Incurred samples were also analysed using an in-house generic extraction procedure based on the principal of using a simple acetonitrile solvent extraction procedure (Robert et al. 2013). The procedure was modified further to include additional clean-up stages, using dispersive solid-phase extraction (dSPE) and hexane defatting (data was acceptable for the single day validation study - results are not presented here). This procedure allowed measurement separately of 'free' FF or FFA levels without the use of a hydrolysis step. Incurred milk samples or controls (2g) were weighed singly into disposable polypropylene 50mL centrifuge tubes. Two negative controls, four recovery samples and seven matrix calibrants were included with each batch. Recoveries and matrix calibrants were prepared at an appropriate level. Recovery samples were fortified prior to extraction with both FF and FFA (in duplicate) at two levels: 1µg kg^{-1} and 5µg kg^{-1}. All samples were extracted by vortex mixing with 2mL

of water and 12mL of acetonitrile. The whole extract was then subjected to dSPE with 0.5g of end-capped C18 material. Extracts were transferred to disposable glass tubes and the matrix matched standards fortified at this stage (with both FF and FFA) to cover a linear range from 0.25-10 or 1-100µg kg^{-1} (appropriate to expected analyte content). Samples were evaporated and reconstituted to a final volume of 2mL with 50% methanol containing 0.5% ammonia, defatted with hexane and then analysed by the UHPLC-MRM^3 procedure described.

2.4 Validation

Bovine raw milks were obtained from cows prior to receiving any treatment and confirmed as negative. Validation of the hydrolysis based method was carried out according to Commission Decision 2002/657/EC (Commission 2002) in bovine raw milk over three separate days, by two analysts. On each day a different raw milk negative control was used. Thirty aliquots of negative milk were weighed into separate 50mL screw cap hydrolysis tubes. Recovery samples (n=21) were then fortified at 0.25, 0.5 or 1 ng mL^{-1} with seven replicates at each level as follows: 10µL, 20µL or 40µL of the 72.25ng mL^{-1} FF secondary standard was added to 2g milk (equivalent to 0.25, 0.5 or 1µg kg^{-1} FFA equivalents). Three negative samples and six matrix calibrants were prepared for each validation lot. Matrix standards were fortified prior to hydrolysis with the FF secondary standard, prepared at FFA equivalent concentrations of 0.25, 0.5, 1.25, 2.5, 3.75 and 5µg kg^{-1} (linear range 0.25-5µg kg^{-1} FFA equivalents). All tubes were then subjected to the sample hydrolysis, extraction and clean-up procedure previously described.

2.5 Incurred sample studies: persistence of residues in milk

Milk samples were available from an ongoing withdrawal study being carried out in three British Friesian dairy cattle (Thompson et al. 2020). Control milk samples were collected from each cow during afternoon milking prior to treatment. Each cow was treated with the recommended therapeutic dose of a FF containing product (Fenflor® 300mg mL^{-1}) according to the manufacturer's instructions, by intramuscular injection (IM) over two sites, at 20mg kg^{-1} bodyweight. This treatment was repeated 48h later. Milk samples were collected during morning and/or afternoon milking for up to 70 days post treatment and stored at -20ºC until analysis. Incurred samples (n=37 per animal; 111 in total) were

analysed with and without hydrolysis, to assess the residue profile over time and the impact of hydrolysis on total FF-related content. Incurred milk samples containing higher FF contents (weeks 1 and 2 withdrawal) were diluted with negative raw milk (x20) and mixed thoroughly prior to weighing of any aliquots.

3. Results and discussion

3.1 Method optimisation

3.1.1 Sample extraction and clean-up

Our previously published work on the development of a method for total FF content in kidney described the optimisation of a sample hydrolysis protocol, subsequent extraction, dSPE and chromatography (Faulkner et al. 2016). However, application of the same procedure to the analysis of milk and fish muscle samples in our laboratory resulted in emulsions forming for these sample types on reconstitution. This is due to the higher fat content in these sample types compared to kidney, with the fatty acids and glycerol's produced on sample hydrolysis being partitioned into the ethyl acetate phase along with the single residue product FFA. A modification of the reconstitution procedure to reduce oil content in the final extracts prior to dSPE was necessary. Hydrolysed milk extracts were instead initially reconstituted in water to remove oils and prevent formation of emulsions. An aqueous portion (0.5mL) was transferred to a dSPE tube containing an equal volume of methanol with 0.5%ammonia and C18 sorbent. The use of end-capped C18 material in combination with ammonia in the reconstitution solvent has been shown to be necessary to prevent significant losses of FFA during the final dSPE step (Faulkner et al. 2016). To ensure solubility was not an issue, experiments were carried out by reconstituting mixed FFA/FF standards initially in water in the range 1-100ng mL^{-1}. Solvent calibration curves were generated by least-squares linear regression analysis with linear regression R^2 values >0.99. In the incurred residue studies in milk using the modified reconstitution procedure, calibration ranges in milk were extended up to 100ng mL^{-1} (µg kg^{-1}) in some cases. Coefficient of determination values (R^2) were >0.98 with residuals below 20% for all calibration points. The excellent linearity demonstrated that solubility was not an issue and that the hydrolysis procedure previously developed (Faulkner et al. 2016) was applicable to milk.

The use of a deuterated (D_3) FF internal standard was assessed for use in the procedure to account for recovery losses during sample extraction/clean-up, and for matrix effects on sample

analysis (deuterium's located on the methyl-sulfone group). Unfortunately, under the strong acidic conditions necessary, loss of deuteriums and conversion to parent FF and D_1/D_2-FF was observed. The use of an internal standard with deuteriums located on the aromatic ring may have been stable under these strong acid conditions, however this was not available for use (cost prohibitive). Matrix effects were assessed by extracting six different negative raw milks and assessing absolute responses against standards prepared in solvent only. They were prepared by taking aliquots of FF/FFA standards and evaporating to dryness, then reconstituting in injection solvent (50% methanol with 0.5% ammonia) or with post d-SPE cleaned negative extract to give milk equivalent concentrations of 12.5µg kg^{-1} FFA and 18µg kg^{-1} FF. Solvent standards were injected followed by matrix standards, with a repeat duplicate injection at the end of the batch. The final method was based on the use of hydrolysed, extracted matrix standards to compensate for analyte losses during extraction and matrix effects.

3.1.2 *Chromatographic separation*

FFA is a hydrophilic compound with a polar amino group which easily ionises at lower pH values. In our previous work, suppression of the polar amino group of FFA through use of a mobile phase pH~10.5 significantly improved the retention and peak shape for FFA, requiring the use of a pH stable C18 column. An investigation into the elution profile of milk matrix components using the chromatographic conditions described in our previous work (Faulkner et al. 2016) showed that it was necessary to modify the gradient method to improve method robustness.

In the investigation of milk matrix phospholipid profiles, data was acquired for hydrolysed milk extracts with a neutral loss MRM experiment for common in source fragment ions with *m/z* 184 and *m/z* 104, using a high in source declustering cone voltage (DP of 120V) and low collision cell energy of 5eV. Phospholipid elution profiles in a hydrolysed milk matrix standard prepared at 10µg kg^{-1}, using the original gradient method ending at 90% methanol, indicated that an extended gradient hold time ending with 100% methanol should improve method robustness. If the chromatographic separation used in our previous work had been applied directly to milk samples, late eluting phospholipids may have remained on the chromatographic column. Late eluting matrix components can appear as 'ghost peaks' in subsequent sample injections. This can lead to poor method robustness due to variable and unpredictable matrix effects resulting in variable responses (most commonly presenting as ion suppression). An extended hold time was therefore added to the end of each gradient cycle in the final

331 method to ensure that late eluting phospholipids were eluted from the chromatographic column prior to
332 subsequent sample injections.

333 Separation of FFA and FF was achieved using the final mobile phase and gradient conditions
334 developed (**supplementary Figure 2**). A retention factor (k) of 6.3 for FFA was calculated (k= retention
335 time FFA-T0/T0; T0 being the retention time equating to the column void volume). This exceeds the
336 minimum criteria required by Commission Decision 2002/657/EC (Commission 2002) of two times the
337 column void volume (equating to a k=1). Excellent peak shape, retention time stability and absence of
338 interfering peaks was achieved for both compounds during all analyses (FF was monitored to ensure
339 complete conversion to FFA in all hydrolysis studies and was necessary for direct comparison against
340 incurred studies lacking a hydrolysis step).

341

342 *3.1.3 MRM^3 optimisation*

343 The MRM conditions used in our previous work were initially applied to the analysis of milk extracts.
344 However, at the lower detection levels required for milk analyses the presence of either isobaric matrix
345 interferences or high MRM baseline signal presented an issue when using MRM experiments,
346 compromising method sensitivity and analyte confirmation. To overcome these matrix interferences,
347 and improve the selective detection and quantitation of lower levels of FFA in milk samples compared
348 to MRM only, the analytical method was optimised based on the use an MRM^3 workflow
349 (parent>product>sub-product fragmentation pathway).

350 **Figure 1** shows a comparison of MRM versus MRM^3 acquired data for a hydrolysed 0.5µg
351 kg^{-1} recovery sample in milk for FFA, along with representative negative milk sample data. Data
352 acquired in MRM mode did not allow for confirmation of FFA in hydrolysed extracts at concentrations
353 lower than 5µg kg^{-1}, because of isobaric interferences or poor response from confirmatory transition
354 product ions. In comparison, the use of an MRM^3 workflow permitted confirmation at much lower
355 concentration levels of <0.25µg kg^{-1} in milk. More than a 9-fold increase in sensitivity was obtained
356 compared to MRM for the quantitative transition, based on the improvement in signal-to-noise (*S/N*). It
357 was necessary to operate the trap at maximum resolution (scan rate 1000 Da/sec) for the second stage
358 fragmentation process (with Q0 trapping enabled) due to a lack of ion fragments and the necessity of
359 achieving sufficient mass resolution of *m/z* 130 and 131 fragments. Operating the trap at this scan rate
360 provides a resolution of around 0.3Da at FWHH. In an MRM^3 quantitative workflow analysis, the analyte

ion is first selected in Q1, then fragmented in Q2 collision cell. Fragment ions are then trapped in the Q3 linear ion trap, with excitation to perform the second fragmentation step. Second-generation fragment ions are scanned out to the detector, with a narrow scan range centred around the selected second-generation product ion. It is notable that the fragmentation pathway fits with that proposed by other authors for FFA (Geis-Asteggiante et al. 2014), where a quadrupole time of flight instrument was used to generate high resolution fragmentation spectra, and proposed structural characterizations for FFA fragment ions along with numerous other veterinary medicines.

3.2 Validation results (hydrolysis method)

3.2.1 Selectivity, specificity, matrix effects and linearity

Negative milk samples were analysed with each of the validation and incurred sample batches to verify the selectivity of the method. Representative MRM^3 chromatograms in milk are shown in **supplementary Figure 2** for a negative, spiked recovery (1µg kg^{-1}) and the lowest matrix fortified calibrant level of 0.25µg kg^{-1}**.** The absence of interfering peaks close to the retention time of FFA demonstrated the selectivity of the analytical method. The retention time in spiked or incurred positive samples also corresponded to calibration standards within the ±2.5% tolerance permitted for LC methods.

The specificity of the method was determined by comparison of the ion ratios in validation spiked controls or incurred tissue samples against matrix standards. Two MRM^3 transitions were monitored per analyte to fulfil the minimum identification criteria required by Commission Decision 2002/657/EC (Commission 2002). Ion ratios were calculated as the ratio of the qualifier ion against the quantifier ion and results for validation controls and incurred study results were within acceptable tolerances. The use of MRM^3 increased the specificity of the method and the number of identification points achieved for FFA to 5. This exceeds the 3 identification points required (Commission 2002).

Evaluation of matrix effect in different raw milk samples was conducted in 6 different hydrolysed milk extracts by preparing matrix matched standards prior to the final drying stage and comparing against solvent standards, at a level equivalent to 12.5µg kg^{-1}. The matrix effect (ME) was calculated and expressed as a %ME as follows (Matuszewski, Constanzer, and Chavez-Eng 2003).

$$\%ME = \frac{matrix\ standard\ response - solvent\ standard\ response}{solvent\ standard\ response} \text{ x } 100$$

Matrix effect suppression was observed with a mean suppression effect of 33% ± SD (%) of 6.8.

The linearity of FFA in hydrolysed matrix fortified standards is shown in **Figure 2a** over a calibration range of 0.25-5µg kg^{-1} and in **Figure 2b** over a wider range of 1-100µg kg^{-1} (used for some incurred study analyses). All calibration plots had coefficient of determination values (R^2) >0.98 with residuals below 20% for each of the three validation days in bovine milk and in the incurred study analyses. As the linear working range of the method was demonstrated up to 100 µg kg^{-1}, incurred milk samples anticipated as containing higher FF contents were diluted with negative raw milk (x20) prior to weighing of any aliquots.

3.2.2 Accuracy and precision

Validation was carried out according to Commission Decision 2002/657/EC by spiking seven replicates for each level to be assessed. Matrix effects and recovery losses were compensated for by using matrix fortified standards. Considering that FF is designated as 'not for use' in milk, validation was carried out at FFA equivalent concentrations of 0.25, 0.5 and 1.0µg kg^{-1}. The accuracy and precision of the method was evaluated by comparing the recoveries of FFA in spiked raw milk samples against the target spiked concentration, at different concentrations over three validation days. Method performance data is summarised in **Table 2**. Accuracy values from 92% to 113% were obtained over the three days, with a mean overall recovery (accuracy) of 103% for FFA over all concentration levels. Precision was estimated at each validation level from intra-day repeatability studies, calculated as the relative standard deviation for repeatability (%RSD_r) obtained within a batch. Intermediate precision was calculated from within laboratory reproducibility studies (%RSD_R), as the relative standard deviation over the three validation days, by two analysts and different lots of reagents. The precision RSD_r values ranged from 10.8% to 18.3% and the RDS_R values from 4.9% at 1µg kg^{-1} level to 8.3% at the lowest validation level of 0.25µg kg^{-1}. For substances without a permitted limit, the RSD_R values achieved should be as low as possible. Values obtained at concentrations less than 100µg kg^{-1} should typically be less than the predicted Horwitz equation target value of 23% for within laboratory reproducibility (Commission 2002). The results demonstrate that method accuracy and precision meet the legislative requirements for validation.

3.2.3 External proficiency testing

Independent method assessment was carried out by testing of milk samples provided by external proficiency test (PT) schemes in 2018 and 2019 (FAPAS®). The schemes covered the amphenicol antibiotic group. In both cases milk samples provided were incurred with chloramphenicol and had been spiked with FF (parent only). The hydrolysis method described herein was applied to the test samples, with total results submitted and expressed as FFA (as is defined in the EU marker residue definition (Commission 2010). Methods applied by other participants did not include a hydrolysis step, participants reporting values for FF or FFA only. FAPAS assigned a value for FF only in the final report (participants were asked to report FF and/or FFA). It is important to note that these samples were indicated in the FAPAS report as being spiked with the parent compound (FF), and not incurred, with the final issued report assigning a FF value only. To assess our method performance in these spiked milk samples it was necessary to convert our submitted FFA results back to FF (by applying a molecular weight correction factor of 1.445). Retrospective calculation gave z-scores of +0.15 and +0.2 in 2018 and 2019 respectively. Performance z-scores of $\leq |2|$ are deemed acceptable, and demonstrate that this procedure is fit for purpose. Although PTs are critical in demonstrating competence for testing laboratories, the use of artificial spiked samples as test materials can lead to assumptions when analysing for certain determinands. For FF, it is appropriate to use incurred materials, to highlight analytical issues, ensure that methods are fit for purpose and fulfil the marker residue definition.

3.2.4 Decision limit (CCα) and detection capability (CCβ)

Measurement uncertainty was determined according to Commission Decision 2002/657/EC (Commission 2002) for substances with no permitted limit by reference to the performance characteristic CCα. An α-error and of 1% and β-error of 5% was applied based on the within laboratory repeatability (RSD_R) data obtained, after plotting the calculated concentration against the added concentration for each of the three days. Calculated CCα and CCβ values are shown in **Table 2**. Representative chromatograms are shown in **supplementary Figure 2** demonstrating sensitivity at 0.25µg kg^{-1} in milk. A non-compliant ("positive") sample can be defined as one containing the identified analyte with at least 99% certainty. In a sample with a reported analyte content equal to CCα, the probability that the analyte is not present is equal to 1%.

3.3 Incurred sample results

Results of the analysis of incurred milk samples obtained from three cows previously treated with FF and collected during the withdrawal period are presented in **Table 3**. Samples were analysed by two separate procedures, either lacking *or* including a hydrolysis step. This was necessary to assess the impact of hydrolysis on the measured residue content in milk, which has not been presented in the literature to date.

The procedure lacking a hydrolysis step monitored for FF or FFA only, these being the only commercially available reference standards and compounds measured to date in milk (Liu, Lin, and Fuh 2016; Power et al. 2014; Rezende, Filho, and Rocha 2012). The extraction procedure was based on a published validated method which included milk analysis for amphenicols within its scope (Robert et al. 2013). The extraction method was modified further to include additional clean-up steps and a single day validation was carried out (results not presented). Matrix effects were compensated for by using matrix matched standards and four spiked recovery samples were analysed with every batch of incurred samples. The overall mean recovery across all batches was 89% for FF and 82% for FFA, with an estimated limit of quantitation (LOQ) of 0.2µg kg^{-1} (based on a peak-to-peak *S/N* of >10:1 for the quantitation MRM). To permit method comparison, the incurred sample results obtained the procedure without hydrolysis were corrected for recovery and the FF results converted to FFA equivalents by applying a molecular weight correction factor of 0.692, similar to that described by Power et al (Power et al. 2014). Results are shown in **supplementary Table 1**.

The hydrolysis procedure accounted for all metabolites as required by the marker residue definition, with the single end product FFA detected only. Matrix effects and recovery losses were compensated for by using matrix fortified standards. All recovery samples were within permitted control limits as determined from the validation data for all incurred sample batches. No correction for recovery was applied.

The results presented in **Table 3** show a comparison of withdrawal milk samples tested with *or* without hydrolysis. Use of the non-hydrolysis method demonstrated an underestimation of FF related content (from days 5-10), increasing with withdrawal period (FF and FFA monitored), and resulting in false negatives from day 24 onwards (<0.2µg kg^{-1}). Levels persisted above 0.5µg kg^{-1} for up to 35 days in the third cow's milk only (samples were not tested beyond this time point by the non-hydrolysis method). Lack of detection of parent FF or FFA residues beyond day 25 was previously reported in a

withdrawal study from 6 milking cows (Power et al. 2014), in a similar dosing study, with an analytical procedure lacking a hydrolysis step and FF method LOQ of 1µg kg^{-1}. The results obtained here, without use of hydrolysis, show a similar withdrawal pattern, but with a lower LOQ it is clear that residues can be detectable beyond day 25 in some cases. Importantly, when compared against data generated by the total hydrolysis procedure, unhydrolysed results may only account for a fraction of FF-related content at a particular time point post treatment.

Analysis of the incurred samples with a procedure including a hydrolysis step resulted in a trend of higher FF-related residue content from day's 5-10 withdrawal onwards. **Figure 3** demonstrates the increasing contribution of other intermediate metabolites in milk (other than FF or FFA) to total FF-related residue content with increasing withdrawal period. At day 5 the percentage of other intermediate metabolites (other than FF or FFA) to total residue content was <10%. This contribution increased steadily to 17-33% at day 10, 44-85% at day 20, and to 100% of measurable content from day 24 onwards. The data demonstrates that inclusion of a hydrolysis step is necessary to ensure accurate estimation of FF related content and avoid false negative results for the longest possible time after administration. Hydrolysis converts intermediate metabolites present in incurred milk samples to FFA (**supplementary Figure 1**). Importantly, the analysis of incurred milk samples from the withdrawal study, using a method including a hydrolysis step versus one lacking hydrolysis, demonstrates that the use of physico-chemical methods lacking a hydrolysis step can result in under-reporting or non-detection of FF related residues in milk.

FF is listed in Commission Regulation (EU) No 37/2010 (Commission 2010) as <u>not</u> permitted for use in the EU in animals from which milk or eggs are produced for human consumption. However, under the veterinary cascade the extra-label use of medicines by veterinary practitioners may occur, according to the provisions of Commission Regulation 2019/6, Article 113 (Commission 2019b). The cascade is risk based, and the prescribing vet must consider known information about the use of the product, and specify an appropriate withdrawal period. Current guidance in the UK by the competent authority – the Veterinary Medicines Directorate (VMD), specifies a minimum of seven days for milk or eggs for the off-label use of veterinary medicinal products (Directorate 2013). Current data in the literature concerning florfenicol withdrawal in milk is based on the use of methods lacking a hydrolysis step (Kawalek et al. 2016; Power et al. 2014). These studies were based on IM administration, determining that FF alone was the major residue in milk, with FF residues being detectable for 5-20

511 days (the latter based on a method LOQ of 1µg kg^{-1}). The data presented here from 3 therapeutically
512 treated dairy cattle, using a method which includes a hydrolysis step, and accounting for other
513 intermediate metabolites, indicates that total FF residues may be detectable for at least 50 days post
514 treatment.
515

516 ## 4. Conclusion

517 A confirmatory UHPLC-MS/MS method has been validated in bovine raw milk that can be used to
518 quantify total FF related content. The results obtained in comparing the total FF content of incurred
519 withdrawal milk samples against results obtained with a method lacking a hydrolysis step indicate that
520 there is a potential for (i) false negative results if applying a physico-chemical method lacking a
521 hydrolysis step and (ii) may lead to a significant underestimation of FF related content.
522 A prolonged withdrawal period is indicated after FF treatment as it has been demonstrated
523 that FF-related residues (other than FF or FFA) can persist in milk for more than 50 days. The data
524 presented shows that intermediate metabolites are a significant contributor to the total FF related
525 content, and are only detectable if methods account for the total FF-related content in samples.
526 Consequently, milk samples taken as part of a monitoring program, and analysed for FF residues by a
527 physico-chemical method, such as LC-MS/MS, should include a hydrolysis procedure, according to the
528 current marker residue definition and MRL status in milk (not permitted).
529 This is the first published method for milk analysis that fulfils the marker residue definition.
530 Data is presented for the first time on the full residue profile in incurred/withdrawal milks. Further work
531 is needed to accurately determine and quantify the total FF residue withdrawal profile in the milk from
532 a wider pool of treated animals, taking into consideration different possible administration routes due to
533 possible veterinary cascade or unauthorised use of FF in dairy cows. The limited data in the literature
534 is based on the use of methods lacking a hydrolysis step (Ruiz B et al. 2010; Power et al. 2014; Kawalek
535 et al. 2016). The data presented here indicates a much longer withdrawal period, and will help inform
536 veterinary practitioners and the dairy industry of appropriate withdrawal times to limit consumer
537 exposure to residues in milk.
538
539
540

Acknowledgements

The authors wish to thank Dr Terence Fodey and Paul Barnes from the Agri-Food and Biosciences Institute for providing the incurred milk samples used for part of this study.

References

Agency, European Medicines. 1996. 'Florfenicol summary report (1)', *Florfenicol summary report (1)*.

Alechaga, E., E. Moyano, and M. T. Galceran. 2012. 'Ultra-high performance liquid chromatography-tandem mass spectrometry for the analysis of phenicol drugs and florfenicol-amine in foods', *Analyst*, 137: 2486-94.

Commission, EU. 2002. 'Commission Decision (EC) no 2002/657 of 12th August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results', *Off J Eur Union.*, 45: 8-36.

———. 2010. 'Commission Regulation (EC) 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.', *Off J Eur Union.*, 53: 1-80.

———. 2019a. 'Commission Delegated Regulation (EU) 2019/2090 of 19 June 2019 supplementing Regulation (EU) 2017/625 of the European Parliament and Council regarding cases of suspected or established non-compliance with Union rules applicable to the use or residues of pharmacologically active substances authorised in veterinary medicinal products or as feed additives or with Union rules applicable to the use or residues of prohibited or unauthorised pharmacologically active substances.', *Off J Eur Union.*, 62: 28-37.

———. 2019b. 'Regulation 2019/6 of the European Parliament and of the Council on veterinary medicinal products and repealing Directive 2001/82/EC', *Off J Eur Union.*, 62: 1-23.

Directorate, Veterinary Medicines. 2013. 'Veterinary Medicines Guidance Note13: Guidance on use of the cascade'.

Faulkner, D., M. Cantley, M. Walker, S. Crooks, D. Kennedy, and C. Elliott. 2016. 'Evidence of non-extractable florfenicol residues: development and validation of a confirmatory method for total florfenicol content in kidney by UPLC-MS/MS', *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 33: 983-94.

Fedeniuk, R. W., M. Mizuno, C. Neiser, and C. O'Byrne. 2015. 'Development of LC-MS/MS methodology for the detection/determination and confirmation of chloramphenicol, chloramphenicol 3-O-beta-d-glucuronide, florfenicol, florfenicol amine and thiamphenicol residues in bovine, equine and porcine liver', *J Chromatogr B Analyt Technol Biomed Life Sci*, 991: 68-78.

Geis-Asteggiante, Lucia, Alberto Nunez, Steven J. Lehotay, and Alan R. Lightfield. 2014. 'Structural characterization of product ions by electrospray ionization and quadrupole time-of-flight mass spectrometry to support regulatory analysis of veterinary drug residues in foods', *Rapid Communications in Mass Spectrometry*: 1061.

Guidi, Letícia R., Patrícia A. S. Tette, Christian Fernandes, Luiza H. M. Silva, and Maria Beatriz A. Gloria. 2017. 'Advances on the chromatographic determination of amphenicols in food', *Talanta*, 162: 324-38.

Horsberg, Tor Einar, Bernt Martinsen, and Kanwal J. Varma. 1994. 'The disposition of 14C-florfenicol in Atlantic salmon (Salmo salar)', *Aquaculture*, 122: 97-106.

Imran, Muhammad, Habib Fazal e, Abdul Tawab, Waqar Rauf, Moazur Rahman, Qaiser Mehmood Khan, Muhammad Rafique Asi, and Mazhar Iqbal. 2017. 'LC–MS/MS based method development for the analysis of florfenicol and its application to estimate relative distribution in various tissues of broiler chicken', *Journal of Chromatography B*, 1063: 163-73.

621 Jianzhong Liu, Ki-Fai Fung, Zhangliu Chen, Zhenling Zeng, and Jie Zhang. 2003.
622 'Pharmacokinetics of Florfenicol in Healthy Pigs and in Pigs Experimentally Infected
623 with Actinobacillus pleuropneumoniae', *American Society for Microbiology*, Vol. 47:
624 820–23.
625 Kawalek, J. C., K. D. Howard, Y. Jones, M. L. Scott, and M. J. Myers. 2016. 'Depletion of
626 florfenicol in lactating dairy cows after intramammary and subcutaneous
627 administration', *J Vet Pharmacol Ther*, 39: 602-11.
628 Liu, H. Y., S. L. Lin, and M. R. Fuh. 2016. 'Determination of chloramphenicol, thiamphenicol
629 and florfenicol in milk and honey using modified QuEChERS extraction coupled with
630 polymeric monolith-based capillary liquid chromatography tandem mass spectrometry',
631 *Talanta*, 150: 233-9.
632 Matuszewski, B. K., M. L. Constanzer, and C. M. Chavez-Eng. 2003. 'Strategies for the
633 Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on
634 HPLC−MS/MS', *Anal Chem*, 75: 3019-30.
635 Pan, X. D., P. G. Wu, W. Jiang, and B. J. Ma. 2015. 'Determination of chloramphenicol,
636 thiamphenicol, and florfenicol in fish muscle by matrix solid-phase dispersion
637 extraction (MSPD) and ultra-high pressure liquid chromatography tandem mass
638 spectrometry', *Food Control*, 52: 34-38.
639 Power, Clare, Ríona Sayers, Martin Danaher, Mary Moloney, Bernadette O'Brien, Ambrose
640 Furey, and Kieran Jordan. 2014. 'Investigation of the persistence of florfenicol residues
641 in bovine milk and fate during processing', *International Dairy Journal*, 39: 270-75.
642 Rezende, D. R., N. F. Filho, and G. L. Rocha. 2012. 'Simultaneous determination of
643 chloramphenicol and florfenicol in liquid milk, milk powder and bovine muscle by LC-
644 MS/MS', *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 29: 559-70.
645 Robert, C., N. Gillard, P. Y. Brasseur, G. Pierret, N. Ralet, M. Dubois, and P. Delahaut. 2013.
646 'Rapid multi-residue and multi-class qualitative screening for veterinary drugs in foods
647 of animal origin by UHPLC-MS/MS', *Food Addit Contam Part A Chem Anal Control*
648 *Expo Risk Assess*, 30: 443-57.
649 Ruiz B, J., M. Zapata N, C. López C, and F. Gutiérrez H. 2010. 'Florfenicol concentrations in
650 milk of lactating cows postreated by intramuscular or intramammary routes', *Revista*
651 *MVZ Cordoba*, 15: 2041-50.
652 Saito-Shida, Shizuka, Kouji Shiono, Jumpei Narushima, Satoru Nemoto, and Hiroshi Akiyama.
653 2019. 'Determination of total florfenicol residues as florfenicol amine in bovine tissues
654 and eel by liquid chromatography–tandem mass spectrometry using external
655 calibration', *Journal of Chromatography B*, 1109: 37-44.
656 Schering-Plough. 1996. 'NADA 141-063 Nuflor Injectable Solution, FDA approval document',
657 *NADA 141-063 Nuflor Injectable Solution, FDA approval document.*
658 Schneider, M. J., S. J. Lehotay, and A. R. Lightfield. 2015. 'Validation of a streamlined
659 multiclass, multiresidue method for determination of veterinary drug residues in bovine
660 muscle by liquid chromatography-tandem mass spectrometry', *Analytical and*
661 *Bioanalytical Chemistry*, 407: 4423-35.
662 Tao, Y., F. Zhu, D. Chen, H. Wei, Y. Pan, X. Wang, Z. Liu, L. Huang, Y. Wang, and Z. Yuan.
663 2014. 'Evaluation of matrix solid-phase dispersion (MSPD) extraction for multi-
664 fenicols determination in shrimp and fish by liquid chromatography-electrospray
665 ionisation tandem mass spectrometry', *Food Chem*, 150: 500-6.
666 Thompson, Colin S., Imelda M. Traynor, Terence L. Fodey, P. Barnes, Dermot V. Faulkner,
667 and Steven R. H. Crooks. 2020. 'Screening method for the detection of residues of
668 amphenicol antibiotics in bovine milk by optical biosensor', *Food Additives &*
669 *Contaminants: Part A*: 1-11.

Thompson, Colin S., Imelda M. Traynor, Terence L. Fodey, Dermot V. Faulkner, and Steven R. H. Crooks. 2017. 'Screening method for the detection of residues of amphenicol antibiotics in bovine, ovine and porcine kidney by optical biosensor', *Talanta*, 172: 120-25.

Varma, K.J., P. E. Adams, T. E. Powers, J. D. Powers, and J. F. Lamendola. 1986. 'Pharmacokinetics of florfenicol in veal calves', *J Vet Pharmacol Ther*, 9: 412-25.

735 **Table 1:** Mass spectrometry conditions**.** EP voltage set at 10V. LIT= linear ion trap (operated at a scan
736 rate of 1000 Da/sec, with a fill time of 48msec, 25msec excitation time, AF3=0.4 and EXB= -154). Note:
737 MS/MS data was also acquired for comparison against LIT data during method validation.
738

Compound	*Polarity*	*Mode*	*Time (min)*	*DP (V)*	*1st Precursor (m/z)*	*CE*	*2nd Precursor (m/z)*	*LIT Product (m/z) (start-stop)*
FFA	Positive	**LIT** (scan rate 1000 Da/sec) Fill time 48msec)	0 – 2.1	40	248.1	18	230.1	129.8 – 130.4 130.8 – 131.4
					Precursor (MS1)		*Product (MS2)*	
FFA	Positive	MS/MS	0 – 2.1	40	248.1	18 31 31	230 130.1 151.1	
FF	Negative	MS/MS	2.1 – 2.8	-40	356	-14 -27	336 185	

739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756

Table 2: Method performance data obtained during method validation for Bovine milk (n=21). Note: %RSDr, relative standard deviation for repeatability, %RSDR, relative standard deviation for within laboratory repeatability. *Legal status as defined in Commission Regulation (EU) 37/2010.

Compound	*Concentration added (µg kg^{-1})*	*Accuracy (%)*	*Intra-day Precision (%RSDr)*	*Inter-day Precision (%RDSR)*	*Status**	*CCα (µg kg^{-1})*	*CCβ (µg kg^{-1})*
FFA	0.25	92	11.8	8.3	Not for use	0.2	0.34
	0.5	105	18.3	7.7			
	1.0	113	10.8	4.9			

Table 3: Comparison of results obtained after extraction and analysis of milk samples with separate procedures lacking (UnHyd) or including a hydrolysis step (Hyd). Samples were obtained from cows previously treated with the recommended dose of Nuflor injectable solution (2 doses, 48 hours apart) and subject to withdrawal. When two successive results fell below the LOQ or CCα, subsequent samples were not tested (NT). Procedure (UnHyd): florfenicol (FF) and florfenicol amine (FFA) results were corrected for recovery and expressed as sum of FFA equivalents (ug kg^{-1}) for comparison. Procedure (Hyd): FFA measured as final hydrolysis product (ug kg^{-1}). No recovery correction applied to results as matrix fortified calibrants are used in the procedure.

Day	Cow 1		Cow 2		Cow 3	
	UnHyd (FFA equiv. ug kg^{-1})	Hyd (FFA ug/kg^{-1})	UnHyd (FFA equiv. ug kg^{-1})	Hyd (FFA ug/kg^{-1})	UnHyd (FFA equiv. ug kg^{-1})	Hyd (FFA ug/kg^{-1})
Pre-treatment	0	0	0	0	0	0
First treatment - IM injection after pm milking						
1	859	752	1114	1057	1166	1451
2 (am)	379	359	611	575	540	645
2 (pm)	309	238	452	539	399	433
Second treatment - IM injection after pm milking						
3	1447	1075	1103	1143	1238	1467
4	484	440	597	547	534	571
5	266	268	436	422	449	499
6	172	161	294	299	243	339
8	66.8	98.5	120	152	171	183
10	39.5	59.2	61.3	81.2	101	121
12	20.7	39.7	32.4	27.6	57.7	67.5
14	10.4	21.5	18.0	23.0	30.5	42.1
16	4.3	13.4	9.2	16.0	14.9	21.9
18	2.4	10.9	6.5	12.1	10.0	15.8
20	1.2	7.9	4.3	7.9	7.6	13.6
22	0.4	5.8	1.6	5.5	6.0	10.4
24	<0.2	4.2	0.9	3.3	4.2	9.3
26	<0.2	3.1	0.3	2.5	2.7	6.3
28	NT	2.6	<0.2	2.1	2.0	4.8
30	NT	1.2	<0.2	1.7	1.5	4.8
32	NT	0.9	<0.2	1.4	1.2	3.4
34	NT	0.8	<0.2	0.9	0.7	2.3
36	NT	0.6	NT	0.7	NT	2.6
38	NT	0.5	NT	0.8	NT	1.9
40	NT	0.3	NT	0.9	NT	1.7
42	NT	0.3	NT	0.7	NT	1.4
44	NT	0.2	NT	0.7	NT	1.0
46	NT	0.2	NT	0.8	NT	0.6
48	NT	0.2	NT	0.7	NT	0.7
50	NT	<0.2	NT	0.8	NT	0.7
52	NT	<0.2	NT	0.8	NT	0.4
54	NT	NT	NT	0.4	NT	0.4
56	NT	NT	NT	0.5	NT	0.3
58	NT	NT	NT	0.3	NT	0.3
60	NT	NT	NT	0.3	NT	<0.2
62	NT	NT	NT	0.3	NT	<0.2
64	NT	NT	NT	<0.2	NT	<0.2
66	NT	NT	NT	<0.2	NT	<0.2

Figure 1a: Negative milk (MRM)

Figure 1b: 0.5ppb recovery in milk (MRM)

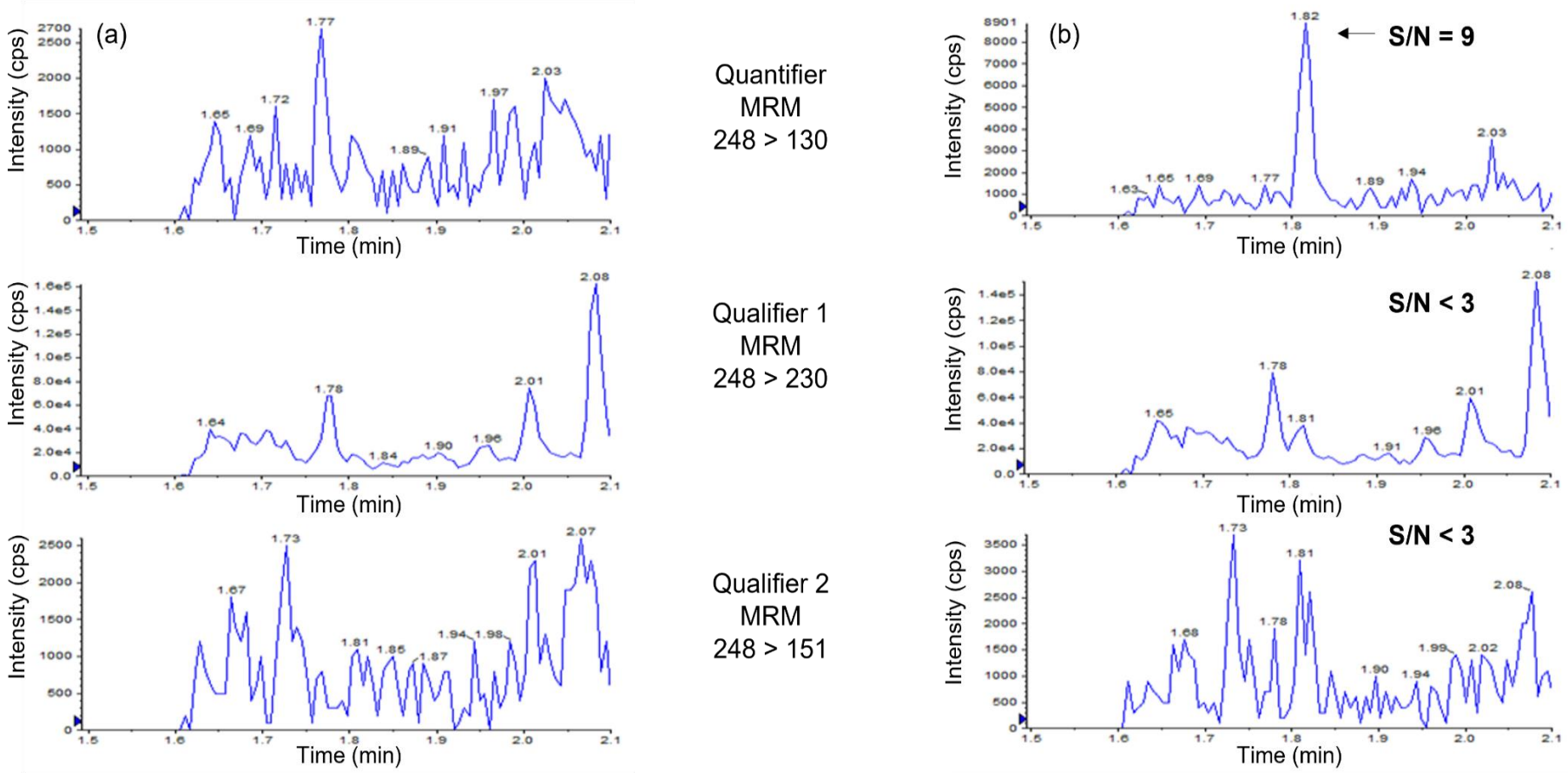


Figure 1 (a-d): Representative **MRM** *versus* **MRM³** chromatogram channel data for florfenicol amine (FFA) in prepared hydrolysed milk samples (unsmoothed). (a) MRM channel data – negative milk (b) MRM channel data for 0.5µg kg^{-1} recovery in milk (c) **MRM³** channel data - negative milk (d) **MRM³** channel data for 0.5µg kg^{-1} recovery in milk.

Figure 1c: Negative milk (**MRM³**)

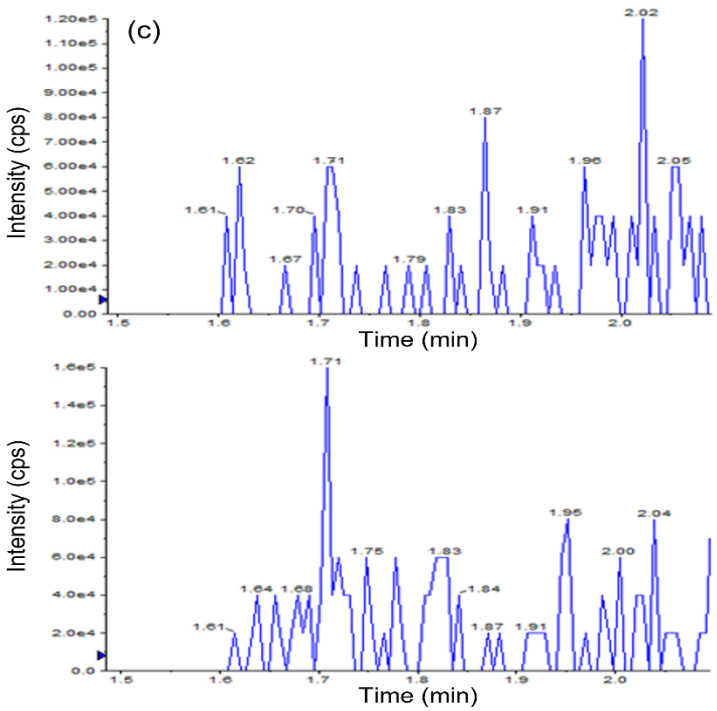


Figure 1d: 0.5ppb recovery in milk (**MRM³**)

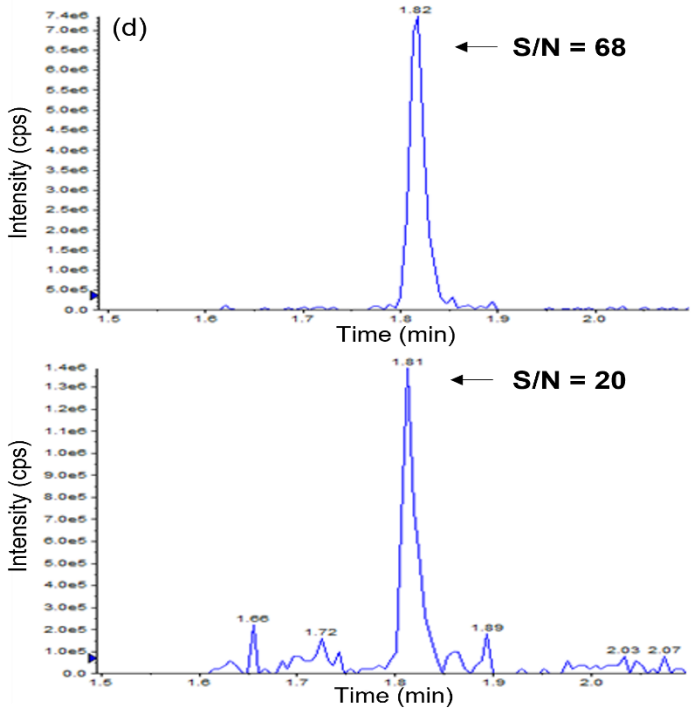


Figure 1 (a-d): Representative **MRM** *versus* **MRM³** chromatogram channel data for florfenicol amine (FFA) in prepared hydrolysed milk samples (unsmoothed). (a) MRM channel data – negative milk (b) MRM channel data for 0.5µg kg^{-1} recovery in milk (c) **MRM³** channel data - negative milk (d) **MRM³** channel data for 0.5µg kg^{-1} recovery in milk.

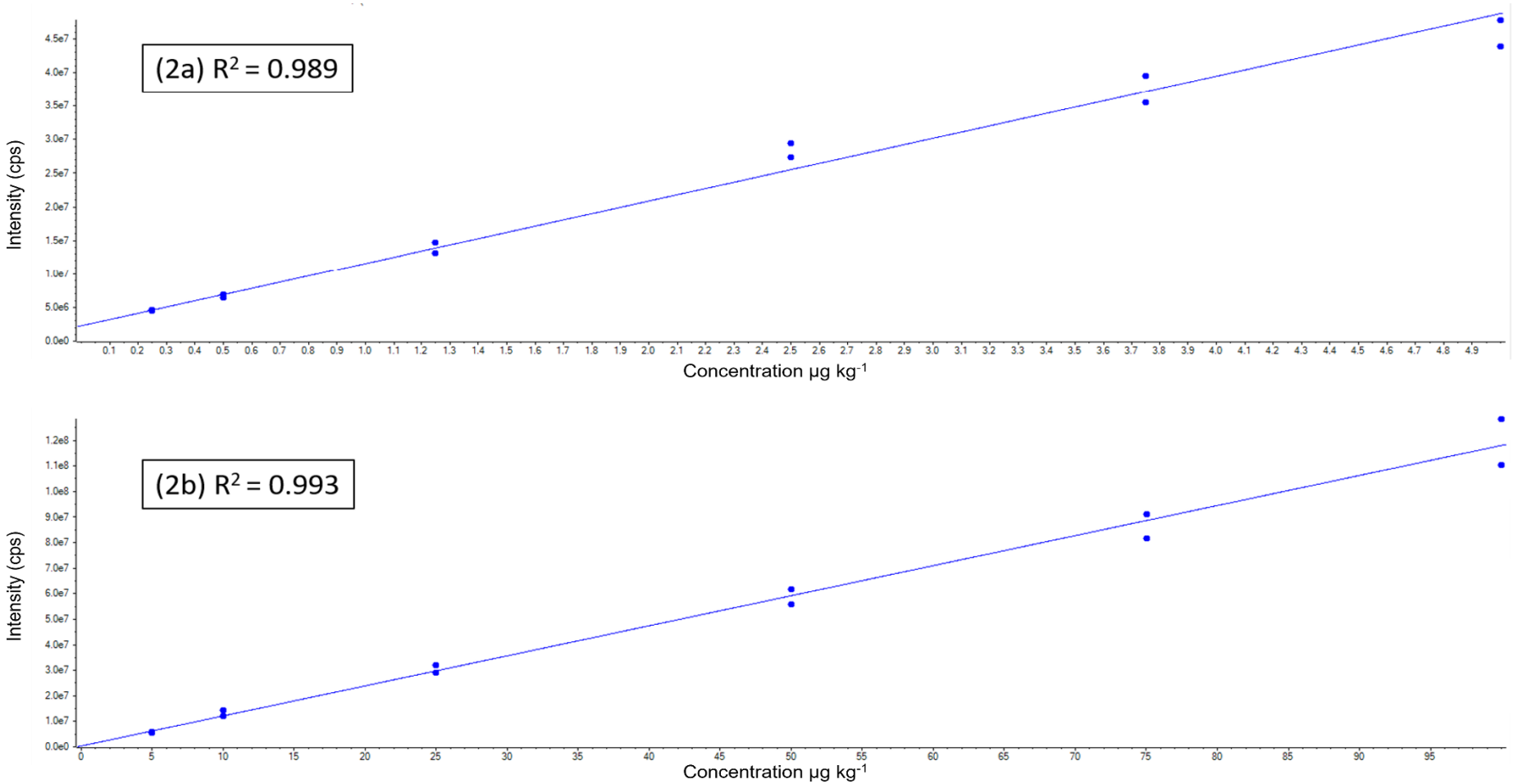


Figure 2: Sensitivity and linearity of florfenicol amine (FFA) in hydrolysed + extracted milk standard curve extracts over a calibration range of 0.25 - 5µg kg^{-1} (Figure 2a) and a range of 5 -100µg kg^{-1} (Figure 2b). Incurred milk sample analyses requiring the use of higher concentration curves (2b: Injected sample volume reduced from 5µl to 0.5µl).

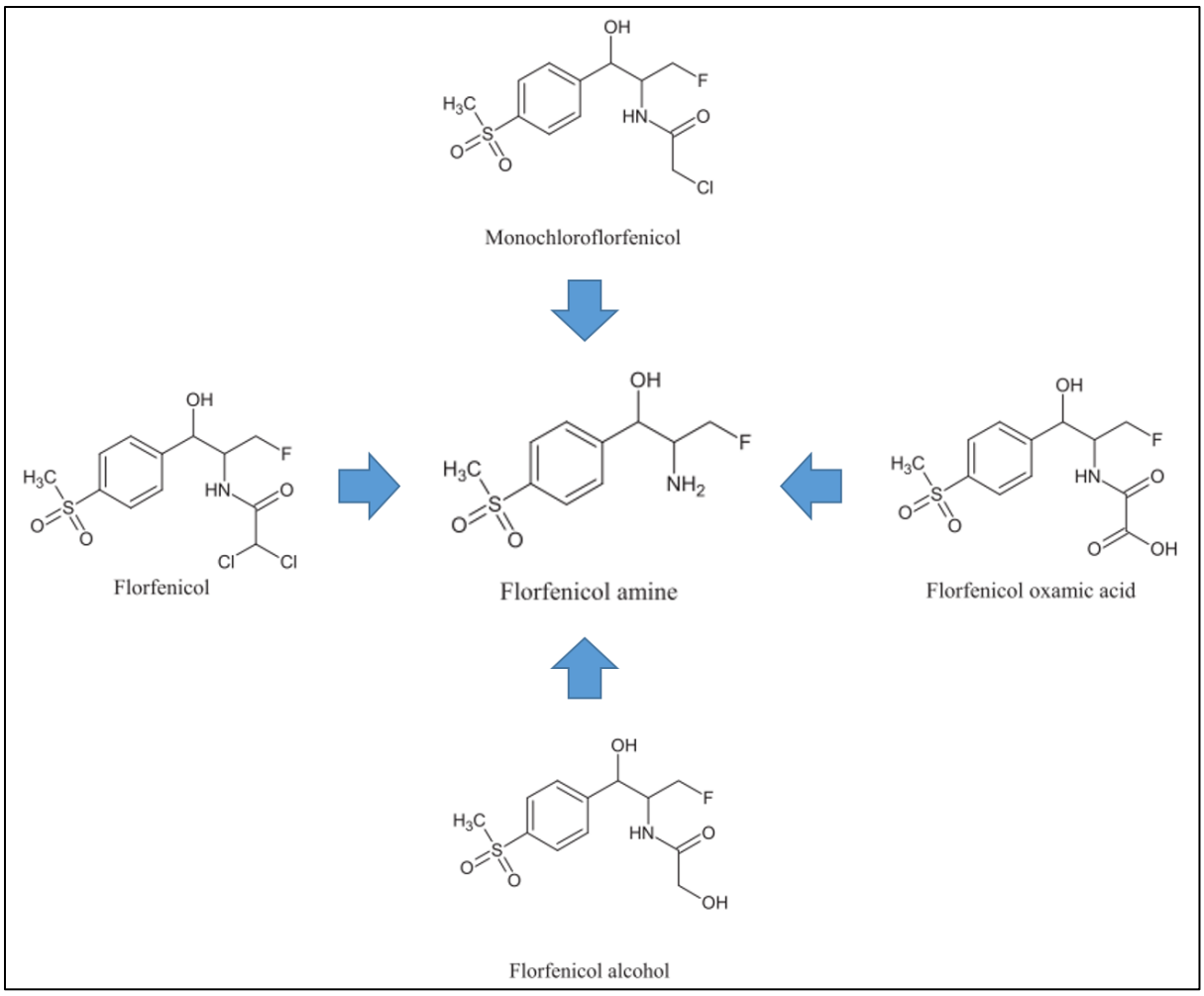
Figure 3: Percentage contribution (%) of intermediate metabolites, other than florfenicol (FF) or florfenicol amine (FFA) to total FF-related residue content in milk. Samples were obtained from 3 cows previously treated with the recommended dose of Nuflor injectable solution (2 doses, 48 hours apart) and then subject to withdrawal.

Figure 3 – Colour print is required

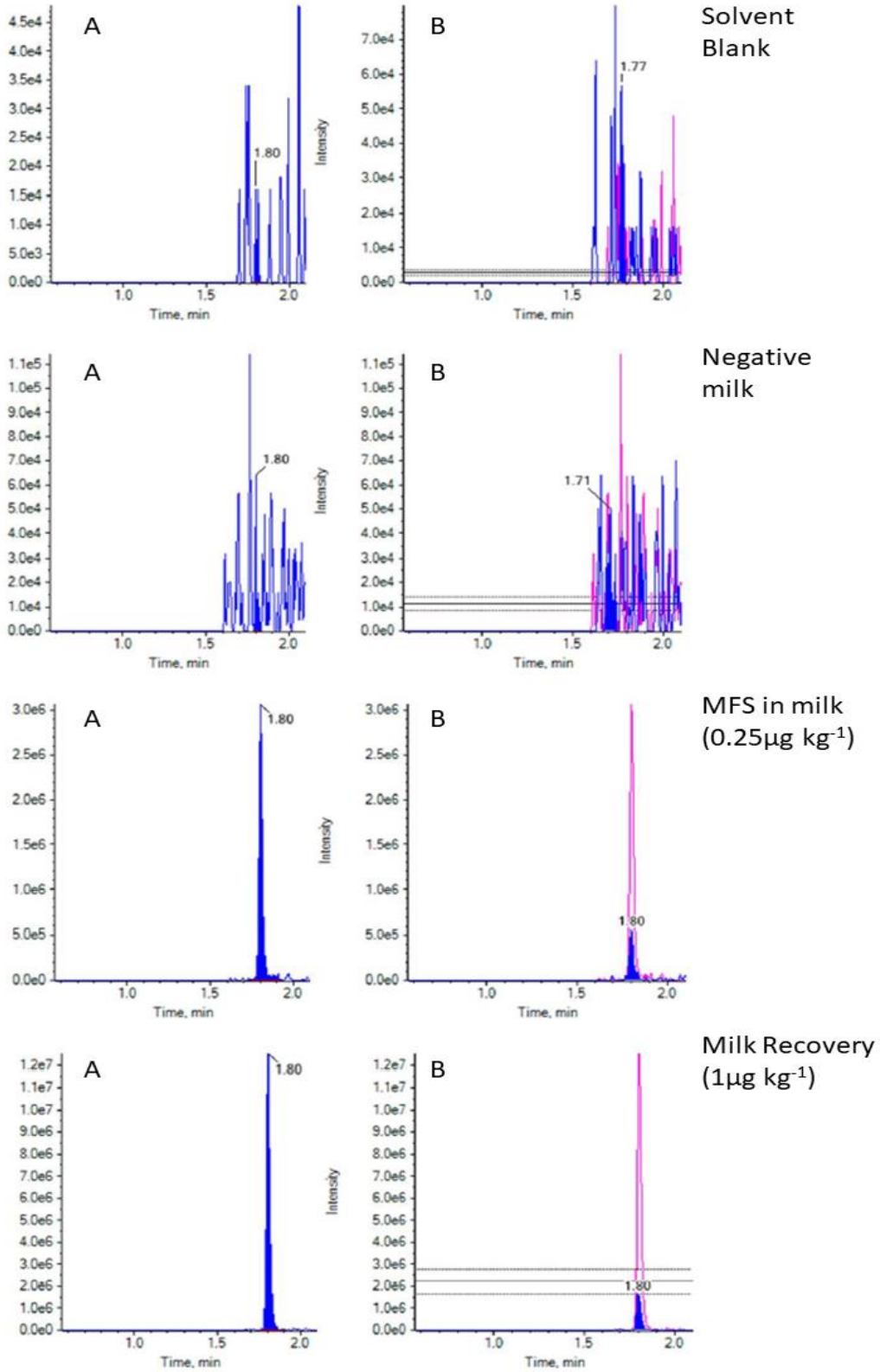
Supplementary materials:

Supplementary Table 1: Incurred sample results obtained using procedure without hydrolysis for florfenicol (FF) and florfenicol amine (FFA). Results were corrected for recovery and the FF results converted to FFA equivalents by applying a molecular weight correction factor of 0.692. The two values were then summed for comparison against the total hydrolysis results for FFA obtained in the same incurred samples (refer to Table 3). NT indicates not tested as levels were below calculated method LOQ.

Day	Cow 1		Cow 2		Cow 3	
	FF (ug kg^{-1})	FFA (ug/kg^{-1})	FF (ug kg^{-1})	FFA (ug/kg^{-1})	FF (ug kg^{-1})	FFA (ug/kg^{-1})
Pre-treatment	0	0	0	0	0	0
First treatment - IM injection after pm milking						
1	1098	100	1333	191	1444	166
2 (am)	476	50	704	123	650	91
2 (pm)	390	39	537	80	486	63
Second treatment - IM injection after pm milking						
3	1885	142	1351	168	1559	159
4	612	61	731	90	676	66
5	327	40	541	62	566	57
6	211	26	345	55	292	41
8	86	7.5	145	20	215	22
10	50	4.7	73	11	125	14
12	27	2.3	38	6.0	70	9.3
14	13	1.3	20	3.9	37	4.9
16	6	0.3	11	2	17	3.0
18	3.5	<0.2	7.1	1.5	12	1.7
20	1.7	<0.2	4.8	1.0	9	1.4
22	0.5	<0.2	1.7	0.4	7.0	1.1
24	<0.2	<0.2	0.9	0.3	5.0	0.7
26	<0.2	<0.2	0.3	<0.2	3.1	0.6
28	NT	NT	<0.2	<0.2	2.2	0.4
30	NT	NT	<0.2	<0.2	1.8	0.3
32	NT	NT	<0.2	<0.2	1.3	0.2
34	NT	NT	<0.2	<0.2	0.8	<0.2
36	NT	NT	NT	NT	NT	NT
38	NT	NT	NT	NT	NT	NT



Supplementary Figure 1: Strong acid hydrolysis is required to release bound residues (in tissue), and convert florfenicol (FF) and intermediate metabolites to the single marker residue florfenicol amine (FFA)



Supplementary Figure 2: Representative MRM^3 chromatograms for florfenicol amine (FFA) in milk showing a solvent blank, negative, spiked recovery (1µg kg^{-1}) and matrix fortified standard (MFS) level of 0.25µg kg^{-1}. **(A)** MRM^3 channel data for quantification (m/z 248 > 230 > 130). **(B)** MRM^3 channel data for qualifier (m/z 248 > 230 > 131) overlaid with quantification channel. Ion ratio tolerance bars of 20% shown.