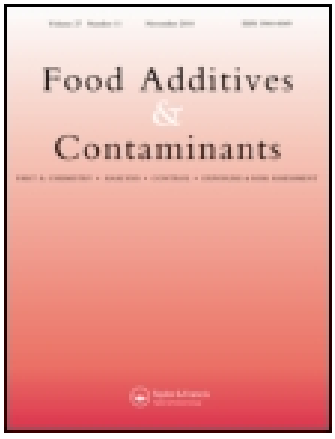

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I.M. Traynor[a], C.S. Thompson[a], L. Armstrong[a], T. Fodey[a], M. Danaher[b], K. Jordan[c], D.G. Kennedy[a] & S.R.H. Crooks[a]

[a] Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, UK

[b] Food Safety Department, Teagasc Food Research Centre, Dublin, Ireland

[c] Moorepark Food Research Centre, Teagasc, Moorepark, Fermoy, Co., Cork, Ireland

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I.M. Traynor[a]*, C.S. Thompson[a], L. Armstrong[a], T. Fodey[a], M. Danaher[b], K. Jordan[c], D.G. Kennedy[a] and S.R.H. Crooks[a]

[a]*Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, UK;* [b]*Food Safety Department, Teagasc Food Research Centre, Dublin, Ireland;* [c]*Moorepark Food Research Centre, Teagasc, Moorepark, Fermoy, Co., Cork, Ireland*

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Imidocarb (IMD) is a veterinary drug that has been used for more than 30 years to treat and prevent parasitic diseases. Pharmacokinetic studies have shown that substantial levels of IMD residues are retained in the edible tissues and milk of cattle and sheep for up to 6 months after administration. This has led to concern regarding the potential adverse effects posed through human consumption of edible tissue or milk from treated animals if the recommended withdrawal periods for the drug are not properly implemented. While MRLs have been established by the European Union, it is important that analytical methods are available to monitor food samples for potentially violative levels of IMD residues. A qualitative biosensor-based immunoassay was developed to allow the detection of IMD at less than the European Union MRLs of 50 μg kg^{-1} for milk and 2 mg kg^{-1} for bovine and ovine liver. Validation of the developed methods provided a detection capability of <25 μg kg^{-1} in milk and <0.75 mg kg^{-1} in liver. A comparison study was undertaken, with IMD incurred milk and ovine liver samples analysed by the newly developed procedures and results compared with those obtained by LC-MS/MS. The newly developed screening method was applied to both incurred milk and liver samples. This faster, cheaper and reliable screening method has potential use in sample analysis to ensure compliance with legislative requirements.

Keywords: imidocarb; liver; milk; residues; immunobiosensor

Introduction

Imidocarb (IMD), administered as either its dipropionate or its dihydrochloride salt, is an antiparasitic veterinary drug with reported therapeutic and prophylactic efficacy (Sevinc et al. 2007) in the treatment of babesiosis and anaplasmosis. The global economic impact of these diseases has been reported to be substantial (Lew & Jorgensen 2005), and so IMD has been used to treat cattle (Haigh & Hagan 1974), sheep (Hashemifesharki 1977) and domestic animals (Price & Dolan 1980) against the diseases for more than 30 years. IMD is licensed in the United Kingdom by the Veterinary Medicines Directorate (2011) and is issued with a withdrawal period of 213 days as a result of the prolonged persistence of the drug in edible tissues (Coldham et al. 1994, 1995). The cause of this persistence has been attributed to the drug's resistance to biotransformation processes, while Moore et al. (1996) indicated that slow elimination from edible tissues *in vivo* was a result of the drug's mechanism at the cellular level. Given this limited metabolism, IMD was identified as the target marker residue for all matrices (Commission Regulation 2001, 2003, 2009). It is important that veterinary drug withdrawal periods are observed, and in Northern Ireland, farmers are required to inform the Department of Agriculture & Rural Development if they have used IMD. This, in turn, leads to an operational requirement for the Department of Agriculture & Rural Development to monitor for the presence of IMD residues in treated herds. MRLs, also known as regulatory limits for the purpose of validation of analytical methods, have been established in milk and a range of edible tissues, and these have been adopted by the EC, on the recommendation of the European Medicines Agency, and by the Codex Alimentarius Commission. The European Union (EU) MRL in bovine and ovine liver is 2 mg kg^{-1}, while the Codex MRL in bovine liver is 1.5 mg kg^{-1}. The MRL in bovine milk (50 μg kg^{-1}) is the same under both authorities, but the EU also prohibits the use of the drug in sheep from which milk is produced for human consumption (Commission Regulation 2003). Codex has not set any MRLs for sheep.

The legislative requirement to analyse bovine and ovine edible tissues and bovine milk for IMD has led to the development of a number of analytical methods to detect the drug in a range of matrices. Crescenzo et al. (2002) assessed a range of different extraction procedures for tissue and milk samples coupled with HPLC technology, while LC-MS/MS was used for the analysis of eggs, honey, meat, milk and seafood (Ishii et al. 2011) with the same technique used to detect the drug in bovine tissues and milk (Inoue et al. 2011). These assays use extensive extraction and clean-up procedures, commonly required for detection by physicochemical methods, which can be, invariably, expensive and time-consuming. Recently, a

*Corresponding author. Email: imelda.traynor@afbini.gov.uk

multi-residue method using UHPLC-MS/MS technology to detect a large range of veterinary drugs (Moloney et al. 2012), including IMD, was developed. This used a simple solvent extraction combined with a concentration step. A similar extraction procedure was used in this study, and to the best of our knowledge, this is the first report of an immunochemical biosensor assay that has been developed to detect IMD in bovine and ovine liver and in bovine milk.

Materials and methods

Instrumentation

An optical biosensor (BIACORE®Q Control Software) was obtained from GE Healthcare (Little Chalfont, UK). Instrument operation and data handling were performed by 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, *N*-hydroxy-succinimide and 1 M ethanolamine (pH 8.5) were obtained from GE Healthcare (Little Chalfont). The reference standard for IMD dipropionate, the IMD mimic (3-[4,5-dihydro-1*H*-imidazol-2-yl] aniline dihydrochloride hydrate), ethylenediamine, human serum albumin (HSA), sodium nitrite and thiophosgene were supplied by Sigma-Aldrich Chemical Company Ltd. (Poole, Dorset, UK). Carboxymethylated dextran sodium salt (D500) was obtained from Ssens BV (Hengelo, The Netherlands), and horseradish peroxidase was purchased rom Roche-Diagnostics Ltd. (Burgess Hill, West Sussex, UK). A biosensor buffer containing HEPES (pH 7.4; 0.01 M), sodium chloride (0.15 M), Ethylenediaminetetraacetic acid (EDTA) (3 mM) and Tween 20 (0.005%) was prepared in-house. A non-specific binding buffer was prepared according to a previously reported method (Situ et al. 2008). Briefly, HEPES (0.238 g), sodium chloride (2.922 g), EDTA (0.127 g), carboxymethylated dextran (0.005 g) and 10% Tween 20 (50 µL) were dissolved in deionised water. The pH was adjusted to 9.0 by using sodium hydroxide (5 M), and the solution was made up to a final volume of 100 mL by using deionised water. All other chemicals were HPLC grade and were supplied by Sigma.

IMD mimic immunogen and corresponding enzyme label preparation

The IMD mimic was reacted with sodium nitrite to create a diazotised species that was coupled with the HSA carrier protein to prepare an immunogenic complex of the hapten. Using the same method, the hapten was also coupled with horseradish peroxidase to prepare a label for use in an enzyme-linked immunosorbent assay.

Antibody production

The prepared immunogen (0.5 mg of HSA in 1 mL of saline) was mixed with 1 mL of Montanide ISA 50V adjuvant to create an emulsion. Two rabbits (New Zealand White) were immunised every 4 weeks with the immunogenic emulsion at four injection sites. Blood samples were collected 10 days after each immunisation and assessed, by a direct competitive enzyme-linked immunosorbent assay, for the presence of IMD-specific antibodies. When a satisfactory titre was obtained, the antiserum was harvested from the rabbits. All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 of the Department of Health, Social Services and Public Safety, UK.

Immobilisation of IMD mimic (diazotised) to biosensor chip surface

The carboxymethyl dextran surface was activated with a 1/1 v/v mixture of 0.2 M *N*-ethyl-*N*′-(3-dimethylamino-propyl) carbodiimide hydrochloride/0.05 M *N*-hydroxy-succinimide for 20 min, and the activated surface was incubated with 1 M ethylenediamine solution for 1 h. Any unreacted sites were deactivated by incubating with 1 M ethanolamine (pH 8.5) for 20 min. The IMD mimic was reacted with sodium nitrite to create a diazotised derivative, and the diazotised IMD mimic was allowed to react with the prepared biosensor chip amine surface overnight at room temperature. The immobilised chip surface was washed with deionised water, dried with nitrogen gas and stored in a refrigerator when not in use.

Preparation of the IMD mimic – the isothiocyanate derivative

The IMD mimic 3-(4,5-dihydro-1*H*-imidazol-2-yl) aniline dihydrochloride hydrate (12 mg) was dissolved in 0.1 M sodium hydrogen carbonate solution (1 mL). Thiophosgene (6 µL) and chloroform (2.5 mL) were added, and the solution was mixed for 1 h. The aqueous layer was removed and washed with chloroform (1 mL). Nitrogen gas was passed through the aqueous layer to remove all traces of chloroform prior to immobilisation.

Immobilisation of the IMD mimic – the isothiocyanate derivative to the biosensor chip surface

The IMD mimic – isothiocyanate (ITC) derivative was coupled with an amine surface on a CM5 sensor chip as previously detailed for the diazotised IMD mimic. The immobilised chip surface was washed with deionised water, dried with nitrogen gas and stored in a refrigerator when not in use.

Sample preparation and extraction procedure (bovine and ovine liver)

Negative and sample livers were weighed (5 g) into glass universal bottles. Calibrants (0, 62.5, 250, 500 and 750 μg kg^{-1} IMD dipropionate) were prepared by adding working standards (50 μL) to aliquots of known negative liver. Acetonitrile (10 mL) was added to all calibrant and sample livers. Each universal was vortexed vigorously for 10 s and mixed on a roller mixer for 30 min before centrifugation at 2295*g* for 10 min. The supernatants were decanted into test tubes and evaporated to dryness by using a Turbo-Vap® LV (Hopkinton, MA, USA) sample concentrator at 50° C under a stream of nitrogen gas and resuspended immediately in biosensor buffer (500 μL) by vortexing vigorously for 1 min. The resulting extracts were microcentrifuged at 11,330 *g* for 10 min and diluted 1:4 with biosensor buffer prior to being transferred (100 μL), in duplicate, to the wells of a microtitre plate for biosensor analysis.

Sample preparation and extraction procedure (bovine milk)

Negative and sample milks were defatted by centrifugation at 2680*g* for 15 min, and the defatted milk (2.5 g) was transferred into glass universal bottles. Calibrants (0, 1, 5, 10 and 25 μg kg^{-1} IMD dipropionate) were prepared by adding working standards (50 μL) to the known negative milk aliquots. To all calibrant and sample milks, 1 M sodium hydroxide (150 μL), acetonitrile (10 mL) and sodium chloride (1.5 g) were added. Each universal was vortexed vigorously for 10 s and mixed on a roller mixer for 30 min before centrifugation at 2050 *g* for 10 min. The supernatants were decanted into test tubes, further centrifuged at 670 *g* for 10 min and transferred (3.5 mL) to clean test tubes. The supernatants were evaporated to dryness using a Turbo-Vap® LV sample concentrator at 60°C under a stream of nitrogen gas. The resulting extracts were reconstituted immediately in the non-specific binding buffer (500 μL) by vortexing vigorously for 1 min. Extracts for analysis (100 μL) were transferred, in duplicate, to the wells of a microtitre plate.

Biosensor assay

Biosensor parameters were evaluated and selected to allow each assay to display optimum performance at half the appropriate regulatory limits. Regeneration solutions were assessed to ensure that the best performance and stability of the chip surface was achieved.

Assay validation

The performance of the developed immunobiosensor assays was assessed in accordance with Commission Decision 2002/657/EC and Community Reference Laboratories Residues (2010) Guidelines for screening methods. IMD free samples (n = 21) of each of the matrix types (bovine and ovine liver and bovine milk) were analysed with and without fortification with IMD in batches of seven samples each on three separate days. Fortification concentrations were half the regulatory limits specific to IMD for each matrix/species combination.

Repeatability of the assays was assessed by determining the inter-assay (n = 3) and intra-assay (n = 10) percentage coefficients of variation (%) following fortification of each of the matrix types with IMD. Within-laboratory reproducibility of the liver assay was determined by calculating the % CV of the positive control (750 μg kg^{-1}) analysed over a period of 12 months from assays (n = 64) performed by various analysts using different batches of matrix, reagents, biosensor chip surfaces and equipment.

Qualitative assay assessment

The assays developed are for use for qualitative screening of samples. A pre-determined cut-off point (F_m) for each assay was calculated. This cut-off point will indicate when a sample contains a substance at or above the screening target concentration (half the regulatory limits) and would be categorised as a "screen positive", subsequently triggering a physicochemical confirmatory test. The cut-off point for each assay was calculated by using the following equation:

$$F_m = M - 1.64\ \mathrm{SD}$$

where M is the mean response and SD is the standard deviation of the 21 fortified validation samples for each matrix type.

To facilitate the comparison of responses obtained from fortified and unfortified samples between days, the response values were converted to index score values by normalising the response data. The use of index scores takes into account both the day-to-day variability in response (in the same way that calculation of B/B_o does) and the "response range" of the test (being the difference in response between the negative and positive standards). The index score is calculated by using the following formula:

$$\frac{\text{Response negative calibrant} - \text{Response sample} \times 100}{\text{Response negative calibrant} - \text{Response positive calibrant}}$$

This formula will give an index score of zero for the negative calibrant and an index score of 100 for the positive calibrant.

Incurred material

Eighteen incurred milk samples, with different concentrations of IMD, were analysed qualitatively by the newly

developed milk assay. Incurred liver samples were prepared by treating two sheep with a single intramuscular injection each of imidocarb diproprionate. One animal received 0.5 mg kg^{-1} body weight, and the other received 0.25 mg kg^{-1} body weight. Both animals were slaughtered 48 h after treatment.

Results and discussion

Antibody production

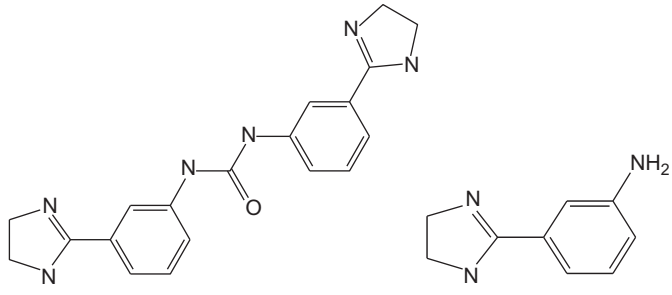
Low-molecular-weight molecules such as IMD would not elicit an immune response in a host animal. To produce an antibody to IMD, it must be chemically conjugated to a large carrier protein. Because the structure of IMD does not lend itself to chemical manipulation, a hapten mimic, 3-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (Figure 1), was selected to produce antibodies that would bind the target analyte. The mimic was successfully conjugated to HSA and antibodies produced.

Antibody and biosensor assay characterisation

Both rabbits (R960 and R965) immunised with the IMD mimic-HSA immunogen produced a polyclonal antibody specific to IMD. These displayed limited cross-reactivity against the IMD mimic (R960, 38% and R965, 3%) and no cross-reactivity against a range of antiprotozoal drugs (metronidazole, dimetridazole, ronidazole, ipronidazole, furazolidone, and trimethoprim) when assessed in buffer. The chip surface immobilised with the diazotised mimic was used initially in this study, and IC_{50} values of 21.5 and 25.6 ng mL^{-1} were obtained in buffer by using R960 and R965 antibodies, respectively. After several hundred assay cycles, the chip surface displayed a rapid decline in assay response values and, because of its unstable nature, was removed from further use in this study. The chip surface produced from the IMD mimic – ITC derivative was assessed with the two antibodies and displayed much higher assay responses and resulted in considerably lower IC_{50} values (5.1 and 10.0 ng mL^{-1} for R960 and R965 antibodies, respectively) than did the previous chip surface. It displayed no loss in surface binding after >1000 assay cycles and was determined as stable and suitable for the development of assays.

Biosensor assay parameters for the analysis of milk and tissue extracts were optimised by using both antibodies. Extracts were mixed with antibody (50/50 v/v) and injected (20 µL) over the sensor chip surface at a flow rate of 20 µL min^{-1}. Report points were recorded before and after each injection to measure the response generated by antibody binding to the IMD mimic immobilised on the sensor chip surface.

As was observed in buffer, R960 displayed more sensitivity than R965 when applied to the analysis of matrix extracts, but, with further evaluation, R960 was deemed unsuitable for the analysis of milk because it showed high levels of variation (38% for R960 compared with 18% for R965) in response values between individual samples (n = 5) when analysed as blanks. These same five samples analysed with the inclusion of non-specific binding buffer in the extraction procedure further reduced the variation to 3%. This high variation in response values was not observed when R960 was used for the analysis of liver, and therefore, it was selected for use with both bovine and ovine liver extracts. It is likely that the difference observed between the two antibodies when applied to the analysis of milk extracts is due to R965 displaying a higher affinity than R960 for the target analyte (IMD) with a subsequent reduction in its susceptibility to matrix interferences within the extracts. Full calibration curves were created for each matrix, and IC_{50} values for ovine liver, bovine liver and milk were calculated as 250, 160 and 3.7 µg kg^{-1}, respectively. To achieve good repeatability of an immunobiosensor assay, it is essential that the chip surface be regenerated effectively between sample injections. To ensure complete regeneration, it was necessary to use two different solutions for the IMD biosensor assays. The chip surface was



Imidocarb 3-(4,5-dihydro-1*H*-imidazol-2-yl) aniline

Figure 1. Molecular structures of IMD and the hapten mimic.

regenerated at a flow rate of 20 μL min^{-1} with a 1-min injection of 100 mM sodium hydroxide for both liver assays and 20% acetonitrile in 250 mM sodium hydroxide for the milk assay.

Liver assay validation

To assess the detection capability of the method, 21 known negative liver samples from each species were extracted by using the method detailed and evaluated separately as blanks, and after fortification with IMD (750 μg kg^{-1}). The detection capability (CCβ) of the liver assay was determined to be <750 μg kg^{-1} for both species (Figure 2a and 2b) as there was no overlap in the concentration values of the fortified and unfortified (blank) sample populations analysed. The cut-off level (F_m) for referring samples for confirmatory analysis was calculated as having an index value of 83.5%. The repeatability of the bovine liver assay was assessed as previously detailed; intra and inter-assay variations were calculated as 10.9% and 18.2% and as 12.1% and 7.6% at concentrations of 200 and 400 μg kg^{-1}, respectively. Similarly, the ovine liver assay repeatability was determined with the intra and inter-assay variations calculated as 18.3% and 11.9% and as 14.6% and 12.4% at concentrations of 200 and 400 μg kg^{-1}, respectively. The within-laboratory reproducibility of the bovine liver assay (n = 64) was ascertained by calculating the % CV of the positive control index values measured and analysed over a 12-month period and was determined as 16.2%.

Milk assay validation

The inclusion of basic conditions for the milk matrix extraction was found to improve the recovery of IMD as had been investigated and determined previously (Crescenzo et al. 2002; Lai et al. 2002). The detection capability of the milk assay was determined as <25 μg kg^{-1} (Figure 2c) and was performed in the same manner as was applied to the liver assays with 21 known negative bovine milk samples extracted by using the method detailed and analysed as blanks, and after fortification with IMD (25 μg kg^{-1}). The cut-off level (F_m) for referring samples for confirmatory analysis was calculated as having an index value of 95.4%. The repeatability of the assay was determined by calculating the intra-assay % CV as 4.5% and 9.9% at concentrations of 2.5 and 25 μg kg^{-1}, respectively, and the inter-assay % CV as 9.6% and 12.3% at the same IMD concentrations, respectively.

Incurred material analysis

Eighteen incurred milk samples of known IMD concentration, previously analysed by LC-MS/MS (unpublished data), were qualitatively evaluated by using the biosensor procedure to assess the ability of the assay to detect samples above the pre-determined F_m value of 95.4%. Ten of these samples (Figure 3) screened above the F_m value while eight screened below. Of the "screened negatives", seven out of eight were confirmed by LC-MS/MS to have concentrations below the IMD MRL (ranging from 3 to 25 μg kg^{-1}) while a single sample was confirmed as containing IMD at 54 μg kg^{-1} and was therefore deemed a false-negative screening result. This same sample was further evaluated quantitatively by the biosensor assay on three separate occasions and gave concentrations of 12.1, 12.2 and 12.9 μg kg^{-1}. Out of the 10 "screened positives", LC-MS/MS analysis identified 8 as containing IMD concentrations in excess of the IMD MRL (ranging from 113 to 966 μg kg^{-1}) and 2 as containing concentrations of 44 and 46 μg kg^{-1}, respectively.

The ovine liver samples from the two treated animals were analysed by using LC-MS/MS (unpublished data) and by the newly developed biosensor assay. These were evaluated against an extended calibration curve (0–3 mg kg^{-1} IMD dipropionate), and IMD liver concentrations were determined as 2.39 and 0.63 mg kg^{-1}, corresponding to the 0.5 and 0.25 mg kg^{-1} body weight dosages, respectively. The corresponding LC-MS/MS results obtained for the two treated animals were 1.27 and 0.75 mg kg^{-1}, respectively. The normal therapeutic dose given to sheep is 1.2 mg kg^{-1} body weight; however, in this study, animals received lower dosages in an attempt to produce incurred ovine tissue samples that would contain concentrations close to the MRL adopted by the EU. The lower dosage produced liver containing 0.63 mg kg^{-1}, which falls below the CCβ of the developed assay, whereas the higher dosage yielded liver containing 2.39 mg kg^{-1}, which is close to the EU MRL and above the CCβ of the assay. These tissues would therefore be suitable for use within laboratories as reference material.

Conclusions

Sensitive, specific and reproducible biosensor assays were developed for the detection of IMD in bovine and ovine liver and in bovine milk. They may be used for screening purposes in conformity with Directive 96/23/EC as they demonstrated a false-compliant rate of <5% at half their relevant regulatory limits, in conjunction with the confirmatory quantification procedure. The developed biosensor assays provide significant savings in terms of time and money, with the extraction and detection of 20 liver or 50 milk samples requiring 25% less time to complete than LC-MS/MS analysis. As staff time equates to 60% of the total analysis cost for both procedures, the financial savings to the regulatory laboratory using this type of screening assay are significant.

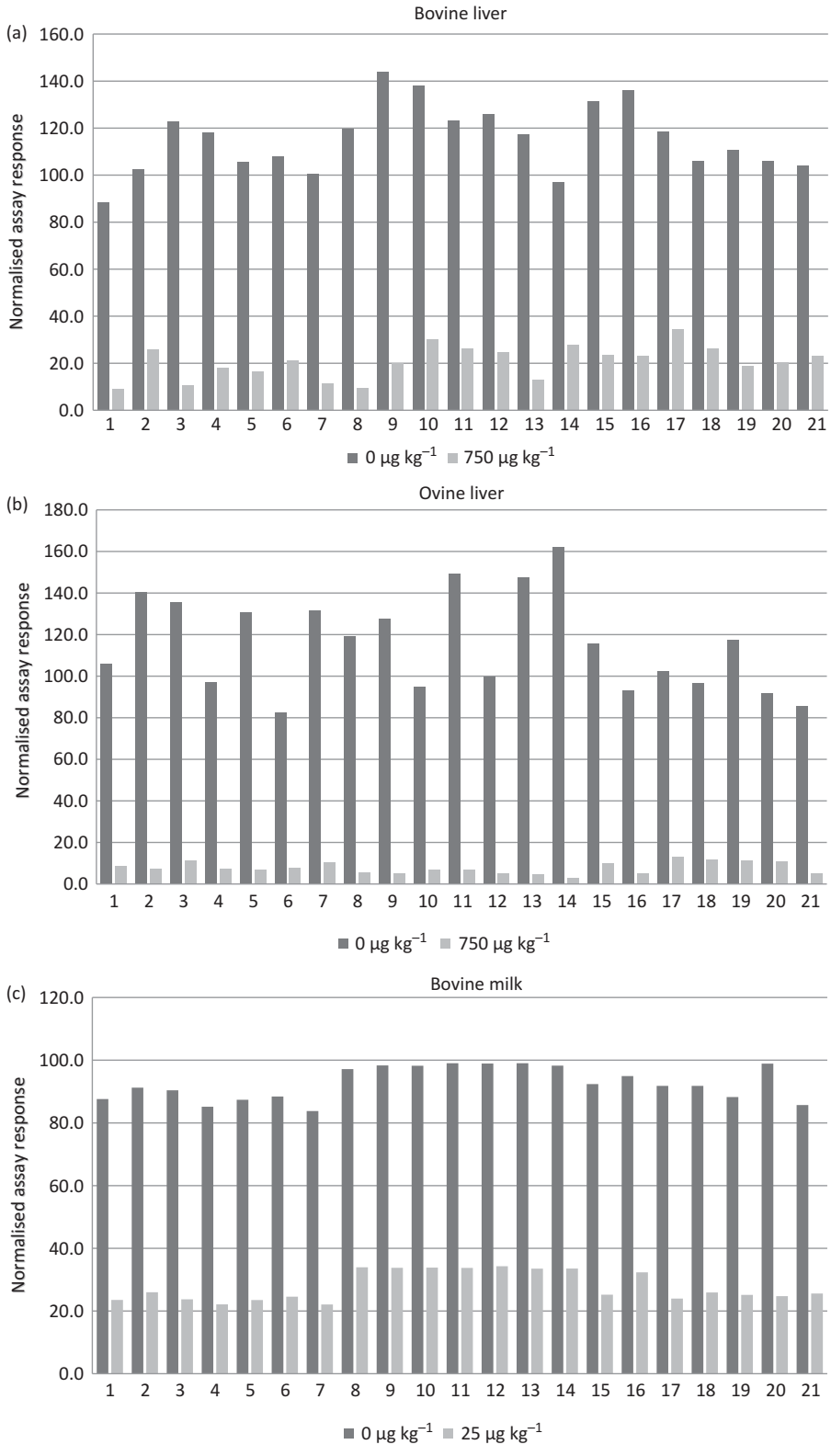


Figure 2. (a) Validation data for 21 bovine liver samples analysed as blank and as fortified with IMD (750 µg kg^{-1}). (b) Validation data for 21 ovine liver samples analysed as blank and as fortified with IMD (750 µg kg^{-1}). (c) Validation data for 21 bovine milk samples analysed as blank and as fortified with IMD (25 µg kg^{-1}).

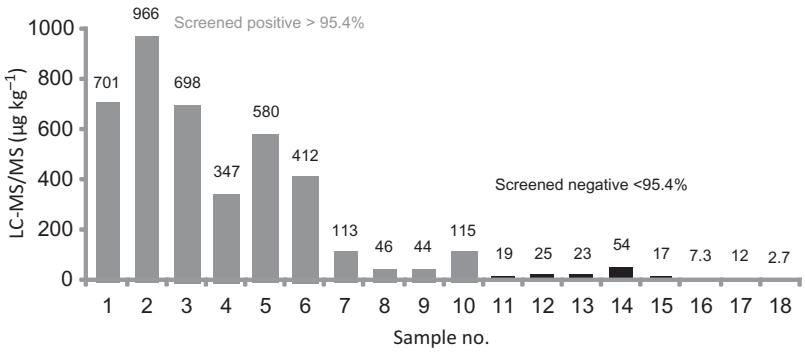


Figure 3. Evaluation of 18 incurred milk samples by LC-MS/MS and immunobiosensor. Samples 1–10 screened positive (>95.4%), and samples 11–18 screened negative (<95.4%). Numbers above bars represent the concentration (µg kg^{-1}) obtained for each sample analysed by using LC-MS/MS.

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