Validation of a monoclonal antibody-based ELISA for the quantification of the furazolidone metabolite (AOZ) in eggs using various sample preparation

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ABSTRACT: A monoclonal-based ELISA, coupled with an assay buffer, solvent and solid phase extraction procedures, was validated for use in the monitoring of egg samples for 3-amino-2-oxazolidinone (AOZ). The procedures allow the detection of protein bound AOZ in the form of 2-nitrophenyl derivative (NPAOZ) in sample supernatant or extract after acid hydrolysis and derivatisation with *o*-nitrobenzaldehyde. The assays were validated according to criteria set down by Commission Decision (2003) for the performance and validation of analytical methods for chemical residues. The detection capability of ELISA's for AOZ in eggs (set on the basis of acceptance of no false negatives) was 0.6, 0.3 and 0.3 μg/kg for buffer, solvent and solid phase extraction, respectively. These values are well below the maximum required performance limit (MRLP) of 1 μg/kg for tissue bound residues of nitrofuran antibiotics. An excellent correlation of results (r = 0.99, n = 14) obtained by the ELISA and LC-MS/MS techniques within the concentration range of 0–5 μg/kg was found in the incurred egg samples. The eggs collected from layer chickens fed 30 and 400 mg/kg of furazolidone for 10 days were monitored by ELISA until AOZ concentrations approached the LoD.

Keywords: monoclonal ELISA; nitrofuran; AOZ; eggs; sample preparation; validation

Furazolidone [N-(5-nitro-2-furfurylidene-3-amino)-2-oxazolidinone] has been used in the treatment of various livestock such as cattle, pig and poultry, as an effective antibiotic both prophylactically and therapeutically for gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp., and as a growth promoter. Furazolidone, as well as the other members of the nitrofuran family have been prohibited within the EU (Commission Regulation, 1995) because of their potentially harmful effects on human health (McCalla, 1983; Vroomen et al., 1990; Van Koten-Vermeulen, 1993; Gottschall and Wang, 1995). Nitrofurans, despite being carcinogenic, are still used in non-EU countries and have been identified in foodstuffs of animal origin and also food commodities imported into the EU (European Commission, 2005). Monitoring the compliance of the EU ban with respect to the import of food products into Europe is therefore of great importance. Furazolidone detection itself has not been effective as the drug is rapidly metabolized after ingestion (Nouws and Laurensen, 1990; McCracken et al., 1995). However, the nitrofuran drugs furazolidone, furaltadone, nitrofurantoin and nitrofurazone, form protein bound metabolites which persist in edible tissue for a considerable amount of time after treatment (Hoogenboom et al., 1991; Horne et al., 1996; McCracken and Kennedy, 1997b).

AOZ (3-amino-2-oxazolidinone) is the metabolite moiety derived from furazolidone. Apart from its long

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term stability in tissue (Nouws and Laurensen, 1990; McCracken et al., 1995, 1997), AOZ is not degraded by common cooking techniques (Hoogenboom et al., 1991) making it practical for monitoring and detection in edible tissues. The AOZ metabolite is known to bind covalently to cellular proteins *in vivo* and is released from tissue under mildly acidic conditions. *O*-nitrobenzaldehyde (*o*-NBA) has been shown to derivatise AOZ *in vitro,* to form the nitro phenyl derivative NPAOZ ([[2-nitrophenyl)methylene]-amino]-2-oxazolidone) (Hoogenboom et al., 1992). The derivatisation step is required to increase the molecular weight of the target compound in order to improve the sensitivity of mass detection (Conneely et al., 2002) and the ability to generate antibodies for immunoassays (Cooper et. al., 2004a).

Although many laboratories have developed methods for the determination of AOZ in edible tissues, only a few studies have focused on the determination of furazolidone or AOZ in eggs (Botsoglou, 1988; Kumar et al., 1994; Draisci et al., 1997). The detection of furazolidone and AOZ in poultry eggs, was carried out by McCracken et al. (2001) using LC-MS, involved the incurred samples being subjected to hydrolysis and the derivatisation process, followed by an extraction step using ethyl acetate. The limit of detection (LoD) for this method was approximately 1.0 μg/kg for both furazolidone and AOZ. Other findings from these authors showed that concentrations of the furazolidone parent drug and AOZ residue in eggs reached a plateau of 360 to 380 μg/kg by the 4th day of treatment with 400 mg/kg furazolidone, coinciding with the results published by Botsoglou (1988). The AOZ residue concentration in egg homogenates was stable when stored at –20°C for 55 days, whereas, the concentration of furazolidone decreased by 44% in identical conditions, emphasising the stability of AOZ and the benefits of its use as a marker in the monitoring of furazolidone use in poultry production.

Liquid-liquid extraction extraction has been utilised by various groups for the isolation of furazolidone or derivatised AOZ released from liver or muscle tissue, combining solvent extraction with liquid chromatography (LC) using ultra-violet (UV) (Kumar et al., 1994; Yoshida and Kondo, 1995; Angelini et al., 1997), or mass spectrometry (MS) detection (McCracken et al., 1995, 2001; McCracken and Kennedy, 1997a). High-performance liquid chromatography (HPLC) combined with MS/MS (O'Keeffe et al., 2004; Mottier et al., 2005) or UV detection (Horne et al., 1996) has also been employed for the analysis of the above analytes. Solvent extraction procedures are effective but the use of a large amount of high grade solvents can be costly, not to mention the health hazards.

Solid-phase extraction (SPE) coupled to LC-MS was used for the determination of the AOZ or furazolidone in liver and muscle (Carignan et al., 1990; Parks and Kubena, 1990; Leitner et al., 2001; Conneely et al., 2002). SPE enables the analyte to be concentrated before its analysis and can reduce the amounts of organic solvents required during sample preparation, as well as being as a clean up step. Moreover, SPE in combination with LC-MS/MS has a high sensitivity and selectivity and allows AOZ to be identified in accordance with EU guidelines (Carignan et al., 1990; Leitner et al., 2001; Conneely et al., 2002). LC-MS/MS can quantify tissue bound nitrofuran metabolites at concentrations of parts per billion (ppb) and satisfies the EU Minimum Required Performance Limit (MRPL) of 1.0 μg/kg (Commission Decision, 2003). On the other hand, SPE is time-consuming and therefore not suitable for testing large series of samples.

Enzyme-linked immunosorbent assay (ELISA) is an effective alternative to analytical detection and monitoring methods offering fast, reliable and relatively cheap determination of low concentrations of target analytes. The first ELISA system capable of determining AOZ was reported by Cooper et al. (2004b) and involved AOZ being released from prawns, derivatised with *o*-NBA, extracted using ethyl acetate and washed in hexane prior to detection. This polyclonal based immunoassay reached a 0.1 μg/kg LoD. A simplified but still sensitive ELISA which used buffer extraction, and was based on monoclonal antibody immunoassay, was developed by Diblikova et al. (2005). This method relied on the use of matrix matched calibration standards to reduce sample interference, and allowed the sensitive determination of AOZ in shrimp, poultry, pork, and beef tissue homogenates without solvent extraction.

The aims of this study were (1) to introduce simplified egg sample preparation for use with ELISA detection, and compare this procedure with conventional preparation methods that use solvent and solid phase extraction techniques; (2) prepare fortified and incurred samples to validate the introduced methods; (3) compare ELISA and LC-MS/MS results in incurred egg samples; and (4) utilise validated ELISA system for the monitoring of AOZ levels in chicken eggs after furazolidone treatment.

MATERIAL AND METHODS

ELISA

Chemicals and biochemicals. 3-amino-2-oxazolidone (AOZ), *o*-nitrobenzaldehyde (*o*-NBA), bovine serum albumin (BSA), 3[[(2-nitrophenyl)-methylene]-amino]-2-oxazolidinone (NPAOZ) was obtained from WITEGA Laboratorien (Berlin, Germany). Peroxidase Type II from horseradish (HRP), dimethyl sulphoxide (DMSO), Tween 20, N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), N,N-dimethylformamide (DMF), and (hydroxymethyl)-aminomethane were obtained from Sigma (St. Louis, USA). 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany), and *n*-hexane and methanol (spectroscopy grade) were provided by Merck (Darmstadt, Germany). Strata SDB-L-Styrene-Divinylbenzene cartridges were obtained from Phenomenex (Aschaffenbburg, Germany). Sephadex-25 and ethyl acetate and furazolidone were purchased from Sigma Aldrich (Heidelberg, Germany). 3([(3-carboxyphenyl)methylene]-amino)-2-oxazolidinone (CPAOZ) and CPAOZ-ethylene diamine-human serum albumin (CPAOZ-ed-HSA) were provided by the Veterinary Science Division, Department of Agriculture and Rural Development (Belfast, Northern Ireland) and previously described (Cooper et al., 2004a). Monoclonal antibody against CP-AOZ-ed-HSA (clone 3B8/2B9) was produced by this laboratory (Vass et al., 2005). All other chemicals were of analytical grade and purchased from Dorapis (Brno, Czech Republic).

Instrumentation. A homogeniser (Ultra-Turax IKA T18, Germany), minishaker (IKA MS2, Germany), centrifuges (Juan CR 3-22, Juan CR 3-I, France), horizontal shaker (Unimax 1010, Germany), manifold (Supelco, Germany) and sample concentrator (Techne DB – 3D, East Port, United Kingdom) were used for sample preparation. Microtitre plates were supplied by NUNC (Roskilde, Denmark). A microtitre plate shaker (IKA MTS4, Germany) and automatic plate washer (ELX50-BIO-TEK Instruments, Vermont, USA) were used for washing microplates. Absorbance was measured using an EL 808 Ultra microplate reader and processed by KC4^{TM} v. 3.1 software (BIO-TEK Instrumentations, Vermont, USA). Conic pipette tip 1:5 ml (cod. EG502) for gel filtration was a product of the BicappaLAB (Rome, Italy).

Buffers and solutions. The following buffers were used in the experiments: (1) 50 mmol/l *o*-NBA in DMSO for the derivatisation of AOZ. (2) 0.1 mol/l phosphate buffer saline (PBS) containing 2 mol/l NaOH for sample neutralisation. (3) 10 mmol/l PBS, containing 145 mmol/l NaCl (pH 7.2), for dilution of the extracts. (4) 0.3 mol/l Na_3PO_4.12 H_2O for pH adjustment of samples. (5) 50 mmol/l carbonate buffer (pH 9.6) as a coating buffer. (6) 10 mmol/l PBS, containing 145 mmol/l NaCl and 0.5% BSA (pH 7.2), for tracer dilution. (7) 10 mmol/l phosphate buffer saline (PBS) with 0.1% Tween-20 as a washing buffer. (8) Substrate buffer contained 0.1 mol/l sodium acetate (pH 5.5, adjusted by addition of 1 mol/l citric acid). (9) Substrate solution for HRP was prepared by the addition of 1 ml of substrate buffer, 200 µl of 1% (w/v) solution of TMB in DMSO and 20 µl of 6% (w/v) H_2O_2 to 20 ml MilliQ-water. (10) Stopping reagent was 2 mol/l H_2SO_4.

Calibration standards. 50 µl of stock NPAOZ solution (1 g/l methanol) was added to 21.56 ml of PBS to obtain the concentration of 2 314 µg/l. Ten µl of this solution was added to 990 µl of PBS or matrix supernatant (refer to paragraph "buffer extraction") to obtain 1 ml of buffer or matrix matched standard of concentration 23.14 µg/l. This standard solution was diluted with PBS or matrix supernatant to obtain standards in buffer or matrix matched standards containing 0.12; 0.23; 0.46; 1.16; 2.31; 4.63 and 9.25 µg of NPAOZ/l. Data were calculated on the basis of underivatised AOZ, a more conventional expression than the derivatised form (NPAOZ). After correcting for the increase in mass, arising from derivatisation with *o*-NBA (using the coefficient 2.314), the respective concentrations (as AOZ equivalents) are as follows: 0.05; 0.10; 0.20; 0.50; 1.0; 2.0; 4.0 µg AOZ /l.

Synthesis of enzyme tracer. CPAOZ (5 µmol, 1.17 mg), N-hydroxysuccinimide ester (5 µmol, 0.58 mg) and DCC (5 µmol, 1.03 mg) were dissolved in DMF (60 µl). The mixture was gently stirred at room temperature for 18 hours. The activated hapten was centrifuged (490 × g for 10 min) and 13.6 µl of supernatant was added whilst stirring to 3.4 mg of HRP in 0.13 mol/l $NaHCO_3$ (300 µl) in order to obtain a molar ratio 5:1 (hapten:HRP). The conjugation mixture was stirred at room temperature for 5 h and the formed tracer was purified by gel filtration using conic pipette tip (upper diameter = 0.9 cm, length = 14.8 cm). The tip was loaded with Sephadex G-25 and 0.01 mol/l $NaHCO_3$ was used as eluent. The tracer obtained was diluted with an equal volume of glycerol and stored at –20°C until use.

Preparation of incurred egg samples. Six clinically healthy chickens were housed in individual wire cages and randomly divided into two equal groups. Drinking water and feed was given *ad libitum* throughout the experiment. Chickens were fed a non-granular conventional layers diet for 3 days and eggs were collected daily. After 3 days, feed was changed to a medicated ration. The first group (3 chickens) was fed a conventional layers ration containing 30 mg/kg of furazolidone over 10 consecutive days. The second group was given a conventional layers ration containing a therapeutic dose of furazolidone (400 mg/kg) for the same duration. After 10 days medicated feed was replaced by non-medicated feed for the remainder of the 90 day experiment. Eggs were collected daily, stored at 4°C in a refrigerator and egg content was homogenised within a week of sample collection. Egg homogenates were stored at –20°C until use.

Buffer extraction (BE). The matrix supernatants from AOZ free (blank) samples were prepared as follows; eggs were homogenised and the homogenate was distributed (1 g each) into 15 ml disposable plastic tubes. At this stage, samples were fortified, when required, by the addition of AOZ standard solution (10 µg/l and 100 µg/l) in H_2O. Three millilitres of H_2O was added into the tubes and the samples vortexed for 10 s. Samples were then placed in boiling water bath for 10 min and then allowed to cool at room temperature. 5 mol/l HCl (150 µl) and 50 mmol/l *o*-NBA in DMSO (25 µl) were added to the samples and incubated at 37°C overnight. After incubation, 0.3 ml of 2 mol/l NaOH in concentrated PBS was added to each tube and the samples vortexed thoroughly for 20 s. Samples were centrifuged at 1 600 × g at 4°C for 15 min and the clear portion of the supernatant was transferred to glass vials using Pasteur pipettes. The final pH of the samples was adjusted to 7.1–7.2 by addition of 1 mol/l NaOH in combination with a pH-monitor. Samples were then ready for ELISA analysis.

Solvent extraction (SE). 1 g of egg homogenates were weighted into 15 ml disposable plastic tubes (with screw caps) and the homogenates were fortified by the addition of AOZ (10 µg/l and 100 µg/l) in H_2O as described above. Three ml of H_2O was added into tubes and vortexed for 10 s. Then, 0.5 ml 1 mol/l HCl and 25 µl 50 mmol/l *o*-NBA in DMSO was added, and the samples were incubated at 37°C overnight. Samples were neutralised by addition of 0.3 ml of 2 mol/l NaOH in 0.1 mol/l PBS and vortexed for 20 s. Five ml of ethyl acetate was pipetted into each sample. Test tubes were then closed and shaken (in the horizontal position) for 15 min followed by centrifugation at 1 600 × g at 4°C for 15 min. Four ml aliquots of the upper ethyl-acetate layer were then transferred into clean glass vials and evaporated to dryness on a heating block at 60°C, under nitrogen. The evaporates were re-dissolved in 0.8 ml of PBS, washed with 2 ml of *n*-hexane and vortexed thoroughly for 30 s. Glass vials were centrifuged (1 100 × g at 4°C), freezed for 15 min at –80°C and then the hexane discarded. The solid portion of sample was thawed and 1 ml *n*-hexane added and the washing step repeated once as described above. The cleaned up sample extract was used for ELISA analysis.

Solid phase extraction (SPE). 1 g of egg homogenate was weighted into 15 ml disposable plastic tubes (with screw caps) and samples were fortified by the addition of AOZ standard solution as described above. Three ml of H_2O, 0.5 ml 1 mol/l HCl and 25 µl 50 mmol/l *o*-NBA in DMSO were added to each sample. Test tubes were vortexed for 10 s and incubated at 37°C overnight. 0.5 ml of 0.3 mol/l Na_3PO_4.12 H_2O, 0.25 ml 2 mol/l NaOH and 4.5 ml H_2O were added to each sample and vortexed for 30 s. Samples were adjusted to pH 7.0–7.2 with 1 mol/l NaOH, centrifuged at 1 100 × g at 4°C for 15 min, and supernatant collected. SDB-L-Styrene-Divinylbenzene cartridges were placed on a vacuum manifold and conditioned using 3 ml ethyl-acetate, followed by 3 ml methanol and 5 × 3 ml H_2O. Eight ml of supernatant was passed through the cartridge, then washed with 6 ml H_2O, and eluted with 2 × 3 ml ethyl acetate. The eluent was collected and centrifuged at 1 100 × g for 10 min. Samples (4.5 ml aliquots) were transferred to clean glass vials and evaporated to dryness on a heating block at 60°C, under nitrogen. The evaporates were re-dissolved in 0.72 ml PBS, vortexed for 30 s and analysed with ELISA. For LC-MS/MS analysis, evaporates were redissolved in 0.5 ml of acetonitrile/water/acetic acid (100:900:1, v/v).

Direct ELISA

200 µl of antibody diluted with coating buffer (1:10 000) was pipetted into wells of microtitre plate and incubated at 4°C overnight. Plates were then washed 3 times using 0.3 ml/well of washing buffer solution. 100 µl of NPAOZ standard or sample and than 100 µl of tracer solution were added to

each well. The plates were incubated for 1 h at 4°C. Unbound compounds were removed by washing 3 times with the washing solution as above. 200 µl of substrate solution was then added to each well and the enzymatic reaction was stopped after a 15 min incubation period at room temperature by the addition of 100 µl/well of stopping solution. Absorbance values were measured at 450 nm.

The concentrations of analyte in samples obtained by SE-ELISA or SPE-ELISA were read directly from the calibration curve generated in buffer. The concentrations of analyte in matrix supernatant, determined by BE-ELISA, were read from the matrix-matched calibration curve. To obtain a result in µg/kg, the concentration (read from matrix-matched calibration curve for AOZ), was multiplied by a final sample volume V (= dilution factor). For V = 4.8 ml, the sample concentration is calculated as follows: Result (µg AOZ/kg) = Result of ELISA (µg AOZ/l) × 4.8 (Diblikova et. al., 2005).

LC-MS/MS analysis

LC-MS/MS was performed with the mobile phase consisting of 0.1% acetic acid in water (A) and acetonitrile (B). The flow rate and temperature of the gradient run was set at 0.2 ml/min and 25°C, respectively. Chromatographic separation was performed on a XTerra MS instrument, C_{18} (5 µm, 3.0 × 100 mm) column, combined with a guard column (2.1 × 10 mm), purchased from Waters. 10 µl of sample was injected. The column was connected to a LC-MS/MS system comprised of a LC pump Surveyor and a TSQ Quantum Discovery Triple quadrupole mass spectrometer equipped with an ESI ion source, (FINNIGAN Termo Electron Corporation, USA). The samples were analysed in a positive polarity mode (ESI+). The mass spectrometer was operated in the selected reaction monitoring mode (SRM) and the settings chosen for optimum sensitivity were spray voltage 4.5 kV, collision energy 13 eV, scan time 0.30 s, scan width 1 000 m/z, sheath gas flow 65 units, auxilary gas flow 5 units and capillary temperature 220°C. Under the given MS/MS fragmentation conditions, the precursor/product (m/z) ion combination for NPAOZ was 236 and, 134 and 104, respectively. Similar fragmentations are found for the internal standard: the precursor/product (m/z) ion combination for NPAOZ-d4 was 240 and 134, respectively. The used collision energy (eV) was 13.

Validation

Twenty egg samples were obtained for the validation study using the standard ELISA protocols to assess the range of matrix interferences in the assay and provide data for the determination of false positive rates. Of the 20 samples, eight were obtained from farms and 12 were purchased from retail outlets within the local area. No measurable AOZ residues were observed in the blank samples from previous ELISA determination and were therefore considered to be free of AOZ.

ELISA analysis was repeated following the fortification with standard AOZ in order to calculate the detection capability (CCβ) at different false positive rates, coefficients of variation, and the percent recovery of the method. The recovery of the added AOZ was calculated as follows: Recovery (%) = AOZ conc. measured/AOZ conc. fortified × 100. Intra-assay and inter-assay variation coefficients for ELISA procedures were determined using duplicate blank samples fortified at the levels 0.5, 1 and 3 (or 5) µg/kg.

RESULTS AND DISCUSSION

Analytical characterisation of ELISA

Three different sample preparation procedures linked with ELISA detection were characterised in terms of basic analytical parameters. Ethyl acetate and solid phase extraction represent a traditional approach to the isolation of the analyte prior to measurement, whereas the detection of AOZ in tissue homogenate enabled a simplification of overall analytical performance. The simplified ELISA method based on the calibration in a matrix environment and the detection of AOZ in sample homogenate was reported for various muscle tissues in our previous work (Diblikova et al., 2005). In this work, a modified procedure was introduced for the determination of AOZ in eggs. Briefly, the assay allows the detection of NPAOZ in tissue supernatant after centrifugation of sample homogenate and derivatisation of AOZ (released from egg proteins) with *o*-NBA. Since matrix supernatant was the subject of analysis, the conventional extraction and clean up steps with organic solvents were omitted from this procedure. High specificity of the monoclonal antibody towards NPAOZ, allows the detection of the analyte in the presence of *o*-NBA without the

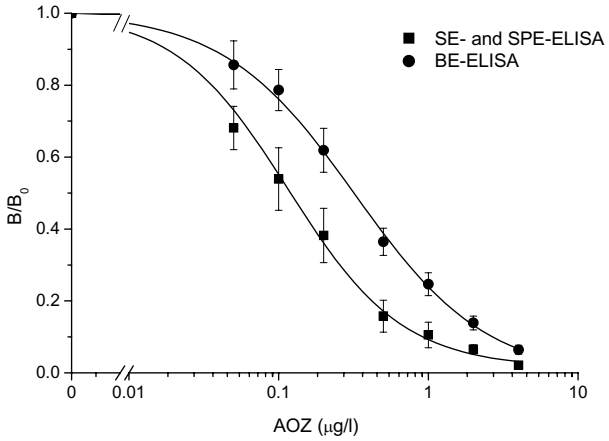

Figure 1. Standard curves of NPAOZ in buffer (used for SE-ELISA and SPE-ELISA) and in the BE supernatant (used for BE-ELISA). Concentrations are expressed as equivalent underivatised AOZ. The bars show the standard deviation for 9 replicate assays performed over 3 months. Reagent dilutions: antibody coating, 1:10 000, tracer CPAOZ-HRP, 1:20 000 (matrix matched curve); antibody coating, 1:10 000; tracer CPAOZ-HRP, 1:80 000 (buffer curve)

subsequent separation of the residual *o*-NBA from the derivatised sample supernatant. The method relies on the use of matrix matched calibration standards to reduce sample interferences. To achieve maximum sensitivity, the procedure was optimised in respect to the reagent and washing volumes, and the minimum volume of derivatisation solution that could be added to the sample supernatant.

Representative standard curves for NPAOZ based on matrix calibration are shown in Figure 1. The standard dose responses are expressed here in the concentration of AOZ equivalent and B/Bo ratio, where B = absorbance at a given concentration of the analyte, Bo = absorbance at zero concentration of the analyte. The ELISA method (with antibody coating for each assay) was performed 9 times over 3 months to establish calibration stability and repeatability. As shown in Figure 1, the sensitivity of AOZ detection in assay buffer is greater than in the egg matrix throughout the calibration range of 0.05–5 µg/l. The mean IC_{50} values for the 9 repeated assays, i.e. the detection levels with the highest sensitivity, were 0.12 µg/l in PBS and 0.324 µg/l in the egg matrix. The variation coefficient for each point on the standard AOZ curve ranged from 8.4 to 33.5% (buffer) and 7.3 to 19.2% (matrix) which indicate good reproducibility. The standard curve generated in buffer was used for calibration of the assays using solvent and SP extraction.

Determination of LoD, detection capability (CCβ) and assay recovery

Twenty chicken eggs, collected from the Southern Moravia region, were analysed by three different ELISA procedures. Figure 2 depicts the assessment of LoD and the CC_{β} for the SE-ELISA performance. The LoD defined here is the concentration of the absorbance value corresponding to the average absorbance at zero AOZ concentration plus 3 times the standard deviation of the absorbance at zero concentration. Dashed line in Figure 2 indicates the average concentration of 20 blanks plus three standard deviations. The CCβ was assessed by using each extraction procedure combined with ELISA to examine 20 blank egg samples fortified at and below the minimum required performance level in equidistant steps. Samples fortified at 0.3 µg/kg AOZ in all cases gave a greater value than the calculated LoD of 0.09 µg/kg. The detection capability of the ELISA using solvent extraction was determined to be 0.3 µg/kg. Similar results were obtained for the BE-ELISA and SPE-ELISA procedures.

Table 1 presents calculated LoDs and measured values of blank samples fortified with AOZ at the level of the detection capability for three ELISA methods. The SPE-ELISA system allowed detection capability and LoD to reach 0.3 µg/kg and 0.0089 µg/kg, respectively, whereas the BE-ELISA showed to be less sensitive but still very effective (0.6 µg/kg and 0.162 µg/kg). Table 2 compares AOZ recovery

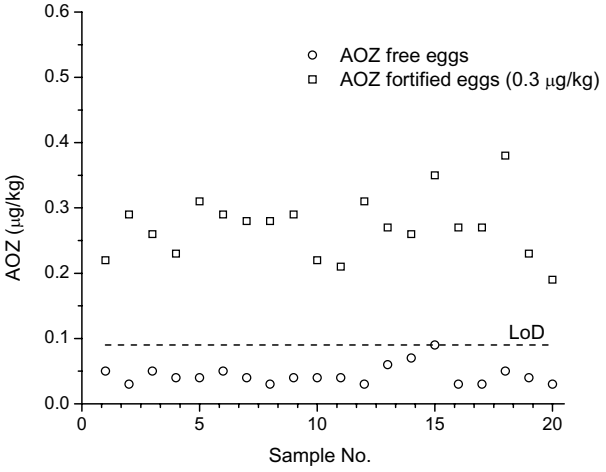

Figure 2. Detection capability (CCβ) of SE-ELISA

Table 1. Limit of detection and determination of AOZ in fortified samples

	SE-ELISA	SPE-ELISA	BE-ELISA
Representative blank samples (n = 20)			
Mean ± SD (μg/kg)	0.044 ± 0.015	0.035 ± 0.023	0.069 ± 0.031
LoD (mean + 3 SD; μg/kg)	0.089	0.104	0162
Fortified samples (n = 20)			
AOZ added (μg/kg)*	0.300	0.300	0.600
Mean ± SD (μg/kg)	0.271 ± 0.046	0.249 ± 0.038	0.377 ± 0.074
Recovery (%)	90.2	82.9	62.8

*The concentrations of added AOZ are identical to the detection capability values determined

Table 2. Precision, recovery and repeatibility of ELISA methods

Method		Fortified concentration (μg/kg)	n	Mean ± SD (μg/kg)	CV(%)	Recovery (%)
Intra-assay coefficient of variation						
	sample 1	0.3	6	0.29 ± 0.03	9.3	97.2
SE-ELISA	sample 2	1	6	0.80 ± 0.11	13.9	79.5
	sample 3	3	6	1.98 ± 0.38	19.6	65.8
	sample 1	0.3	6	0.40 ± 0.03	8.2	132.2
SPE-ELISA	sample 2	1	6	0.97 ± 0.13	13.3	97.2
	sample 3	3	6	3.04 ± 0.72	23.7	101.2
	sample 1	0.5	8	0.34 ± 0.07	21.3	67.0
BE-ELISA	sample 2	1	8	0.75 ± 0.07	9.5	74.6
	sample 3	5	8	4.13 ± 0.32	7.7	82.5
Inter-assay coefficient of variation						
	sample 1	0.3	6	0.27 ± 0.03	10.3	90.0
SE-ELISA	sample 2	1	6	0.76 ± 0.11	14.0	75.8
	sample 3	3	6	2.15 ± 0.15	7.1	71.7
	sample 1	0.3	3	0.32 ± 0.04	11.9	105.5
SPE-ELISA	sample 2	1	3	0.92 ± 0.07	7.7	92.4
	sample 3	3	3	2.52 ± 0.63	25.3	84.1
	sample 1	0.5	4	0.43 ± 0.12	27.1	86.3
BE-ELISA	sample 2	1	4	0.82 ± 0.12	14.3	82.1
	sample 3	5	4	4.20 ± 0.53	12.8	82.9

n = numer of measurements; R = numer of repeated assays

values obtained in blank samples fortified with AOZ, at levels of 0.3, 1, and 3 μg/kg for SE and SPE procedures, and 0.5, 1, and 5 μg/kg for the BE-ELISA. The average recovery for the SPE method was higher than for the SE-ELISA and BE-ELISA. The procedures provided comparable intra-assay variation coefficients (CV) in the range of 7.7 to 23.7%. Inter-assay variation coefficients (assay repeatability) of the procedures were calculated from the assay data obtained for the fortified samples within an 8 week period. Measurements were carried out using standard assay protocols for the re-

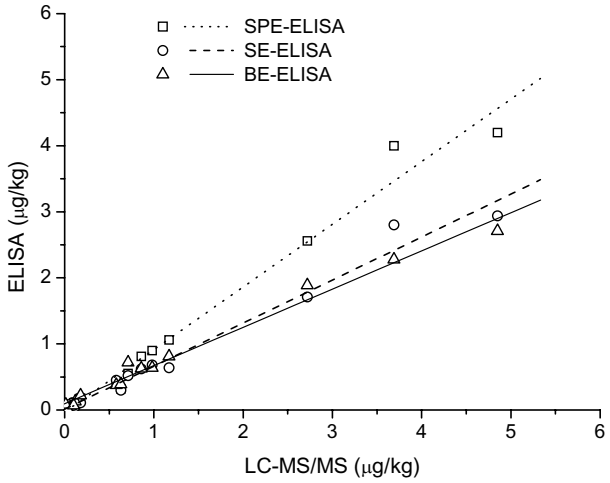

Figure 3. Correlation of the results obtained by ELISA and LC-MS/MS. The regression equations for the SPE-ELISA, SE-ELISA and BE-ELISA are $y_1 = -0.03 + 0.94x$, $y_2 = 0.015 + 0.65x$, $y_3 = 0.097 + 0.58x$, respectively

spective ELISA methods. As shown in Table 2, the inter-assay variability had similar characteristics as for the intra-assay measurement.

Comparison of ELISA with LC-MS/MS

The accuracy of the validated ELISA procedures were determined by the comparison of results with LC-MS/MS data. The procedures were tested using the same incurred egg samples as prepared within this study. The results obtained by the ELISA methods and LC-MS/MS are compared in Figure 3. A good correlation between the respective ELISA procedures and LC-MS/MS results was found in the identical concentration range (0–5 μg/kg) (r = 0.99, n = 14). In all cases, the ELISA showed more or less, an underestimation of AOZ concentration in comparison to the reference method. The slope of the graph showing the SPE-ELISA method, indicates less of an underestimation than for the SE- and BE-ELISA methods. The ELISA underestimation may be due to the incomplete derivatisation of AOZ, extraction efficiencies, matrix effects or other factors. However, these results are consistent with those found by Cooper et al. (2004b) and Diblikova et al. (2005).

AOZ monitoring in eggs using ELISA

The results of the AOZ monitoring in eggs collected from six chickens are shown in Figures 4 and 5. The samples were analysed using the SE-ELISA procedure over a course of 3 months. The AOZ concentrations in egg samples from chickens treated with 30 mg/kg of furazolidone, determined 2 days after ending treatment, ranged from 11–17 μg/kg (Figure 4). AOZ concentrations of egg homogenates from chickens treated with 400 mg/kg of furazolidone, also determined 2 days after ending treatment, ranged from 142–293 μg/kg (Figure 5). A decrease in AOZ concentration towards the LoD (0.09 μg/kg) for chickens treated with 30 and 400 mg/kg occurred for 40 and 86 days after ending each treatment, respectively. This lengthy withdrawal period and the stability of AOZ residues

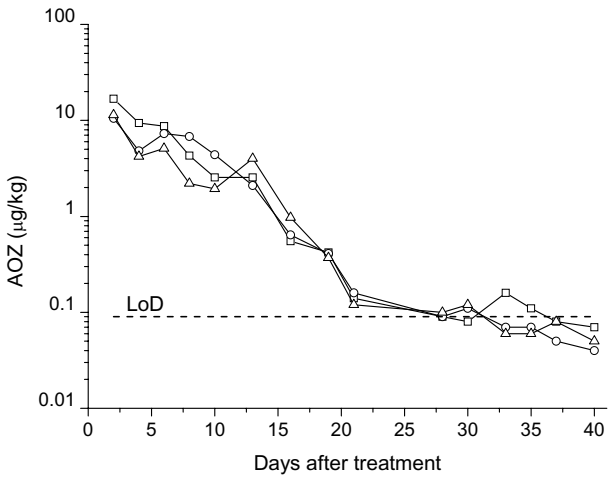

Figure 4. AOZ in eggs from 3 chickens after treatment for 10 days with 30 mg furazolidone per kg of feed

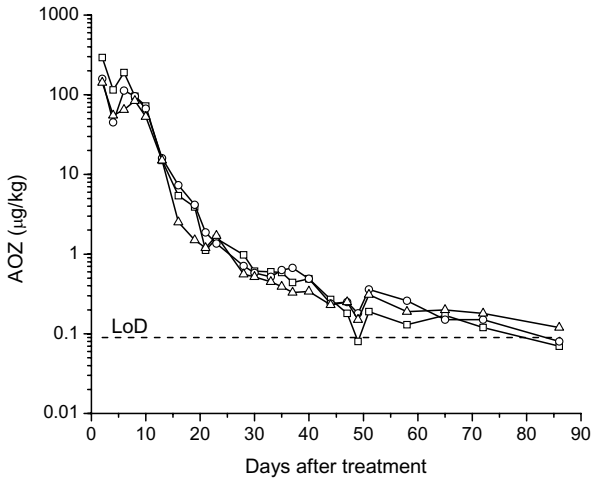

Figure 5. AOZ in eggs from 3 chickens after treatment for 10 days with 400 mg furazolidone per kg of feed

confirms that AOZ is a more relevant marker of the furazolidone ban than parent drug and also demonstrates the long-term stability of this metabolite in eggs.

CONCLUSIONS

Three different sample preparation methods linked with ELISA detection were investigated and compared with LC-MS/MS for the determination of protein bound AOZ in eggs. The simplified ELISA, developed in this study, allowed the detection of AOZ in sample supernatant, and offers the analysis of high sample numbers in comparison with the methods based on traditional extraction procedures. The detection capability of the ELISA methods were between 0.3 µg/kg and 0.6 µg/kg and the LoD ranged between 0.09 and 0.16 µg/kg. This is well below the criteria set down by the Commission Decision (2003) that states the MRPL for tissue bound residues of nitrofurans is 1 µg/kg. With respect to the slight drop in detection capability, the simplified BE-ELISA approach performs comparably to other validated extraction methods and offers the advantage of time and cost efficiency. The results from the three ELISA methods and LC-MS/MS were highly correlated for incurred egg samples. When the AOZ concentrations determined by these ELISAs are corrected by control samples, the methods can be used as a reliable analytical means for the quantification of AOZ in eggs. The simplified ELISA with the matrix-matched calibration can be less effective if small numbers of various matrices are to be analysed. On the other hand, ELISAs in combination with conventional extraction procedures are better usable to a broader scale of food matrices such as muscle, liver, or honey samples.

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