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In-house validation of an ELISA method for screening of semicarbazide in eggs

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1 In-house validation of an ELISA method for screening 2 of semicarbazide in eggs

3 4 **Abstract**

5 An enzyme-linked immunosorbent assay (ELISA) method is described for the
6 semi-quantitative determination of semicarbazide (SEM), the marker residue for the
7 banned nitrofurantoin drug nitrofurazone, in chicken eggs. The sample homogenate is
8 subjected to acid hydrolysis and derivatisation with o-nitrobenzaldehyde, followed by
9 ethyl acetate / hexane extraction and detection by ELISA. The ELISA procedure has been
10 validated using 0.3, 1.0 and 3 $\mu\text{g kg}^{-1}$ of SEM in fortified samples. Detection capability
11 (CC_B) was set on the basis of the acceptance of 5 % false compliant results for a given
12 concentration level according to Commission Decision 2002/657/EC, and was
13 determined to be 0.3 $\mu\text{g kg}^{-1}$ with a respective limit of detection of 0.13 $\mu\text{g kg}^{-1}$. A
14 validated LC-MS/MS method was used for the analysis of incurred egg samples and the
15 results compared with ELISA. A good correlation between the results obtained from
16 ELISA and LC-MS/MS within the concentration range 0.12 to 20.3 $\mu\text{g kg}^{-1}$ was observed
17 in samples collected from chickens fed with a medicated ration of nitrofurazone
18 ($r = 0.992$, $n = 14$). Validated ELISA enabled reliable monitoring of SEM levels in eggs
19 collected from incurred chickens for a 90 day period.

20
21 **Keywords:** *Nitrofurazone; semicarbazide; egg homogenate; ELISA; LC-MS/MS;*
22 *validation; incurred samples; SEM depletion*

1 Introduction

2
3 Nitrofurazone (NFZ) belongs to the nitrofuran family of broad spectrum antibiotics which
4 are banned in the EU due to concerns about their carcinogenic and mutagenic effects on
5 human health. The global nitrofuran crisis which came to light during 2002/03 revealed a
6 frequent presence of nitrofuran residues in poultry and aquaculture products imported
7 into Europe, especially from many Asian countries and Brazil (Diblikova et al. 2005).
8 This led to import restrictions and mandatory testing. Member states of the European
9 Union are now required to monitor compliance of the prohibition of nitrofuran antibiotic
10 use through their annual national residue control plans. The minimum required
11 performance limit (MRPL) for nitrofurans (and respective residue markers) in poultry
12 muscle and shrimp has been set at $1 \mu\text{g kg}^{-1}$ by the EU (Commission Decision
13 2002/181/EC 2003). The presence of protein bound metabolites in eggs after the
14 administration of nitrofuran drugs was demonstrated by McCracken et al. (2001).
15 However, an MRPL for nitrofurans in eggs has not been laid down within the EU. Some
16 countries, such as Germany, have translated the MRPL of $1 \mu\text{g kg}^{-1}$ from muscle and
17 aquaculture products to egg as stated in the predefined German National Residue Control
18 Plan (Bock et al. 2007).

19 Effective control of nitrofuran drug use in livestock is based on measuring the
20 concentration of tissue bound nitrofuran metabolites after their release from tissues and
21 derivatisation (Hoogenboom et al. 1992). Semicarbazide (SEM) is the tissue bound
22 metabolic product of NFZ persisting in animal tissues for considerable periods after
23 administration of the parent drug to livestock and was therefore established as a marker
24 for nitrofurazone detection. Recently, the presence of SEM was confirmed in jars of
25 processed baby food, bread and other commodities. The source of this contamination was
26 azodicarbonamide, which is used in the manufacture of the plastic gaskets inside the lids
27 of glass jars. It was found that SEM is a breakdown product of this commonly used
28 foaming agent. As a consequence, the use of SEM as a definitive marker for the
29 monitoring of illegal NFZ administration has become questionable (Pereira et al., 2004).
30 However, according to EU legislation, SEM remains the marker residue for NFZ in foods
31 of animal origin (Cooper et al. 2007).

1 The common method of SEM analysis involves acid hydrolysis for the release of
2 tissue bound residues in a sample, followed by derivatisation with o-nitrobenzaldehyde
3 (o-NBA). The formed structure is the derivatised analyte nitrophenyl semicarbazide
4 (NPSEM). Analysis based on this approach provides data on total SEM content (bound
5 and free) in a tested sample. Chromatographic methods, predominantly LC-MS/MS, used
6 for the determination of nitrofurans residues in poultry tissues have been well established
7 for monitoring marker metabolites in chicken eggs (Kumar et al. 1994; Yoshida and
8 Kondo 1995; Draisci et al. 1997; McCracken et al. 2001; Finzi et al. 2006; Szilagyi and
9 de la Calle 2006; Bock et al. 2007). These methods are highly sensitive and specific,
10 however, they are also time consuming and quite expensive. Therefore, a method offering
11 efficient routine analysis which enables the pre-selection of positive samples prior to
12 confirmatory analysis is required. ELISA technology provides a very convenient way to
13 reach the sample frequency requirements with a reduced cost in terms of both time and
14 resources.

15 A recently developed ELISA method was validated as a screening procedure for
16 the determination of SEM in chicken muscle (Cooper et al. 2007). In our study, a
17 competitive ELISA for SEM, previously developed in this laboratory, was validated for
18 its suitability for the screening of egg samples, a more available matrix for the monitoring
19 of NFZ abuse on chicken farms. Validation was performed using analysis of fortified
20 blank samples and by the comparison of results obtained by ELISA and LC-MS/MS
21 using incurred egg samples. In addition, ELISA was used to monitor levels of SEM in
22 eggs for long term periods after medication.

24 **Experimental**

25 *Chemicals and Reagents*

26 Nitrofurazone (NFZ), semicarbazide (SEM, hydrazinecarboxamide) hydrochloride
27 and o-nitrobenzaldehyde (o-NBA) were obtained from Sigma-Aldrich (Steinheim,
28 Germany). 3[(2-Nitrophenyl)methylene]-hydrazinecarboxamide (NPSEM) was obtained
29 from WITEGA (Berlin, Germany). Dimethyl sulphoxide (DMSO), horse-radish
30 peroxidase (HRP) and polyethylene-sorbitan-monolaurate (Tween20) were obtained from

1 Sigma (St. Louis, USA). 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from
2 Serva (Heidelberg, Germany). Ethyl acetate, n-hexane, methanol and all other chemicals
3 (HPLC or gradient grade) were purchased from Dorapis (Brno, Czech Republic). The
4 internal standard (^{13}C - $^{15}\text{N}_2$ -) SEM and derivatised analyte NPSEM used for LC-MS/MS
5 analysis were supplied by Sigma-Aldrich, Czech Republic, and by Riedel-de Haen from
6 VETRANAL, Czech Republic, respectively. Rabbit antibody MVK31 (IgG) raised
7 against NPSEM and 3[(3-Carboxyphenyl)methylene]-hydrazinecarboxamide-horse radish
8 peroxidase (CPSEM-HRP) conjugate were prepared by this laboratory and described in
9 detail elsewhere (Vass et al. 2007).

10 11 *Instrumentation*

12 A homogeniser (Ultra-Turax IKA T18, Germany), vortex machine (IKA MS 3 basic,
13 Germany), waterbath (Memmert WB14, Germany), centrifuge (Juan CR 3-22, France),
14 horizontal shaker (Unimax 1010, Germany), and sample concentrator (Techne DB – 3D,
15 East Port, United Kingdom) were used for sample preparation. Microtitre plates were
16 supplied by NUNC (Roskilde, Denmark). An automatic plate washer (ELX50-BIO-TEK
17 Instruments, Vermont, US) was used for washing microtitre plates. Absorbance was
18 measured using an EL 808 Ultra microplate reader and processed by KC4TMv3.1 software
19 (BIO-TEK Instrumentations, Vermont, US). MCX cation exchange SPE columns were
20 obtained from Waters OASIS (Massachusetts, US). The LC-MS/MS equipment,
21 comprising of a LC pump Surveyor and a TSQ Quantum Discovery triple quadruple mass
22 spectrometer equipped with an APCI ion source, was purchased from FINNIGAN
23 Thermo Electron Corporation, US.

24 25 *Reagents and calibration standards*

26 The following buffers and solutions were used: (1) 10 mmol L⁻¹ phosphate buffered saline
27 (PBS) containing 145 mmol L⁻¹NaCl (pH 7.2) for antibody dilution, sample extract and
28 calibration standard preparation. (2) o-NBA solution comprised of 50 mmol L⁻¹ o-NBA in
29 DMSO and 100 mmol L⁻¹ o-NBA in methanol for the derivatisation reaction in ELISA
30 and LC-MS/MS, respectively. (3) 2 mol L⁻¹ NaOH in 0.1 mol L⁻¹ PBS for sample

1 neutralisation. (4) 50 mmol L⁻¹ carbonate buffer (pH 9.6) as a coating buffer. (5) PBS
2 with 0.5 % (w/v) BSA (pH 7.2) for HRP conjugate dilution. (6) PBS with 0.1 % Tween-
3 20 as washing buffer. (7) Substrate buffer was 0.1 mol L⁻¹ sodium acetate adjusted to pH
4 5.5 by addition of 1 mol L⁻¹ citric acid. (8) Substrate solution was prepared by the
5 addition of substrate buffer (1 mL), 1 % (w/v) solution of TMB in DMSO (200 µL) and
6 20 µL of 6 % H₂O₂ to 20 ml MilliQ-water. (9) 2 mol L⁻¹ H₂SO₄ was used as stopping
7 reagent. (10) LC stock SEM (¹³C-¹⁵N₂-) internal standard (1 mg mL⁻¹) was prepared in
8 methanol and stored at 2 to 8 °C for a maximum of three months.

9 Stock NPSEM solution in methanol (1 mg mL⁻¹) was diluted with PBS to obtain a
10 100 µg L⁻¹ NPSEM working solution. Serial dilution with PBS yielded NPSEM
11 calibration concentrations of 0, 0.09, 0.19, 0.37, 0.75, 1.49, 1.87 and 7.47 µg L⁻¹. To
12 account for the mass increase arising from the derivatisation of SEM to NPSEM
13 concentrations were divided by the coefficient 1.867 to obtain SEM equivalent
14 concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1 and 4 µg L⁻¹.

16 ***Preparation of incurred egg samples***

17 Twelve clinically healthy chickens were randomly divided into three groups and housed
18 in individual wire cages. Drinking water and feed was given *ad libitum* throughout the
19 experiment. All chickens were fed a non-medicated ration of a non-granular conventional
20 layer diet for 3 days prior to medication, and their eggs collected. Group 1 (n = 2) was fed
21 the non-medicated ration for the entire course of the experiment, whereas Group 2 (n = 5)
22 and Group 3 (n = 5) were fed medicated rations throughout a 10 day medication period,
23 containing 30 mg kg⁻¹ and 400 mg kg⁻¹ of NFZ, respectively, followed by the non-
24 medicated ration for the remainder of the 90 day experiment. Eggs were collected
25 regularly, homogenised within a week of sample collection, and the homogenates stored
26 at - 20 ° C until use.

28 ***Sample preparation for ELISA***

29 Egg homogenate was thawed and 1 g aliquots weighed into 15 mL plastic tubes. When
30 required, blank samples were fortified by the addition of SEM. Distilled water (3 mL)
31 was added to the sample homogenate and vortexed thoroughly. Then, 1 mol L⁻¹ HCL

1 (0.5 mL) and o-NBA solution (50 μ L) were added successively to the homogenate. The
2 mixture was vortexed for 30 sec and incubated overnight for 16 hr at 37 °C. After cooling
3 to room temperature, 2 mol L⁻¹ NaOH in 0.1 mol L⁻¹ PBS (0.3 mL) was added into each
4 tube to neutralise the pH level and vortexed thoroughly for 20 sec. Ethyl acetate (5 mL)
5 was then added gently to prevent foaming, shaken horizontally on a low setting (10 min),
6 and the mixture centrifuged (1330 x g, 10 min, 4 °C). The upper ethyl acetate layer (4
7 mL) was transferred into a glass test tube and evaporated under nitrogen at 60°C. Hexane
8 (2 mL), was added to the evaporate, vortexed thoroughly, followed by the addition of
9 PBS (0.8 mL). Samples were vortexed again, centrifuged (1330 x g, 5 min, 4°C) and
10 stored at -80 °C for 10 min (or 25 min at -20 °C). The hexane layer was removed under
11 vacuum, and the solid phase residue left to thaw. To remove remaining lipid components
12 from sample extract, a further 1 mL hexane was added, the sample was centrifuged and
13 the hexane layer separated as described above. The thawed sample extract in PBS was
14 pipetted directly into the wells of the microtitre plate for analysis by ELISA.

15

16 ***ELISA detection***

17 Microtitre wells were coated with antibody diluted with coating buffer 1:8000
18 (200 μ L per well) and left to incubate overnight (16 hr, 4 °C). Plates were washed three
19 times with washing buffer (0.3 mL per well) and then 100 μ L of standard analyte or
20 sample, followed by 100 μ L of CPSEM-HRP (diluted to 1:40000) was pipetted to each
21 well. Plates were incubated (1 hr, 4 °C) and the washing step repeated. Substrate solution
22 (200 μ L per well) was added and after 15 min the enzymatic reaction stopped by the
23 addition of 2 mol L⁻¹ H₂SO₄ (100 μ L per well). Absorbance was measured at 450 nm.

24

25 ***ELISA validation procedure***

26 The study was performed in accordance with Commission Decision 2002/657/EC,
27 Chapter 3 of technical Annex (Commission Decision 2002/657/EC 2002), applying
28 selected performance characteristics to validate the immunoassay screening method.
29 Twenty egg samples, 10 from farms in the Brno area and 10 from retail outlets, were
30 collected for validation experiments. Because no measurable SEM residues were found
31 by ELISA and LC-MS/MS analysis, the samples were considered free of SEM. The blank

1 samples were fortified with SEM and carried out to investigate the detection capability of
2 the method at different false positive rates. The limit of detection (LoD) was calculated
3 on the basis of standard deviation of the blank response by the equation $B + 3 SD_B$ where
4 B is the mean response of blanks and SD_B is standard deviation for 20 blank samples
5 taken for the investigation. Variation coefficients were calculated from the repeated
6 measurement of recovery using blank samples fortified at 0.3, 1 and 3 $\mu\text{g Kg}^{-1}$.
7 Specificity of the assay towards NPSEM and potential cross-reactants is described
8 elsewhere (Vass et al. 2007).

10 *Sample preparation for LC - MS/MS analysis*

11 A modified procedure according to McCracken and Kennedy, 1997 was carried out as
12 follows: Homogenised eggs (1 g) were transferred into a 15 mL centrifuge tube and an
13 aliquot of internal standard dissolved in methanol (^{13}C - $^{15}\text{N}_2$ - SEM, 40 μL) was added to
14 the homogenates while vortexing thoroughly. After an equilibration period (15 min),
15 0.1 mol L^{-1} HCL (10 mL) and o-NBA solution (250 μL) were added to samples, followed
16 by an overnight incubation at 37 °C under gentle mixing. Samples were cooled to room
17 temperature, neutralised with 0.3 mol L^{-1} tri-sodium phosphate (500 μL) and pH was
18 adjusted to 7 (\pm 0.5) using 2 mol L^{-1} NaOH. Samples were then centrifuged (1800 x g,
19 15 min, 24 °C) and the supernatant (10 mL) applied to cation exchange SPE columns
20 previously conditioned with methanol (2 mL) and water (2 mL). The SPE columns were
21 dried (2 min) under a vacuum and eluted with ethyl acetate (3 mL) into glass test tubes.
22 The sample eluent was evaporated under nitrogen at 45 °C and re-dissolved in 0.5 mL of
23 water:acetonitrile (1:1 v/v). The final solution was filtered through a 0.45 μm syringe
24 filter into a HPLC vial and injected into the LC-MS/MS equipment (10 μL). A calibration
25 curve was prepared using blank samples fortified with a SEM standard solution and
26 internal standard (^{13}C - $^{15}\text{N}_2$ -SEM) over the range of 0.2 to 5.0 $\mu\text{g Kg}^{-1}$.

28 *LC – MS/MS conditions*

29 The mobile phase consisted of 0.1 % acetic acid in water (A) and acetonitrile (B) using a
30 gradient run at a flow rate of 0.2 mL per minute at 25°C. Chromatographic separation was
31 performed on an XTerra MS, C_{18} column (3.5 μm , 2.1 x 150 mm), in combination with a

1 guard column (2.1 x 10 mm), both purchased from Waters. The injection volume (10 μ L)
2 was analysed using positive polarity (APCI +). The mass spectrometer was operated
3 using the selected reaction monitoring mode (SRM) and settings were optimised for
4 sensitivity as follows: spray voltage = 8.5 kV, collision gas pressure = 0.5 mTorr, scan
5 time = 0.30 sec, scan width = 0.500 m/z, sheath gas flow = 30 units, aux gas flow
6 = 5 units and capillary temperature = 220 $^{\circ}$ C.

8 **Results and discussion**

9 *Calibration curve*

10 The representative ELISA curve for SEM (equivalents) in assay buffer is shown in Figure
11 1. Dose responses are expressed as B/B₀ ratio, where B = absorbance at a given
12 concentration of the analyte and B₀ = absorbance in the absence of the analyte in the
13 reaction mixture. The ELISA was performed 6 times over a two month period to assess
14 calibration stability and repeatability. The variation coefficients (CV) calculated for
15 individual calibration points of the standard curve ranged from 7.2 to 14.5 % which
16 indicated good calibration stability. The mean IC₅₀ value (corresponding to 50 % binding
17 inhibition) for the 6 repeated assays was $0.18 \pm 0.035 \mu\text{g L}^{-1}$. Calibration curve generated
18 in buffer was used for all SEM determinations carried out in egg samples in this study.

19
20 [Insert Figure 1]

22 *Detection capability (CC _{β})*

23 While screening procedures need not be fully quantitative, they should measure the
24 presence or absence of the target analyte at or below regulatory levels. Thus, performance
25 data including the detection capability of the assay were assessed with respect to the
26 declared MRPL (1 $\mu\text{g kg}^{-1}$) for nitrofurans metabolites in edible tissues (Commission
27 Decision 2002/181/EC 2003). Figure 2 demonstrates the assessment of the CC _{β} by
28 ELISA investigation of 20 blank samples fortified with SEM at a 95 % confidence limit.
29 Based on these experiments, CC _{β} derived from the standard curve generated in assay
30 buffer was determined to be $0.3 \mu\text{g kg}^{-1}$. At this level of fortification, the assay recovered

1 the analyte at values between 0.12 to 0.39 $\mu\text{g kg}^{-1}$, fulfilling the maximum permissible
2 false negative rate (5 %). The LoD obtained by ELISA investigation of 20 blank samples
3 was calculated to be 0.13 $\mu\text{g Kg}^{-1}$ with values ranging from 0.09 to 0.15 $\mu\text{g kg}^{-1}$.

4
5 [Insert Figure 2]

6 7 *Assessment of assay recovery*

8 To ascertain information on matrix interferences and assay variability, a set of blank
9 samples were fortified with the analyte (SEM) at concentrations of 0.3, 1.0 and 3.0
10 $\mu\text{g kg}^{-1}$ and carried through the ELISA procedure. The results of this study are shown in
11 Table 1. The mean recovery values were calculated from six individual sample
12 measurements at each concentration level. The recovery for 0.3 $\mu\text{g kg}^{-1}$ of added SEM to
13 egg homogenates was found to be approximately 110 %, whereas recovery values for 1.0
14 and 3.0 $\mu\text{g kg}^{-1}$ were lower (around 80 %). This difference in recovery can be caused by
15 non-linearity of peripheral parts of calibration curve. Intra-assay CV, calculated from six
16 recovery measurements carried out using one microtitre plate simultaneously, ranged
17 from 7.4 to 28.2 %. Inter-assay CV, obtained from six repeated analysis carried out over a
18 one month period, were calculated to be 13.7 to 18.2 %.

19
20 [Insert Table 1]

21 22 *Specificity*

23 Specificity of ELISA detection is determined by the binding properties of the antibody
24 incorporated into the assay system. High specificity of the antibody towards NPSEM
25 allows reduction of interference with structurally related chemicals in a sample, without
26 use of additional clean up steps. Cross-reactivity (CR) values were calculated using
27 equation $\text{CR (\%)} = (50 \% \text{ binding inhibition by standard (NPSEM)} / 50 \% \text{ binding}$
28 $\text{inhibition by related compound}) \times 100$. CR results for the antibody used in this assay
29 against 18 potential cross-reactants are presented elsewhere (Vass et al. 2007). No
30 measurable CR was found with SEM, nitrofurans antibiotics, their metabolites, their NP
31 derivatives, pesticides or nonylphenol. Additionally, the negligible assay response to

1 o-NBA (CR < 0.01 %) eliminated the need for separation of this reagent from the
2 reaction mixture after derivatisation.

4 ***Comparison of ELISA and LC-MS/MS analysis***

5 In order to compare the analytical performance of the screening ELISA against the
6 LC-MS/MS reference method, the concentration of derivatised SEM was determined by
7 analysing extracts from eggs using the respective methodologies. Data concerning the
8 LC-MS/MS fragmentation of SEM are presented in Table 2.

10 [Insert Table 2]

12 A total of 14 incurred egg samples from 5 different chickens were selected at
13 random, analysed by both ELISA and LC-MS/MS procedures, and compared using the
14 correlation graph in Figure 3. A strong correlation between the ELISA and LC-MS/MS
15 results within the concentration range 0.12 to 20.3 $\mu\text{g kg}^{-1}$ was found. The determined
16 regression equation for these samples was $y = -0.055 + 1.038x$ ($r = 0.992$, $n = 14$). A
17 good correlation between the two methods was also obtained when the results were
18 correlated using low values within the concentration range 0.1 to 1.3 $\mu\text{g Kg}^{-1}$. The
19 respective equation $y_1 = 0.12 + 0.1513x$ ($r = 0.882$, $n = 10$) shows an underestimation by
20 ELISA in comparison with the broader concentration range. The validation results of the
21 introduced ELISA demonstrated its use as a reliable screening tool for semi-quantitative
22 analysis of SEM in real egg samples.

24 [Insert Figure 3]

26 ***SEM depletion in incurred egg samples***

27 The validated ELISA was utilised to study the depletion of SEM in incurred egg samples
28 collected from experimental chickens for a 90 day period. Results obtained from five
29 chickens fed with feed containing 400 mg of NFZ per kilogram of feed (Group 3) during
30 the medication period (day 1 to 10) are shown in Figure 4a. As expected, SEM

1 concentration reached a plateau around day 12, followed by a successive decline toward
2 the detection capability of the method. A similar experiment was also performed using
3 five chickens fed with feed containing 30 mg of NFZ per kilogram (Group 2, Data not
4 shown). On average, the time span of contamination in eggs after ingestion of NFZ in low
5 dose chickens was detectable above and including 0.3 $\mu\text{g kg}^{-1}$, up to day 40 of the
6 experiment, whereas contamination in high dose chickens was detectable as late as day
7 80 of the experiment.

8
9 [Insert Figure 4]
10

11 Surprisingly, ELISA also detected the presence of SEM (0.32 to 1.0 $\mu\text{g kg}^{-1}$) in
12 five egg homogenates which were collected during and after the medication period (days
13 6 to 16) from the control group of birds (group 1, Figure 4b). Analysis of these samples
14 using LC-MS/MS confirmed the validity of the ELISA findings. The results suggest that
15 a low nitrofurazone concentration could circulate in the air. Ingestion of NFZ by the
16 control chickens that were located in the same room resulted in the bioavailability of
17 SEM in the egg homogenates. Other mechanisms of contamination (i.e. feed, water,
18 faeces or physical contact) were ruled out as possibilities.

19 20 **Conclusions**

21 The ELISA method described in this study offers a reliable screening tool for the
22 determination of total (extractable and tissue-bound) SEM content in egg homogenates.
23 Sensitivity of the immunoassay was comparable to confirmatory LC-MS/MS technique.
24 The method was validated using fortified egg homogenates at levels of 0.3, 1.0 and
25 3.0 $\mu\text{g kg}^{-1}$ of SEM, satisfying the 1 $\mu\text{g kg}^{-1}$ EU MRLP requirement for nitrofurans
26 detection. Detection capability, specificity, recovery and assay variability are compatible
27 with the EU requirements for qualitative and/or semi-quantitative assays as described by
28 Commission Decision 2002/657/EC. A good correlation of results was found for incurred
29 samples measured by screening ELISA and LC-MS/MS, although different sample
30 preparation methods were used. Practical applicability of the ELISA assay was

1
2
3 1 demonstrated by monitoring SEM in eggs collected from chickens treated with
4 nitrofurazone.
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FIGURES LEGEND

Figure 1. ELISA standard curve for the determination of NPSEM. Concentrations are expressed as underivatised SEM equivalents Bars indicate the standard deviation for six replicate assays performed over a one month period. Reagent dilutions were 1:8000 of antibody MVK31 (IgG) and 1:40000 of the HPR conjugate.

Figure 2. Determination of CC_{β} in egg homogenates.

Figure 3. Correlation of the results for the extracted incurred egg samples analysed by ELISA and LC-MS/MS, respectively. a) Within the concentration range 0 – 20 $\mu\text{g kg}^{-1}$, n = 14, b) within low analyte concentrations, n = 10.

Figure 4. Depletion of SEM measured by ELISA in eggs from a) Five chickens fed with meal containing NFZ at a concentration of 400 mg kg^{-1} (Group 3), b) Un-medicated Group 1 control chickens (n = 2). Bars show the standard deviations for the groups of experimental birds. Days 1 to10 indicated represent the medication period.

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Figure 1

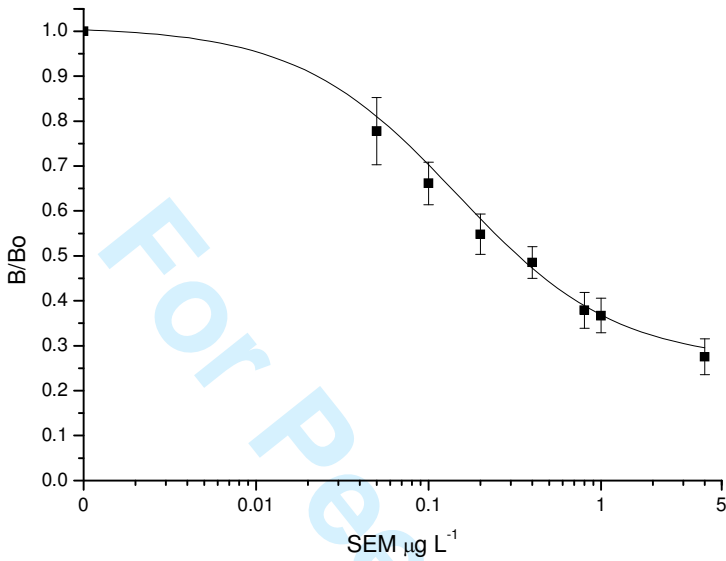


Figure 2

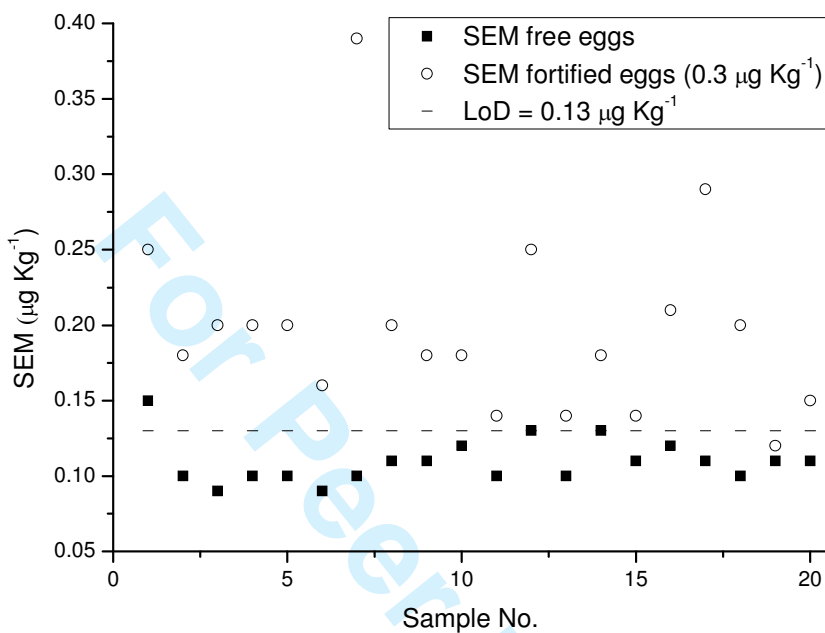


Figure 3

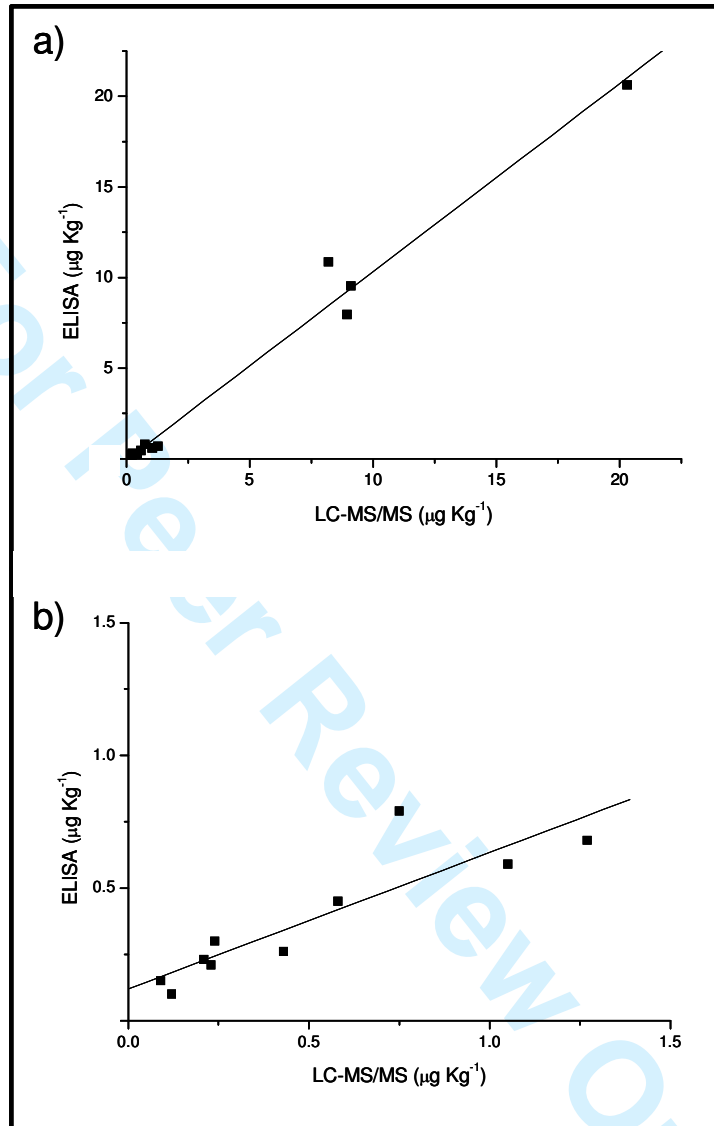
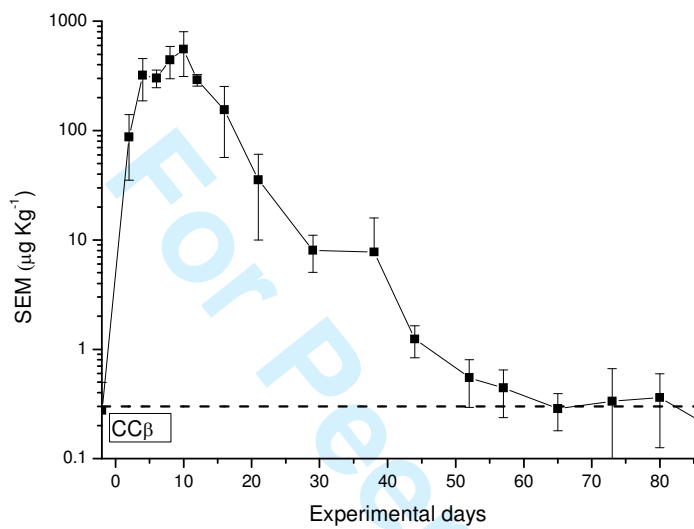


Figure 4

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b)

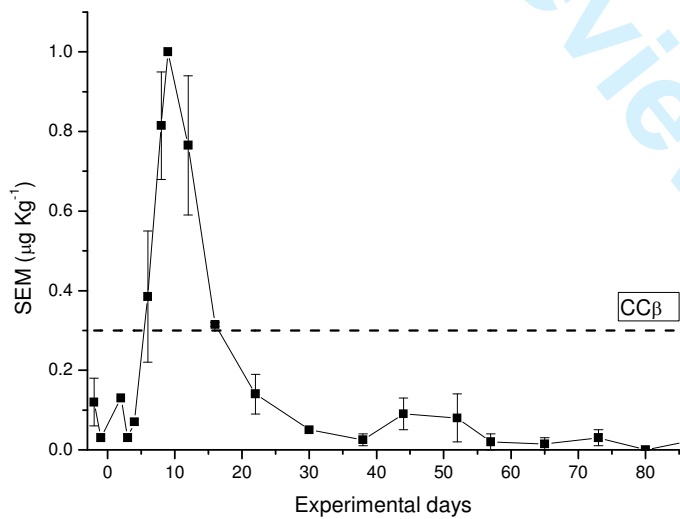


Table1. SEM recovery obtained from fortified egg homogenates by ELISA

	Fortified conc. ($\mu\text{g kg}^{-1}$)	Recovered conc. ($\mu\text{g kg}^{-1}$) \pm SD	Recovery \pm SD (%)	CV (%)
Intra-assay	0.3	0.33 \pm 0.09	110.0 \pm 28.2	28.2
	1.0	0.78 \pm 0.06	77.8 \pm 7.4	7.4
	3.0	2.38 \pm 0.53	79.4 \pm 22.2	22.2
Inter-assay	0.3	0.33 \pm 0.06	109.3 \pm 18.2	18.2
	1.0	0.82 \pm 0.11	81.5 \pm 13.7	13.7
	3.0	2.54 \pm 0.41	84.6 \pm 16.1	16.1

Table2. MS/MS fragmentation data for semicarbazide

Components	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
2NP-SEM	209	166, 192	13
2NP-(¹³ C- ¹⁵ N ₂)-SEM	212	168, 195	13

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