Screening method for the detection of residues of amphenicol antibiotics in bovine milk by optical biosensor

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Abstract

An immunobiosensor assay was developed for multi-residue screening of the parent amphenicols, thiamphenicol and florfenicol, along with the metabolite florfenicol amine, in bovine milk. A polyclonal antibody raised in a rabbit after immunisation with a florfenicol amine-protein conjugate was employed in the assay. Milk samples were subjected to acetonitrile extraction, reconstituted in buffer and diluted prior to biosensor analysis. Validation data obtained from the analysis of fortified samples has shown that the method has a detection capability of less than 0.25 µg kg^{-1} for florfenicol and less than 0.5 µg kg^{-1} for florfenicol amine and thiamphenicol. The cross-reactivity profile and validation data for the detection of these amphenicols is presented together with results obtained following the analysis of florfenicol incurred samples using the developed screening method along with a comparison of results obtained from the analysis of the same incurred samples using an MRM^3 UPLC-MS/MS confirmatory method. Results obtained from the analysis of samples from both treated and non-treated animals which were co-housed and which show the potential for cross-contamination are also presented.

Keywords: ***Screening; detection; amphenicols; milk; optical biosensor***

Introduction

Amphenicols, including chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FF) are synthetic broad spectrum antibiotics which have been used extensively as veterinary medicines in the treatment of various diseases. They inhibit protein synthesis in susceptible bacteria by binding to ribosomal sub units, thereby preventing the transfer of amino acids and further protein formation. Their low cost, high potency and ready availability under such brand names as TAF Spray®, Fenflor®, Nuflor®, Florocol® and Resflor Gold® have led to them being a popular choice for those involved in animal husbandry in Europe and throughout the world.

The use of CAP, however, was banned in all food producing animals because of concerns that emerged over its haematological toxicity and the potential risks to human health through the consumption of food containing residues of the drug. As a result of the ban on the use of CAP, FF has become increasingly popular as an antibiotic of choice in the livestock, poultry and aquaculture industries. FF is a fluorinated derivative of TAP (itself a CAP analogue) and has a range of activity which is broadly similar to that of CAP and demonstrates efficacy against both gram positive and gram negative bacteria, although it does not carry the risk of inducing aplastic anaemia that CAP does (Lobell et al. 1994; Shen et al. 2002; Switala et al. 2007; Andree et al. 2010). Unlike TAP which remains largely unchanged, FF is rapidly metabolised *in vivo* to several intermediates including florfenicol amine (FFA), florfenicol alcohol (FA), florfenicol oxamic acid (FO) and monochloroflorfenicol (MCF) (FDA 1996) as shown in Figure 1. While the ratios of these metabolites vary between species, FFA is predominant in most food producing animals. The use of FF and TAP in foodstuffs is, however, strictly regulated in many countries including the European Union, the United States and China with the EU establishing MRLs for FF and TAP in the tissues of livestock. The European Medicines Agency has also established an MRL for TAP in milk of 50 µg kg^{-1} however none exists for FF and consequently the use of FF, identified as the sum of FF and its metabolites measured as

FFA, is not permitted in animals producing milk for human consumption (European Commission 2009). Any method employed for the detection of FF in milk should therefore be extremely sensitive with much lower detection limits required (at least in the low µg kg^{-1} range) than for those analytical methods employed for tissue analysis where MRLs have been set, since any confirmed level of the drug within a milk sample breaches stipulated EU legislation.

Milk and other dairy products, including milk powders for infant consumption, are staple food sources throughout the world, being high in many nutrients including protein and lactose. With demand for dairy products increasing, because of the world's ever rising population, the production of milk and milk products has become a vital multi-billion pound industry. At the same time, the widespread use of antibiotics as both growth promoters and for therapeutic reasons in dairy animal production poses a potential risk to human health through the consumption of adulterated milk as well as the overuse of such antibiotics increasing the possibility of the emergence of bacterial resistance; a subject which is becoming an ever more serious concern to authorities worldwide.

While a substantial number of methods have been reported for the determination of amphenicols in edible tissue and animal feed (Zhang et al. 2008; Luo et al. 2009; Luo et al. 2010; El-Banna and El-Zorba 2011; Luo et al. 2009; Tao et al. 2012; Tao et al. 2014; Schneider et al. 2015; Faulkner et al. 2016; Thompson et al. 2017), far fewer have addressed the unauthorised use of FF in dairy cattle and the potential contamination of milk bound for human consumption through its use (Rezende et al. 2012; Samanidou et al. 2015; Hsiang-Yu et al. 2016; Kawalek et al. 2016). The majority of these methods employ sensitive and time-consuming physicochemical procedures which are often prohibitively expensive as screening tools for most laboratories. Furthermore, it has been reported that to be certain of avoiding an underestimation of total FF content in milk, it is necessary to include an acid hydrolysis step within these physicochemical methods. Biosensor technology by contrast is cheap, sensitive and rapid (Thompson et al. 2011; McGrath et al.

2013; Eggeling et al. 2015; Gaudin 2017) and is the ideal tool for screening large numbers of samples for potentially non-compliant levels of FF in milk. Almost all immunoassay development with regard to amphenicol detection in milk has been concerned with the presence of CAP rather than FF, indeed a critical review of screening methods for the determination of amphenicols in milk lists 17 immunoassays, all of which are concerned with CAP only (Samsonova et al. 2012). A fluorescence-based lateral flow immunoassay has been reported (J. Wang et al. 2018) with limits of detection for TAP and FF in milk of 0.8 and 1.9 µg kg^{-1} being achieved while a competitive binding technique with a horseradish peroxidase-FF conjugate and employing molecularly imprinted polymer nanoparticles provided a limit of detection for FF in milk of 90 - 100 µg kg^{-1} (Caro et al. 2020). The developed immunoassay reported here compares favourably to these methods and utilising this biosensor technology has the added advantage of having no requirement for the acid hydrolysis of samples that is necessary for physicochemical methods due to the favourable cross-reactivity profile of the antiserum employed.

The discovery of non-compliant FF concentrations in kidney samples taken from dairy herds in Northern Ireland in 2017 prompted the current study to determine if there was an issue with the misuse of FF in dairy cattle producing milk for human consumption. A simple and cost effective method previously developed at this institute for the determination of amphenicol residues in bovine, ovine and porcine kidney (Thompson et al. 2017) was therefore modified and successfully applied to the analysis and determination of these compounds in bovine milk with limits of detection in the low µg kg^{-1} range being achieved. The method was fully validated according to Commission Decision 2002/657/EC (European Commission 2002) and was then applied to a study which was designed to determine the persistence of FF residues in milk over time following treatment of dairy cattle with a therapeutic dose of the drug. A further study was designed to determine if detectable levels of FF can be produced in the milk of untreated animals as a result of cross-contamination through being housed with treated animals. Validation data is

presented along with biosensor results obtained from both experimental studies as well as corresponding MRM^3 UPLC-MS/MS confirmatory results following acid hydrolysis of the same incurred samples.

Materials and methods

Instrumentation

An optical biosensor (BIACORE®Q) was obtained from GE Healthcare/Biacore (Uppsala, Sweden). Instrument operation and data handling was performed using BIACORE®Q Control Software (Version 3.0.1).

Reagents and chemicals

CM5 sensor chips and an amine coupling kit containing N-ethyl-N'-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS) and ethanolamine (1 M) were obtained from GE Healthcare (Little Chalfont, England). Reference standards for FF, FFA, TAP and CAP were supplied by Sigma-Aldrich Chemical Company Ltd. (Poole, Dorset, U.K.). Fenflor® was purchased from Veterinary Surgeons Supply Co. Ltd. (Lisburn, U.K.). Biosensor assay buffer contained HEPES pH 7.4 (0.01 M), sodium chloride (0.15 M), EDTA (3 mM) and Tween 20 (10%). The solution was degassed and filtered prior to use.

All other chemicals were HPLC grade and were supplied by BDH (Poole, Dorset, U.K.).

Biosensor assay development

Immobilisation of florfenicol amine

FFA was immobilised on the surface of a CM5 sensor chip as described in a previously published paper (Thompson et al. 2017). Briefly, the carboxymethyl dextran surface was activated by contact with a 1/1 mixture of 0.2 M EDC/0.05 M NHS (50 μL) for 30 min.

Reactants were removed and FFA (3 mg) dissolved in 1 mM borate buffer pH 8.5 (1 mL) was applied to the prepared surface (50 µL). This was allowed to react overnight at room temperature. Any unreacted sites were deactivated by incubating with 1 M ethanolamine pH 8.5 (50 µL) for 30 min. The chip surface was then washed with deionised water and dried with nitrogen gas. The immobilised sensor chip was stored refrigerated in the presence of a desiccant when not in use.

Immunogen and antibody production

A FFA-BTG (bovine thyroglobulin) immunogen was produced and anti-FFA polyclonal antiserum raised in a rabbit according to the method outlined in a previous publication (Fodey et al. 2013).

Antibody specificity and selectivity

The ability of the polyclonal antibody to cross-react with the compounds of interest and representative compounds from other antibiotic families was assessed by production of calibration curves and determination of the cross-reactivity using the formula:

$$\text{Cross-Reactivity} = \frac{IC_{50} \text{ of Florfenicol}}{IC_{50} \text{ Other Compounds}} \times 100$$

If significant cross-reactivity of a compound in buffer was observed then the cross-reactivity was evaluated in sample matrix when fortified milk was subjected to the developed extraction procedure as shown in Figure 2.

Sample preparation and extraction procedure for biosensor analysis

Initially, a direct assay was assessed whereby fortified milk samples were diluted with deionised water, mixed with antibody and subjected to biosensor analysis, however the resulting limit of detection achieved (52.7 µg kg^{-1}) was deemed to be insufficiently sensitive for the screening of a banned substance. An extraction procedure previously

developed by the authors for the detection of amphenicols in kidney was therefore modified for application to milk samples. Various extraction parameters were adjusted during method development to achieve optimum sensitivity including sample and buffer resuspension volumes and extract dilution factors. Antibody to extract mix ratios along with flow rates and injection volumes were also assessed during biosensor analysis to determine the lowest limits of detection possible, with IC50 values in the low µg kg^{-1} range being achieved for all three amphenicols as shown in Table 1.

Negative and sample milks were defatted by centrifuging at 4000 rpm for 15 min and weighed (2.5 g) into glass universal bottles. Calibrants (containing 0, 0.125, 0.25, 0.5, 1.0 and 2.5, 5.0 and 10.0 µg kg^{-1} FF) were prepared by adding working standards (50 µL) to the known negative aliquots to produce a calibration curve. All calibrants and samples were allowed to stand for 10 min at room temperature and were then treated identically. Acetonitrile (5 mL) was added to each universal, vortexed vigorously for 10 s and mixed on a roller mixer for 30 min. All universals were then centrifuged at 3500 rpm for 10 min and supernatants (6 mL) were carefully pipetted into test tubes ensuring that no milk residue at the bottom of the universals was transferred. Supernatants were evaporated to dryness using a TurboVap® LV sample concentrator at 60°C under a stream of nitrogen gas. The resulting extracts were reconstituted immediately in biosensor assay buffer (250 µL) by vortexing vigorously for 1 min and transferred to microcentrifuge tubes. Following microcentrifugation at 13,000 rpm for 10 min, all extracts were diluted 1:1 by adding 200 µL extract to 200 µL biosensor buffer and vortexing for 1 min prior to being transferred (160 µL), in duplicate, to the wells of a microtitre plate.

Experimental study 1: Persistence of FF residues in milk

Three British Friesian dairy cattle (518 - 645 kg bodyweight), belonging to this institute and known to be free from exposure to amphenicols, were treated with an injectable formulation of FF (Fenflor® 300 mg mL^{-1}) according to the manufacturer's instructions. A

control milk sample was collected from each animal during afternoon milking prior to treatment. Fenflor® was then administered by intramuscular injection over two sites at 20 mg kg^{-1} bodyweight. This treatment was repeated 48 hours later. Milk samples were collected during morning and/or afternoon milking each day following the initial treatment for a period of 71 days post initial treatment. These were stored frozen at -20°C until analysed.

Experimental study 2: Presence of FF in the milk of untreated animals

Twelve British Freisian cattle were divided into three groups, each containing three steers and one dairy cow. For each group, the three steers were treated with a therapeutic dose of a FF containing product (Fenflor® 300 mg mL^{-1}) according to the manufacturer's instructions and housed together in a pen measuring 12 m x 12 m. A female British Friesian producing milk and known to be free from FF residues was then introduced to the pen for seven days and remained untreated. A milk sample was taken immediately prior to her introduction to the pen then twice daily for a seven day period. These were stored frozen at -20°C until analysed. The study was repeated on two further occasions with new animals introduced each time.

Results and discussion

Antibody characterisation

The ability of the rabbit polyclonal antiserum to cross-react with the compounds of interest was assessed along with representative compounds from other antibiotic families. If significant cross-reactivity of a compound in buffer was observed then the cross-reactivity displayed following acetonitrile extraction was evaluated in the presence of milk (assay cross-reactivity). The antiserum displayed good sensitivity for FF, TAP and FFA (FFA in milk displaying the highest IC_{50} concentration at 1.71 µg kg^{-1}) however CAP displayed a

more limited cross-reactivity which was insufficient to meet the MRPL requirement for this compound as shown in Table 1.

The cross-reactivities were calculated relative to the FF calibration curve and while assay cross-reactivities were found to be lower for the other members of the amphenicol group, it can be seen from the IC_{50} values obtained following extraction from milk that detection of all compounds in the low µg kg^{-1} range was shown to be easily achievable. This was particularly important in the case of FFA as the marker residue for FF is described as the sum of FF and its metabolites measured as FFA.

Biosensor assay validation

The developed immunobiosensor assay was validated in accordance with Commission Decision 2002/657/EC.

The methods used for the calculation of index scores and subsequent cut-off values have been detailed in a previously published paper (Thompson et al. 2017). Briefly, twenty one known negative bovine milks were analysed in three batches of seven on three successive days both unfortified and fortified with FF at 0.25 µg kg^{-1} and the results used to determine the cut-off value as shown in Table 2. The calculated cut-off value was 76.8 for FF. Table 3 shows the results obtained when a further twenty one known negative bovine milks were then analysed in three batches of seven on three successive days both unfortified and fortified separately with FFA and TAP at 0.5 µg kg^{-1} and the results used to determine the cut-off values. The calculated cut-off values on this occasion were 52.3 for FFA and 118.5 for TAP and, being the lowest of the three calculated cut-off values, the cut-off for FFA was adopted to reduce the risk of missing a non-compliant sample.

One sample (FFA POS 3) gave an index value of less than 52.3 which is less than a 5% β-error at the level of interest and therefore meets the criteria for an acceptable false compliant rate for screening assays as determined by Commission Decision 2002/657/EC.

To give further confidence of avoiding any possibility of obtaining false compliant results, the cut-off value was reduced by 20 index units to 32.3 at this laboratories own risk, even though it may increase the number of false non-compliant screening results obtained. As statutory testing progresses, this figure will be regularly reviewed in conjunction with confirmatory analysis results and adjusted as further data becomes available. All fortified samples gave index values above the adopted cut-off level of 32.3 and in addition, all unfortified samples gave index values below 32.3 providing false compliant and non-compliant rates of 0% for all populations as shown in Figure 3.

Experimental study 1: Persistence of FF residues in milk

All milk samples were analysed by both the developed biosensor based procedure and by a UKAS accredited MRM^3 UPLC-MS/MS confirmatory method also developed within this institute. Although during normal statutory analysis of samples only negative (0 µg kg^{-1}) and positive (0.25 µg kg^{-1}) calibrants are employed to provide a "compliant/non-compliant" screening result based upon an index cut-off point, on this occasion, for the biosensor analysis of incurred samples, a full calibration curve was extracted in an attempt to provide a more definitive correlation between results obtained from the two technologies in terms of µg kg^{-1}. Initial analysis of the incurred samples showed that the concentrations obtained were outside the dynamic range of the calibration curve and further analyses at both 1/100 and 1/1000 dilutions of samples in negative extract was required as shown in Table 4.

This study has shown that there is an extended withdrawal period for FF in milk with detectable (and therefore non-compliant) levels being found more than 50 days after therapeutic treatment by both technologies in two of the treated animals while they were still at detectable levels up until day 41 in the other animal. Concentrations did not drop below the MRM^3 UPLC-MS/MS ccα of 0.20 µg kg^{-1} until day 49 for animal 1 and day 65 for both animals 2 and 3. The two methodologies showed excellent correlation with a

calculated correlation coefficient (r) of 0.99 for all three animals. Milk samples collected prior to treatment tested compliant using both methods.

Experimental study 2: Presence of FF in the milk of untreated animals

All milk samples were analysed using the developed biosensor screening method and the MRM^3 UPLC-MS/MS confirmatory procedure which has a ccα of 0.20 µg kg^{-1} for FFA (although the method can detect levels below this concentration). Milk samples from the lactating animals displayed low levels of FFA in all three studies 36 hours after being housed with the treated steers. These levels persisted throughout the study. While the majority of the samples clearly displayed trace levels of drug using both methods, most were below the ccα of the confirmatory method. A sample taken from cow 1 in the afternoon of day three however displayed a level of 0.28 µg kg^{-1} FFA while samples taken in the morning of day five from cow 2 and cow 3 displayed levels of 0.31 and 0.30 µg kg^{-1} FFA, respectively which are all non-compliant levels of drug as shown in Table 5.

Conclusions

A screening procedure employing biosensor technology has been developed through the modification of a method previously developed at this institute. It is capable of detecting amphenicol concentrations in the low µg kg^{-1} range in bovine milk. This is particularly important for the analysis of FF (identified as the sum of FF and its metabolites measured as FFA) as no MRL has been set and the use of this drug is therefore not permitted in animals from which milk will be produced for human consumption. The cross-reactivity data obtained has shown that the antiserum is suitable for the detection of at least three of the amphenicol parent drugs and metabolites.

The developed immunobiosensor assay has advantages over existing methodologies, most of which employ expensive and time-consuming physicochemical techniques. Many laboratories employ these inexpensive and reliable immunobiosensor

methods for the routine screening of large numbers of samples which would otherwise be prohibitively expensive if based solely upon procedures such as HPLC and LC-MS/MS. Furthermore, it has been demonstrated that a hydrolysis step must be included in physicochemical methods for the quantification of FF residues in milk to avoid the significant risk of reporting false compliant results through the underestimation of total FF residue content. The current study has provided a fully validated screening method allowing the complete extraction and analysis of at least 30 samples within 12 h and has demonstrated, through the analysis of incurred milk, that this immunoassay does not require an acid hydrolysis step, with detection of incurred residues in the low μg kg^{-1} range being easily achieved.

It would appear likely that the FF metabolites previously described (FO, FA, FFAG and MCF), or conjugates thereof, display significant cross-reactivity to the polyclonal antiserum employed in the screening test and occur in the extracts as a significant percentage of the total residue concentration present, thereby increasing the sensitivity of the screening assay and eliminating the need for acid hydrolysis of samples. The antiserum used in this study was raised to FFA by conjugating the drug via its amine group to a carrier protein and using the resulting complex as an immunogen in the host animal (Fodey et al. 2013). Therefore, antibodies would have been produced to bind the remainder of the structure not used in the conjugation reaction, as indicated by the high cross-reactivity obtained for TAP and FF. Consequently, it can be assumed that antibodies have been produced that predominantly bind the methylsulfonyl-benzene-alcohol part of the structure with little or no influence from the fluorine moiety, whose presentation to the immune system may have been sterically hindered by the carrier protein. This assumption is supported by the fact that the antiserum displays superior cross-reactivity to TAP than to FFA itself. This part of the structure is also common to the metabolites shown in Figure 1. Although lack of availability meant that it was not possible to assess the cross-reactivity of the metabolites themselves, it would be expected to be considerable, based on the high

degree of similarity between their structures and that of the antigen. Furthermore it is also possible that *in vivo* moieties, incapable of being hydrolysed to FFA and which cross-react with the antibody, are present in milk and are isolated by solvent extraction alone.

In Northern Ireland in 2017, kidney samples taken from four bovines as part of the routine sampling program, each originating from a different herd, were confirmed as containing non-compliant FF concentrations. Of the four non-compliant samples, three were taken from dairy cattle. Although not conclusive in itself, these results along with other anecdotal evidence gave rise to the suspicion that FF misuse may be an issue in milk producing cattle, hence the development of this screening method. Subsequently, routine milk analysis was introduced and bulk tank samples from nine different farms were found to contain FF residues ranging from 0.38 - 4.6 µg kg^{-1}. Following these findings, and with little published data available in literature, it was decided to carry out two experimental studies to determine the FF withdrawal period for therapeutically treated dairy cattle and secondly to determine if cross-contamination can occur between treated and non-treated animals when housed together. All samples were analysed using both the developed biosensor screening procedure and the MRM^3 UPLC-MS/MS confirmatory method also developed at this institute. Data obtained from both technologies has shown that there is an extended withdrawal period for FF in milk, with detectable (and therefore non-compliant by EU law) levels being found more than 50 days following treatment. Furthermore, it has shown that concentrations of FF residues greater than the ccα (0.20 µg kg^{-1}) of the confirmatory method can be present in the milk of untreated animals when in close contact with treated animals suggesting that it would be advisable for dairy farmers to keep treated animals housed separately from untreated milk producers to avoid any possibility of cross-contamination.

In summary, the results from the studies undertaken would suggest that the concentrations of FF residues detected in milk samples from dairy cattle, as part of the Northern Ireland residue testing programme, are much more likely to be related to misuse

of the drug rather than from any form of cross-contamination, although not exclusively so. The data produced strongly suggests that care should be taken by veterinary practitioners with regard to withdrawal time advice given when administering FF and by milk producers when housing treated cattle. It is clear from the experimental results obtained that, when applied to incurred samples, the developed method was shown to be capable of successfully screening potentially non-compliant samples from FF-treated animals while confirmatory analysis of the same samples by MRM^3 UPLC-MS/MS showed excellent correlation between the two technologies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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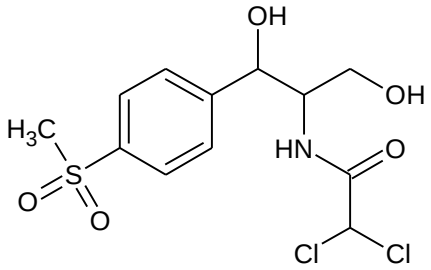
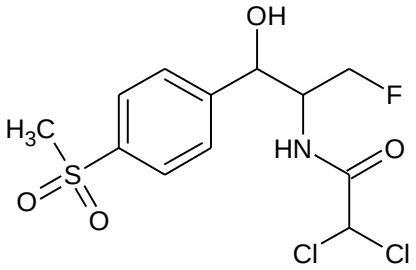
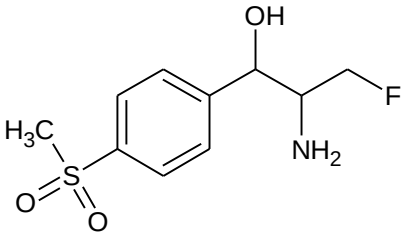
Figure 1: Structures of chloramphenicol, thiamphenicol, florfenicol and the major metabolites of florfenicol.



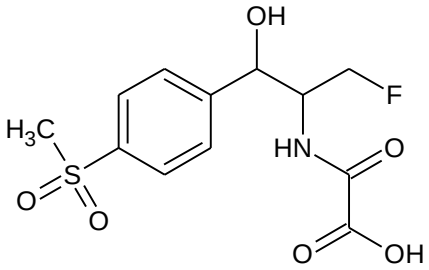
Thiamphenicol



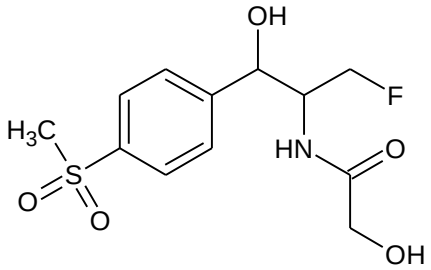
Florfenicol



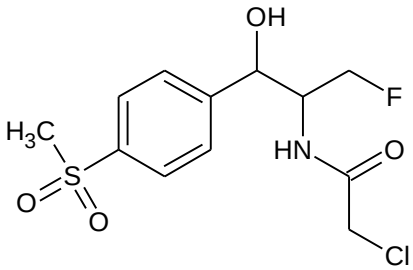
Florfenicol amine



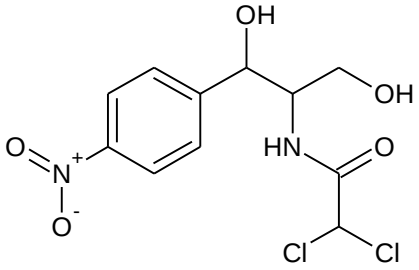
Florfenicol oxamic acid



Florfenicol alcohol



Monochloroflorfenicol



Chloramphenicol

Figure 2: Typical amphenicol calibration curves obtained following extraction from bovine milk.

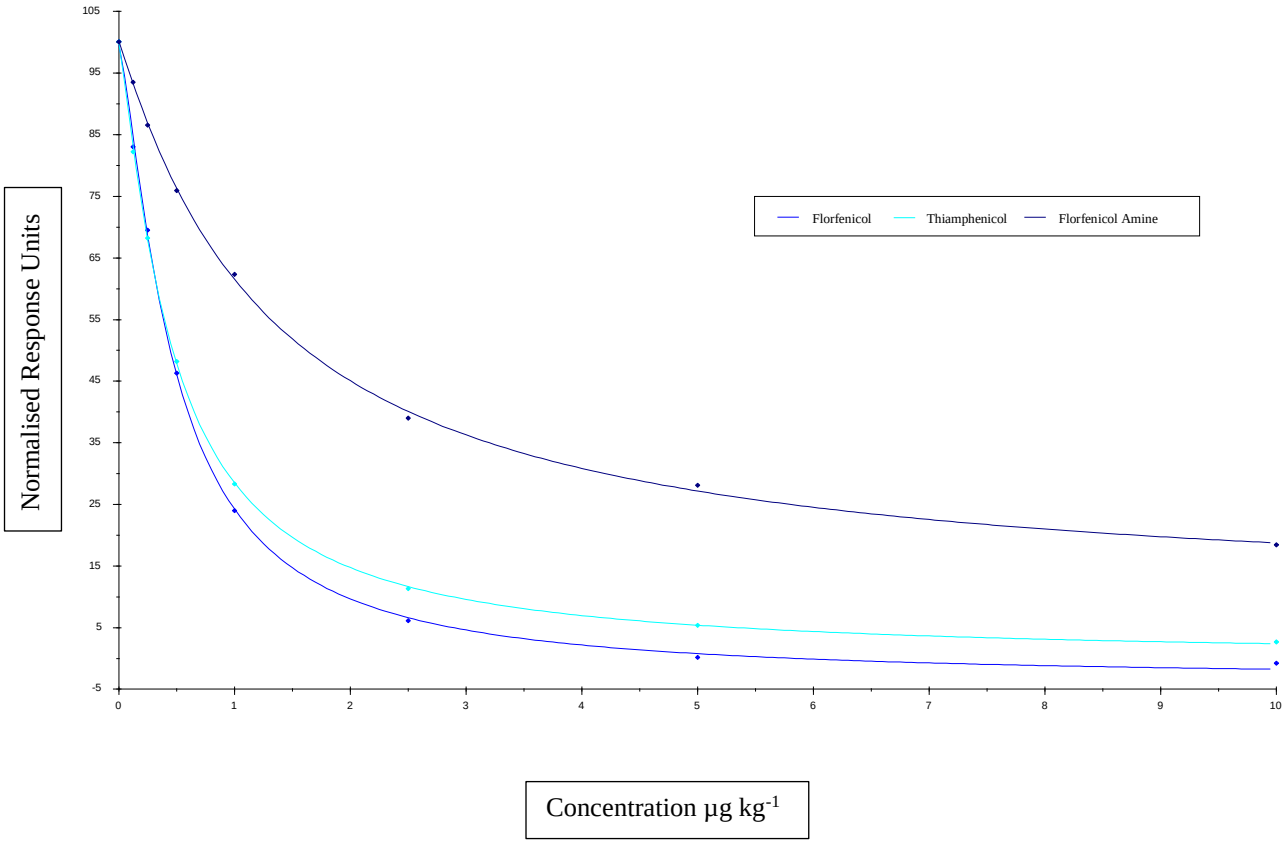
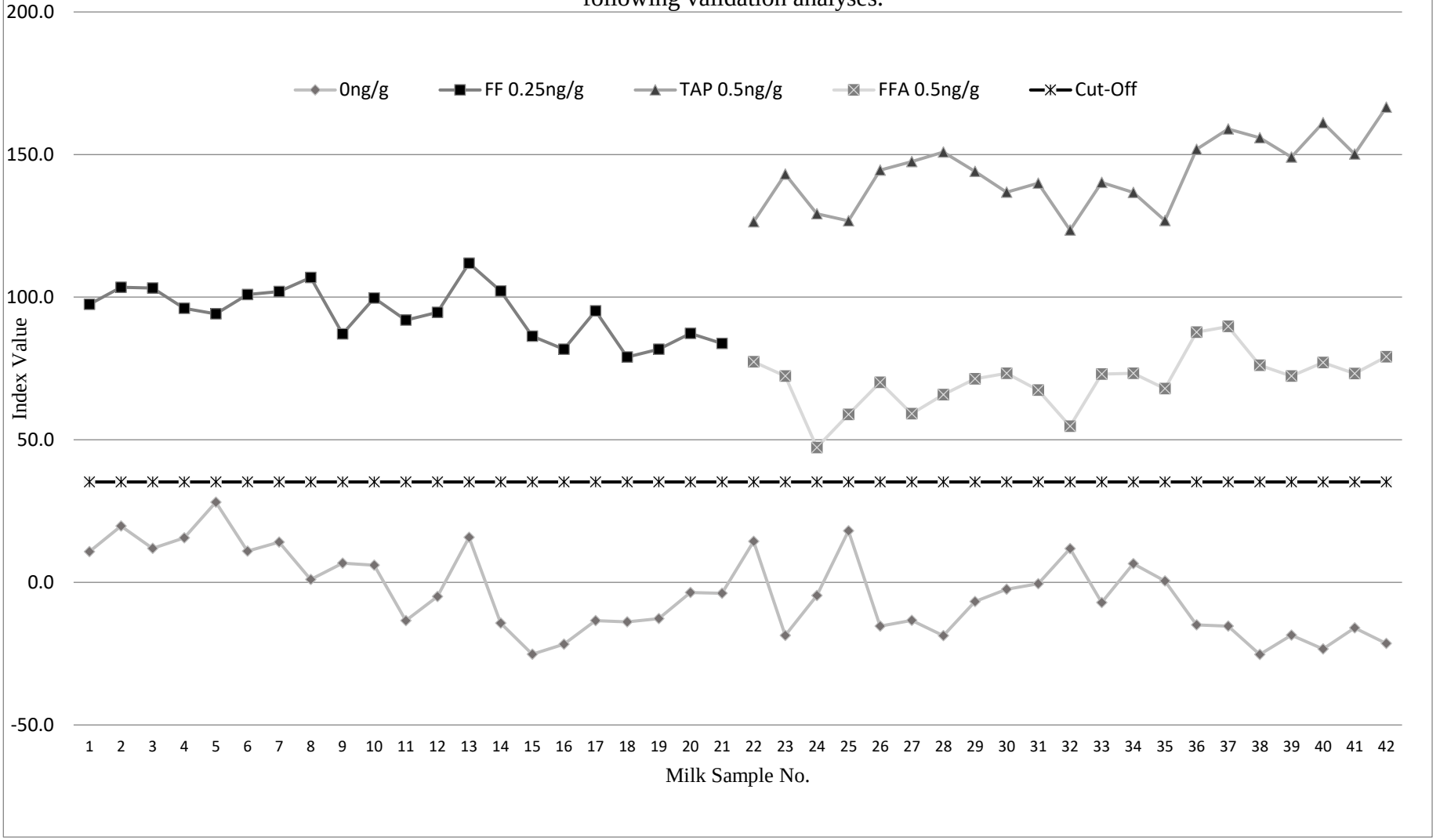


Figure 3: Index values obtained for unfortified and fortified bovine milk samples and the cut-off value adopted following validation analyses.



Compound	% Cross Reactivity in Buffer	IC_{50} Buffer (µg kg^{-1})	% Cross Reactivity in Milk	IC_{50} Milk (µg kg^{-1})
Florfenicol	100.0	0.39	100.0	0.44
Thiamphenicol	114.7	0.34	93.6	0.47
Florfenicol Amine	47.0	0.83	25.7	1.71
Chloramphenicol	15.7	2.49	6.5	5.20
Chlortetracycline	0	None	Not Assessed	Not Assessed
Erythromycin	0	None	Not Assessed	Not Assessed
Lincomycin	0	None	Not Assessed	Not Assessed
Kanamycin	0	None	Not Assessed	Not Assessed
Cephalexin	0	None	Not Assessed	Not Assessed
Sulphamethazine	0	None	Not Assessed	Not Assessed
Enrofloxacin	0	None	Not Assessed	Not Assessed
Ampicillin	0	None	Not Assessed	Not Assessed

Table 1: Rabbit polyclonal antiserum % cross-reactivities and IC_{50} concentrations in buffer and bovine milk.

Assay 1	Sample	RU Diff	Index Score	Sample	RU Diff	Index Score
	NEG 1	27.0	**10.8**	FF POS 1	243.9	**97.5**
	NEG 2	49.3	**19.7**	FF POS 2	259.0	**103.5**
	NEG 3	29.8	**11.9**	FF POS 3	258.3	**103.2**
	NEG 4	39.1	**15.6**	FF POS 4	240.5	**96.1**
	NEG 5	70.2	**28.1**	FF POS 5	235.4	**94.1**
	NEG 6	27.3	**10.9**	FF POS 6	252.4	**100.9**
	NEG 7	35.2	**14.1**	FF POS 7	255.3	**102.0**
Assay 2	**Sample**	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**
	NEG 8	2.2	**1.0**	FF POS 8	244.5	**106.9**
	NEG 9	15.4	**6.7**	FF POS 9	199.1	**87.1**
	NEG 10	13.7	**6.0**	FF POS 10	228.1	**99.7**
	NEG 11	-30.7	**-13.4**	FF POS 11	210.2	**91.9**
	NEG 12	-11.5	**-5.0**	FF POS 12	216.5	**94.7**
	NEG 13	36.1	**15.8**	FF POS 13	255.9	**111.9**
	NEG 14	-32.8	**-14.3**	FF POS 14	233.4	**102.1**
Assay 3	**Sample**	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**
	NEG 15	-46.7	**-25.2**	FF POS 15	159.6	**86.3**
	NEG 16	-40.2	**-21.7**	FF POS 16	151.1	**81.7**
	NEG 17	-24.7	**-13.4**	FF POS 17	176.2	**95.2**
	NEG 18	-25.7	**-13.9**	FF POS 18	146.1	**79.0**
	NEG 19	-23.5	**-12.7**	FF POS 19	151.1	**81.7**
	NEG 20	-6.6	**-3.6**	FF POS 20	161.5	**87.3**
	NEG 21	-7.0	**-3.8**	FF POS 21	155.1	**83.8**
Mean						**94.6**
SD						**9.1**
Mean-1.96*SD						**76.8**

Table 2: Index scores for unfortified bovine milk samples and for the same samples fortified at 0.25 µg kg^{-1} florfenicol.

Negatives (0µg kg^{-1})			Thiamphenicol (0.5µg kg^{-1})			Florfenicol Amine (0.5µg kg^{-1})		
Sample	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**
NEG 1	27.0	**14.4**	POS 1	236.9	**126.4**	POS 1	144.8	**77.3**
NEG 2	-34.8	**-18.6**	POS 2	268.2	**143.1**	POS 2	135.4	**72.3**
NEG 3	-8.7	**-4.6**	POS 3	242.2	**129.2**	POS 3	88.7	**47.3**
NEG 4	33.9	**18.1**	POS 4	237.6	**126.8**	POS 4	110.4	**58.9**
NEG 5	-28.9	**-15.4**	POS 5	270.8	**144.5**	POS 5	131.3	**70.1**
NEG 6	-24.9	**-13.3**	POS 6	276.5	**147.5**	POS 6	110.7	**59.1**
NEG 7	-35.0	**-18.7**	POS 7	282.7	**150.9**	POS 7	123.3	**65.8**
Sample	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**
NEG 8	-12.8	**-6.7**	POS 8	274.6	**144.1**	POS 8	136.1	**71.4**
NEG 9	-4.5	**-2.4**	POS 9	260.7	**136.8**	POS 9	139.8	**73.3**
NEG 10	-1.0	**-0.5**	POS 10	266.8	**140.0**	POS 10	128.5	**67.4**
NEG 11	22.5	**11.8**	POS 11	235.3	**123.5**	POS 11	104.2	**54.7**
NEG 12	-13.6	**-7.1**	POS 12	267.3	**140.2**	POS 12	139.2	**73.0**
NEG 13	12.3	**6.5**	POS 13	260.6	**136.7**	POS 13	139.8	**73.3**
NEG 14	1.0	**0.5**	POS 14	241.9	**126.9**	POS 14	129.5	**67.9**
Sample	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**	**Sample**	**RU Diff**	**Index Score**
NEG 15	-29.4	**-14.9**	POS 15	299.9	**151.8**	POS 15	173.2	**87.7**
NEG 16	-30.5	**-15.4**	POS 16	314.1	**159.0**	POS 16	177.2	**89.7**
NEG 17	-50.0	**-25.3**	POS 17	307.9	**155.9**	POS 17	150.3	**76.1**
NEG 18	-32.5	**-16.5**	POS 18	294.4	**149.1**	POS 18	142.8	**72.3**
NEG 19	-46.2	**-23.4**	POS 19	318.3	**161.2**	POS 19	152.3	**77.1**
NEG 20	-31.6	**-16.0**	POS 20	296.7	**150.2**	POS 20	144.6	**73.2**
NEG 21	-42.3	**-21.4**	POS 21	329.0	**166.6**	POS 21	156.3	**79.1**
Mean					**141.4**			**70.3**
SD					**11.7**			**9.2**
Mean-1.96*SD					**118.5**			**52.3**

Table 3: Index scores for unfortified bovine milk samples and for the same samples fortified at 0.5 µg kg^{-1} thiamphenicol and florfenicol amine.

Days Post Treatment Cow 1	Biosensor µg kg^{-1}	MRM^3 UPLC-MS µg kg^{-1} FFA	Days Post Treatment Cow 2	Biosensor µg kg^{-1}	MRM^3 UPLC-MS µg kg^{-1} FFA	Days Post Treatment Cow 3	Biosensor µg kg^{-1}	MRM^3 UPLC-MS µg kg^{-1} FFA
Control	0.00	0.00	**Control**	0.00	0.00	**Control**	0.00	0.00
1	837	752	**1**	1420	1451	**1**	1220	1057
2	535	359	**2**	513	645	**2**	784	575
3	806	771	**3**	1650	1467	**3**	1510	1143
4	604	440	**4**	620	570	**4**	751	547
5	369	268	**5**	627	498	**5**	369	422
6	244	161	**6**	375	339	**6**	191	299
7	110	93.8	**7**	277	270	**7**	158	225
9	50.5	84.0	**9**	241	183	**9**	183	152
11	33.3	47.4	**11**	211	120	**11**	78.8	81.2
13	14.6	21.5	**13**	68.1	67.5	**13**	51.5	27.6
15	13.0	19.8	**15**	30.6	42.1	**15**	30.5	23.0
17	7.1	14.8	**17**	10.3	21.9	**17**	12.3	16.0
19	3.5	8.6	**19**	24.6	15.8	**19**	16.2	12.1
21	2.5	5.5	**21**	7.8	13.6	**21**	9.6	7.9
23	2.1	5.1	**23**	6.9	10.4	**23**	4.8	5.5
25	1.9	4.3	**25**	6.1	9.3	**25**	2.5	3.3
33	1.0	1.1	**33**	1.6	3.4	**33**	1.2	1.4
41	0.42	0.31	**41**	1.1	1.7	**41**	0.54	0.90
49	0.18	<0.20	**49**	0.50	0.74	**49**	0.73	0.69
57	0.11	<0.20	**57**	0.25	0.34	**57**	0.34	0.48
65	0.02	<0.20	**65**	0.16	<0.20	**65**	0.21	<0.20
71	0.00	<0.20	**71**	0.08	<0.20	**71**	0.02	<0.20

Table 4: Comparison of biosensor screening and MRM^3 UPLC-MS/MS confirmatory results for florfenicol incurred bovine milk samples.

Day/Time Post Treatment	Cow 1 Biosensor µg kg^{-1}	Cow 1 MRM^3 UPLC-MS/MS µg kg^{-1}	Cow 2 Biosensor µg kg^{-1}	Cow 2 MRM^3 UPLC-MS/MS µg kg^{-1}	Cow 3 Biosensor µg kg^{-1}	Cow 3 MRM^3 UPLC-MS/MS µg kg^{-1}
1 am	0.00	ND	0.01	ND	0.00	ND
1 pm	0.00	ND	0.03	<0.20	0.00	ND
2 am	0.00	ND	0.06	<0.20	0.00	ND
2 pm	0.04	<0.20	0.03	<0.20	0.00	<0.20
3 am	0.10	<0.20	0.08	<0.20	0.06	<0.20
3 pm	0.18	**0.28**	0.24	<0.20	0.07	<0.20
4 am	0.16	<0.20	0.24	<0.20	0.10	<0.20
4 pm	0.18	<0.20	0.23	<0.20	0.11	<0.20
5 am	0.12	<0.20	0.33	**0.31**	0.40	**0.30**
5 pm	0.19	<0.20	0.22	<0.20	0.21	<0.20
6 am	0.07	<0.20	0.12	<0.20	0.20	<0.20
6 pm	0.04	<0.20	0.11	<0.20	0.06	<0.20
7 am	0.06	<0.20	0.15	<0.20	0.06	<0.20
7 pm	0.07	<0.20	0.11	<0.20	0.05	<0.20

Table 5: Comparison of biosensor screening and MRM^3 UPLC-MS/MS confirmatory results obtained during cross-contamination study.

ND = Not Detected

<0.20 = FFA trace detected but below ccα of 0.20 µg kg^{-1} while figures in bold text = FFA concentration >ccα