# BIOCHIP BASED IMMUNOASSAY AND ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE SCREENING OF MULTIPLE AVERMECTINS IN BEEF MUSCLE AND LIVER

N. O'Loan, L. Farry, B. Bell, J. Mahoney, J. Porter, M. McGarrity, M. Grecco, M.L. Rodríguez*, R.I. McConnell, S.P. FitzGerald

Randox Food Diagnostics, Crumlin, United Kingdom

*scientific.publications@randox.com

**Abstract – Avermectins are macrocyclic lactone derivatives with potent anthelmintic activity. For consumer protection it is necessary to monitor the levels of these residues in food. The availability of immunoanalytical methods enabling broad recognition of avermectins to facilitate the screening of batches of samples is advantageous. This study reports the analytical performance of a biochip based immunoassay and an enzyme-linked immunosorbent assay (ELISA) for the screening of multiple avermectins in beef muscle or liver. Competitive immunoassays were employed. Biochip array technology is based on the biochip, the solid phase and the vessel where miniaturised chemiluminescent immunoassays take place. The chemiluminescent signals were detected on the Evidence Investigator analyser. For the ELISA the capture antibodies were immobilized and stabilized on the 96-well microtitre plate surface. Absorbances were read at 450nm. On the biochip platform the assay detected emamectin benzoate, eprinomectin, abamectin, ivermectin and doramectin (% cross-reactivity ranging from 75 to 254%); with the ELISA the same compounds were detected (%cross-reactivity ranging from 40 to 174%). The limit of detection in beef muscle was 0.75ppb on the biochip and 5 ppb in beef liver with the ELISA. These immunoassays represent useful analytical tools for the screening of these compounds.**

## I. INTRODUCTION

The avermectins are a group of chemically related compounds originally isolated from the actinomycete *Streptomyces avermitilis*. They are macrocyclic lactone derivatives with potent anthelmintic activity, but lack antibacterial or antifungal activity (1). The avermectins include the compounds ivermectin, abamectin, doramectin, emamectin, and eprinomectin. The extensive use of anthelmintic drugs in food-producing animals can cause the presence of residues in food. For consumer protection it is necessary to monitor the levels of anthelmintic residues to ensure that they remain within the legally permitted maximum acceptable concentrations. Analytical methods have been developed for the detection of avermectins in different matrices using a range of technologies including high performance liquid chromatography, liquid chromatography-mass spectrometry, liquid chromatography-tandem mass spectrometry, ELISAs (2,3,4,5). Screening methods facilitate the detection of residues in samples at border inspection points, slaughter and import houses as only positive results need to be confirmed with a confirmatory method. The availability of rapid and simple immunoanalytical methods enabling broad recognition of avermectins to facilitate the screening of batches of samples is advantageous and this study reports the analytical performance of a biochip based immunoassay and an ELISA for the screening of multiple avermectins in beef muscle and liver.

## II. MATERIALS AND METHODS

*Sample preparation*

*Biochip based immunoassay*. Acetonitrile was added to 5 grams of pre-homogenised beef muscle and mixed before addition of sodium chloride and anhydrous magnesium sulphate. The sample was then mixed and centrifuged. After centrifugation, half the volume of the upper solvent layer was transferred to a clean glass test tube and evaporated at +50°C. The sample was then reconstituted with diluted wash buffer and mixed. The reconstituted samples were then diluted to provide an overall sample dilution of 1. The sample volume added to each biochip was 50µl.

*ELISA*. Acetonitrile was added to 5 grams of pre-homogenised beef liver and mixed before addition of sodium chloride and anhydrous

magnesium sulphate. Following shaking, hexane was added. The sample was then mixed and centrifuged. After centrifugation, 2ml of the lower acetonitrile layer was transferred to a clean glass test tube, dimethyl sulfoxide (DMSO) was added then dried down at +50$^0$C. The sample was then reconstituted with sample buffer and mixed. The reconstituted samples were then diluted 5-fold. The sample volume added to each well was 25µl.

*Biochip based immunoassay*

The biochip based immunoassay for the determination of avermectins is competitive. The avermectins present in the standard and/or sample compete with horseradishperoxidase labelled conjugate for specific sites in the antibodies on the biochip surface. The biochips (9mm x 9mm) were supplied in carriers (3x3 biochips per carrier); a carrier handling tray is provided with the system and allows the simultaneous handling of 6 carriers (54 biochips). 150µl of assay diluent was applied to the biochips, followed by 50µl of calibrator or sample. After incubation for 30 minutes at +25°C and 370 rpm, 100µl of conjugate was added into each biochip. After incubation for 60 minutes at +25°C and 370rpm, the biochips were washed. Signal reagent was then added and the chemiluminescent signal output generated on the biochips was then captured using digital imaging technology on the Evidence investigator analyser. The system incorporates dedicated software, which automatically processes, reports and archives data generated.

*ELISA*

The avermectins ELISA presented the 96-well microtitre plate precoated with the capture antibody. 25 µl assay buffer/sample buffer was applied to each well, followed by 25 µl of calibrator/sample and 75 µl of conjugate. The duration of the assay was 1 hour and 30 minutes at a temperature of +15-+25$^0$C.

The assay is a direct competitive ELISA where the avermectins present in the standard and/or sample compete with horseradishperoxidase labelled conjugate for specific sites in the antibodies coated to the plate. The colorimetric visualization of the reaction is carried out by addition of tetramethylbenzidine (TMB) as substrate reagent and the absorbance measured at 450 nm is inversely proportional to the concentration of the analyte.

*Performance evaluation parameters*

*Specificity / Cross-reactivity.* Specificity, expressed as %cross-reactivity(%CR) was calculated as follows:

% CR = [(IC50 (analyte) / IC50 (cross-reactant)] x 100. The half maximal inhibitory concentration (IC50) for each analyte and cross-reactant tested was calculated by taking 50% of the signal of the zero calibrator and reading this value from the x-axis (ppb) off the corresponding calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

*Limit of Detection (LOD).* LOD was calculated as the mean + 3 SD from the data for a minimum of 20 negative beef muscle or liver samples. This represents the lowest concentration of each analyte that can be distinguished due to matrix effects.

*Precision.* For the biochip intra-assay precision was determined for each analyte by fortifying controls at 3 levels spanning the calibration range and then assaying 20 replicates of the three different levels over three runs. For the ELISA the intra-assay precision was determined at 6 levels and assaying 12 replicates. Results were expressed as %C.V.

## III. RESULTS AND DISCUSSION

*Performance evaluation of the biochip based immunoassay and ELISA for avermectins.*

Both immunoassays were standardised to ivermectin and presented broad cross-reactivity profile (Table 1). The avermectins emamectin benzoate, eprinomectin, abamectin, ivermectin and doramectin were detected with % cross-reactivity ranging from 75 to 254% for the biochip based immunoassay and from 40% to 174% for the ELISA. This increases the screening capacity of the tests for these compounds.

Table 1. Specificity data of the biochip based immunoassay and ELISA for avermectins

| Compound | Biochip % Cross-reactivity | ELISA % Cross-reactivity |
|---|---|---|
| Ivermectin | 100 | 100 |
| Emamectin benzoate | 254 | 174 |
| Eprinomectin | 191 | 137 |
| Abamectin | 178 | 148 |
| Doramectin | 75 | 40 |

The LOD in beef muscle was 0.75ppb (calibration range 0-96 ppb) for the biochip based immunoassay. The LOD in beef liver was 5 ppb for the ELISA (calibration range: 0-10 ppb). These values are below Maximum Residue Limits (MRLs) available for different countries, as for instance 10ppb-40ppb in muscle and 70-100ppb in liver (6).

The intra-assay precision data, expressed as %C.V., showed values typically ≤12% for different concentration levels with the biochip immunoassay and <10% with the ELISA (Tables 2 and 3).

Table 2. Intra-assay precision of the biochip based immunoassay for avermectins

| Compound | Coefficient of variation (%) |
|---|---|
| Level 1 | 9 |
| Level 2 | 12 |
| Level 3 | 11 |

Table 2. Intra-assay precision of the ELISA for avermectins

| Level | Coefficient of variation (%) |
|---|---|
| Level 1 | 3.7 |
| Level 2 | 2.6 |
| Level 3 | 3.1 |
| Level 4 | 3.7 |
| Level 5 | 8.1 |
| Level 6 | 3.1 |

The biochip based immunoassay was applied to the Evidence Investigator analyser and with this system fifty four biochips' including nine calibrators and up to forty five samples can be handled at a time. The time to result for forty five samples is 4 hours including sample preparation. With the ELISA forty samples can be analysed at a time and the time to result for the forty samples is 4 hours including sample preparation.

## IV. CONCLUSION

In this study two immunoassays for the screening of avermectins on different platforms were evaluated with regard to their applicability to the broad recognition of these compounds in beef matrices. Both immunoassays detected multiple avermectins: abamectin, doramectin, emamectin, ivermectin and eprinomectin. The data indicates optimal analytical performance. These immunoassays represent useful analytical tools for the screening of avermectins in batches of beef muscle or liver samples and reduce the quantity of samples to be assessed by confirmatory analysis.

## REFERENCES

1. Burg RW, Miller BM, Baker EE, Birnbaum J, Currie SA, Hartman R, Kong YL, Monaghan RL, Olson G, Putter I, Tunac JB, Wallick H, Stapley EO, Oiwa R, Omura S. (1979). Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob Agents Chemother. 15(3): 361-7.

2. Sams, R. (1993). Chemical assay of avermectins by high performance liquid chromatography with fluorescence detection. Vet. Parasitol. 48(1-4): 59-66.

3. Rübensam, G., Barreto, F., Barcellos Hoff, R., Pizzolato, T.M. (2013). Determination of avermectin and milbemycin residues in bovine muscle by liquid chromatography-tandem mass spectrometry and fluorescence detection using solvent extraction and low temperature clean-up. Food Control. 29: 55-60.

4. Shi, W., He, J., Jiang, H., Hou, X., Yang, J., Shen, J. (2006). Determination of multiresidue of avermectins in bovine liver by indirect competitive ELISA. J. Agric. Food Chem. 54: 6143-6146.

5. Rudik, I., Cummings, M.R., Poppenga, R.H. (2002). Isolation and multiresidue detection of macrolide endectocides present in animal matrices. J. Vet. Diagn. Invest. 14: 295-302.

6. International Maximum Residue Level Database http://www.mrldatabase.com