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Application of total error approach to assess the performance of a biological method (ELISA) to detect nicarbazin residues in eggs☆

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ABSTRACT

Nicarbazin, a coccidiostat, is used as a feed additive in poultry but not in laying hens. Feed contamination may however occur resulting in residues being present in eggs. As a Maximum Residue Limit (MRL) does not exist for nicarbazin residues in eggs a "Differential Action Level" (DAL) of 100 μg/kg has been established by the Veterinary Medicines Directorate (VMD). We have studied a commercial ELISA kit validated to detect and quantify nicarbazin in eggs with a sensitivity of 3 μg/kg. We used the total error approach to assess the performance of and validate the kit at the DAL level. The accuracy profile has been successfully obtained for the ELISA kit. The method cannot however be validated as a semi-quantitative method and we have consequently determined a cut-off based on 5% false negative rate according to European Decision 2002/657 on blank and spiked samples (70 μg/kg). The cut-off value established was 20 μg/kg using the 95th percentile.

1. Introduction

Nicarbazin belongs to the family of coccidiostats and is used as a zootechnical feed additive for poultry. Coccidiostats are widely used compounds to prevent and treat coccidiosis, a contagious parasitic disease affecting livestock, particularly poultry, that is associated with warm and humid conditions [1].

According to Regulation 1831/2003/EC [2], anticoccidials are licensed as feed additives. Nicarbazin is authorised for use in broilers but not in laying hens [3]. Accidental cross-contamination of feed, however, has been shown to result in residues of the compounds in eggs [4,5]. Relatively high nicarbazin residue levels have also been found in the liver of poultry and a clear cause–effect relationship has been established between contaminated feed supplied from the feed mill and contaminated feed on the farm. Birds can also be exposed to alternative sources of nicarbazin near to slaughter including older nicarbazin-medicated feed from the feeding system or from litter. According to the Veterinary Medicines Directorate (VMD, United Kingdom), the likely cause of these residues is contamination at the feed mill, during transport and/or inadequate cleaning of hoppers and lines between batches of feed in farms [6].

Nicarbazin is an equimolar mixture of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6 dimethylpyrimidine (HDP). Its marker residue is DNC. Most of the residue analyses for nicarbazin are based on methods detecting the DNC molecule.

The European Commission has concerns over consumer health and has set Maximum Residue Limits (MRLs) for number of veterinary drugs in different matrices (Regulation 2377/90/EC) [7]. There is, however, no MRL for nicarbazin residues in eggs in Europe. In the absence of an MRL a zero tolerance approach should be used.

Several immunoassays for nicarbazin, using different platform technologies as ELISA, Biacore™, lateral flow device or dry chemistry immunoassay have been developed [8–11]. In particular, the developer of the ELISA kit (CER, Laboratory of Hormonology, Marloie, Belgium) has published the development and validation of this ELISA kit for nicarbazin in eggs according to decision EC/2002/657 [12]. The detection capability (Cc β), i.e. the smallest amount of the substance that can be detected, identified and/or quantified in a sample with an error probability of β, for the egg nicarbazin kit has been established as being 3 μg/kg [8].

In practice, some European Member States have used an alternative approach. In 1998, the VMD in the United Kingdom set a "Differential Action Level" (DAL) of 100 μg/kg body weight as a decision threshold for follow-up action.

Because the kit has only been validated in house by the producer, before it can be used in routine analysis by National Reference Laboratories (NRL), we wished to validate it in our laboratory at a DAL level of 100 μg/kg to verify its performance.

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We have analysed the validation data using the total error approach in this work. The total error approach is based on constructing an accuracy profile [13–15], and has been widely discussed for physicochemical methods as described in this special review and in a previously published work [16]. In 2003, the American Association of Pharmaceutical Scientist (AAPS) and the Food and Drug Administration (FDA) [17] recommended that this approach be used for macromolecule ligand binding assays.

We therefore proposed to use this approach to assess the performance of an ELISA method in this study.

To construct an accuracy profile a validation study determining trueness of fit and precision simultaneously is required. From the results of the validation experiments a two-sided β expectation tolerance interval was calculated for each concentration level and the accuracy profile was constructed. We needed to define acceptance limits and the risk of the procedure resulting in a $(1-\beta)$ proportion of measurements falling outside of these limits. The risk was set at 5% and acceptance limits at ±50% in accordance with European Decision 2002/657 [12] and as described previously [16].

2. Materials and methods

2.1. immunoassay procedure

We used the CER ELISA kit (ref E.E.2, Laboratory of Hormonology, Marloie, Belgium).

The test principle and sample extraction methods have been extensively described by Huet et al. [8].

We used different apparatus to that described by Huet et al: a micro-titre plate washer ELP40 (ADIL Instruments, France) and micro-titre plate reader SpectraCount™ (Packard, France) to wash and read the micro-titre plate.

2.2. Total error profile

2.2.1. Calibration standards

Calibration standards were contained ready-to-use in the kit box from the manufacturer. Vials containing standard solutions of DNC of 0.2, 0.5, 2, 5, 10 and 20 μg/l in buffers were provided.

Six series of analysis were performed and calibration samples were analysed in duplicate. The total number of calibration standards are 72 and are sufficient to establish the response function including 4p or 5p logistic models as recommended by Hubert et al. [14].

2.2.2. Validation standards

Validation standards were prepared at 70, 140 and 210 μg/kg in homogenised eggs and were diluted after extraction (1/10), to obtain similar levels to those of the calibration standards before loading onto the micro-titre plate.

Six series of analyses were performed for the validation standards and were measured in triplicate. The total number of validation standards are 54 and are sufficient to establish the accuracy profile as recommended by Hubert et al. [14].

2.2.3. Validation analysis

The validation data were processed on e.noval software, version 2.0, and Seelva Version 1.0 beta 8, for logistic functions (Arlenda, Liège, Belgium).

2.3. Cut-off assessment

In a second process we used the kit as a qualitative tool. To do this we set cut-off criteria and determined concentration values based on positive or negative responses by testing 52 blank samples and 40 samples spiked to 70 μg/kg. We used the 95th percentile value to establish the cut-off, i.e. the value at which samples are deemed to be positive.

3. Results and discussion

3.1. Response function and accuracy profile

The relationships between response and concentrations were analysed using different regression models: linear, weighted linear, quadratic, weighted quadratic, 4 or 5 parameter logistic functions, weighted logistic functions and log–log regression.

We used a number of indexes reflecting the major validation criteria to select the best model. These were defined in a recent paper by Rozet et al. [18] and are contained in the software used. The first is the accuracy index (I_A). This is a global indicator of method performance depending on dosing range index (I_{DR}), trueness index (I_T) and precision index (I_P). The I_{DR} indicates that fraction of the range which is valid; when $I_{DR} = 1$, the whole range studied is accepted. The trueness index is an index describing method bias. An index close to 1 implies that the method is almost unbiased. The precision index describes random variation. An index close to 1 indicates that the method offers good precision.

Table 1 summarises the different indices obtained for the response functions tested. The best I_A was obtained for quadratic regressions with or without weighting. The index values, however, were low, ranging from 0.63 to 0.50. Considering the I_{DR}, the best model was the weighted $(1/X^2)$ quadratic regression with a value of 0.72, i.e. only 70% of the dosing range is acceptable.

Several models have an I_A value of 0 indicating that trueness or precision are poor, outside of the acceptance limits and consequently these models cannot be used directly.

Accuracy profiles for seven response functions tested are shown in Fig. 1. Visual examination confirms considerable variation and lack of trueness.

No calculation was possible for three models (unweighted five parameter logistic regression, weighted linear regression, and linear regression).

3.2. Trueness

Examination of I_T values (Table 1) shows that the best value (unweighted quadratic regression) obtained from a different model to that which had the best I_A (weighted $(1/X^2)$ quadratic regression). This indicates that the choice of model must be based not only on I_A but must take all indices into account.

Considerable lack of trueness is seen for the logistic and log–log models with bias values of close to −50, implying that the I_T and consequently I_A indices are 0.

This bias may be due to a matrix effect between the calibration and validation standards: the calibration standards are provided in buffer but the validation standards are in a complex matrix (homogenised eggs).

3.3. Precision

Two sets of precision values were calculated: repeatability and intermediate precision. Precision was better for the weighted $(1/X^2)$ quadratic regression model than for other quadratic models (Table 1). Considerable variability however was seen at the lowest concentration (7 ng/ml). The relative standard deviation (R.S.D.) of repeatability and the R.S.D. of the intermediate precision are shown in Table 2 for three models. These findings show acceptable repeatability but the intermediate precision which did not comply with the limits set in Decision 2002/657 [12].

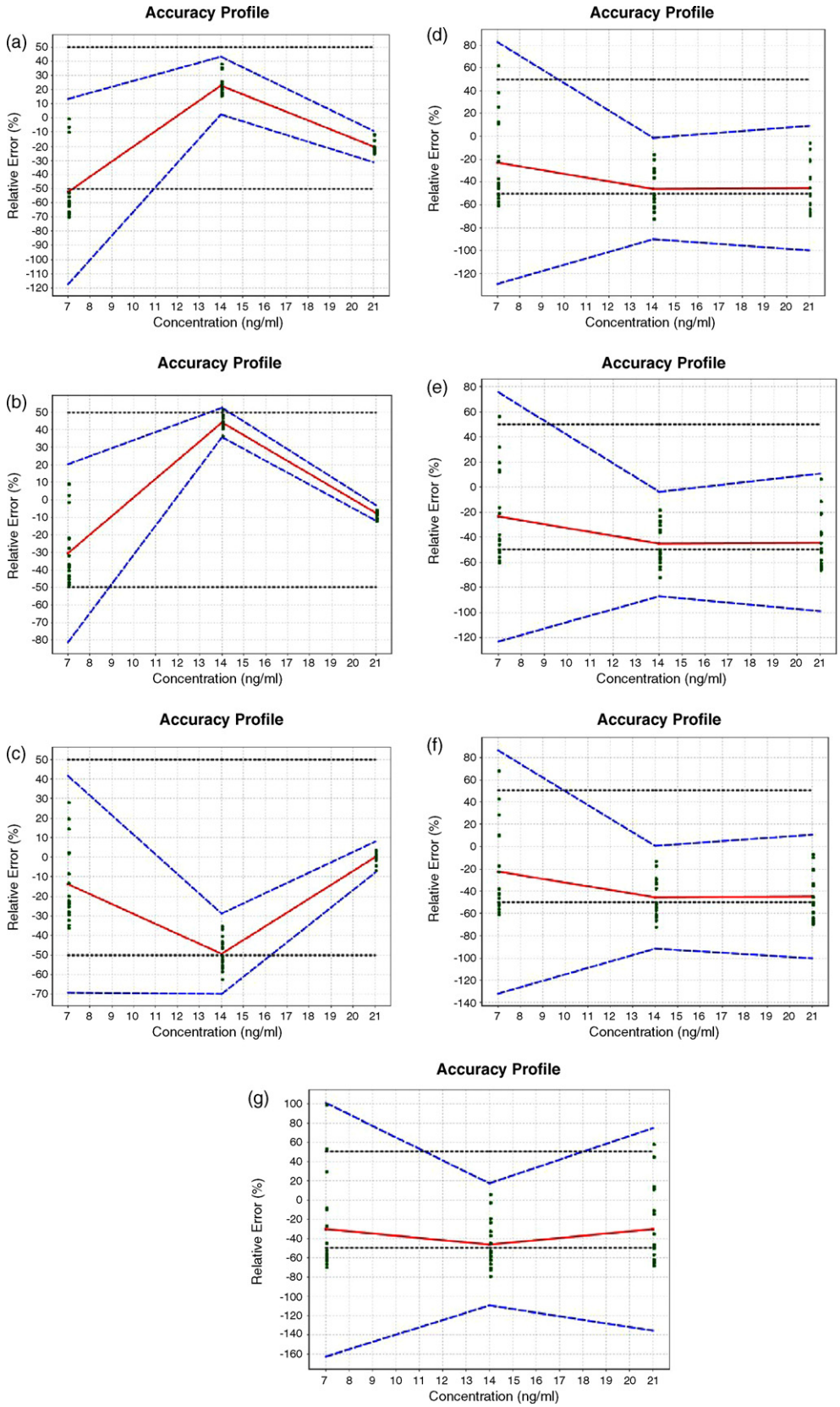

Fig. 1. Accuracy profile obtained for the measurement of the level of nicarbazin in eggs with (a) weighted ($1/X^2$) quadratic regression, (b) weighted ($1/X$) quadratic regression, (c) unweighted quadratic regression, (d) weighted four parameter logistic regression, (e) unweighted four parameter logistic regression, (f) weighted five parameter logistic regression, (g) log–log regression. Plain lines are the relative bias, the dashes lines are the β-expectations tolerance limits, the dotted curves represent the acceptance limits, the dots represent the relative back-calculated concentrations of the validation standards.

Table 1
Indices of the different regression models tested ranked by accuracy index (I_A).

Model	I_A	I_{DR}	I_P	I_T	Figure
Weighted ($1/X^2$) quadratic regression	0.64	0.72	0.70	0.52	1a
Weighted ($1/X$) quadratic regression	0.56	0.48	0.61	0.61	1b
Unweighted quadratic regression	0.50	0.34	0.57	0.65	1c
Weighted four parameter logistic regression	0	0	0	0	1d
Unweighted four parameter logistic regression	0	0	0	0	1e
Weighted five parameter logistic regression	0	0	0	0	1f
Unweighted log–log regression	0	0	0	0	1g
Unweighted five parameter logistic regression	NC	NC	NC	NC	
Weighted linear regression	NC	NC	NC	NC	
Linear regression	NC	NC	NC	NC	

I_A: accuracy index; I_{DR}: dosing range index; I_T: trueness index; I_P: precision index; NC: not calculated.

Table 2
Precision (repeatability and intermediate precision) obtained for models used, by concentration level tested.

Models	Concentration level (ng/g)					
	7		14		21	
	Repeat. (R.S.D. %)	IP (R.S.D. %)	Repeat. (R.S.D. %)	IP (R.S.D. %)	Repeat. (R.S.D. %)	IP (R.S.D. %)
Weighted ($1/X^2$) quadratic regression	2.72	23.62	1.36	7.49	1.01	4.11
Weighted ($1/X$) quadratic regression	4.23	18.69	2.26	3.53	1.34	1.90
Unweighted quadratic regression	6.30	20.75	3.52	7.98	2.09	3.27

Repeat: repeatability, IP: intermediate precision, R.S.D.: relative standard deviation (precision) (%).

3.4. Solutions tested

A number of solutions can be used to resolve the trueness problem. The first is to use a correction coefficient as described by Hubert et al. [19] to correct the matrix effect. The correction coefficient was computed from the slope of the linear equation linking theoretical spiked concentration to recovered concentration computed by inverse prediction. The correction coefficient used is the reciprocal of the slope achieved with the validation standards.

The equation of the line is:

$$[\text{Recovered}] = 1.867 + 0.454\ [\text{Added}]$$

and the correction coefficient to be applied to the instrument response is therefore: new results = (old results − 1.87)/0.45.

Fig. 2 shows the new accuracy profile then obtained. For trueness the I_T was close to 1, i.e. bias was close to 0 although precision was amplified at all levels. This solution cannot therefore be used.

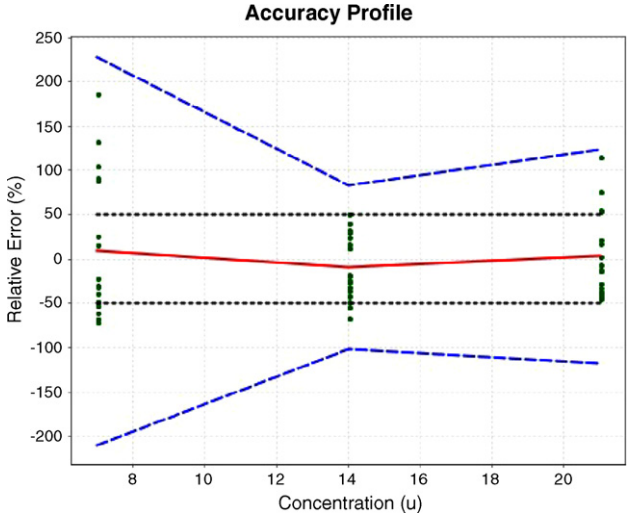

Fig. 2. Accuracy profile obtained with corrected results. Plain lines are the relative bias, the dashes lines are the β-expectations tolerance limits, the dotted curves represent the acceptance limits, the dots represent the relative back-calculated concentrations of the validation standards.

The second solution is to work in a dosing range from 14 to 21 ng/ml without correction. In this case the best response function is the weighted ($1/X$) quadratic regression.

The third solution is to use the method as a qualitative test.

3.5. Cut-off determination

A cut-off value was determined using blank samples and samples spiked to 70 μg/kg. Because the high variability seen in the validation and according to Decision 2002/657 [12] it is reasonable to set a false negative rate of 5%. A cut-off value of 20 μg/kg was established by calculating the 95th percentile of our samples. This value is acceptable as it is well below the DAL of 100 μg/kg. The false positive rate at this cut-off is less than 1%.

3.6. Comparison with initial data published

It is difficult to compare our validation results with those obtained by Huet et al. [8]. This author has not really calculated trueness and precision so the point by point comparison on these criteria is not possible. However, Huet has compared results obtained by an LC–MS–MS method and its ELISA method from incurred or spiked eggs and not from quantitative validation study with standard curves. The results have shown a high variability, i.e. accuracy, depending on concentration as shown by our validation results.

4. Conclusion

We have used the accuracy profile successfully to assess the performance of the ELISA kit for nicarbazin in eggs. The performance of the method was compared to the acceptance limits set in accordance with European Decision 2002/657 [12]. Under our conditions, however, the method cannot be validated for use as a semi-quantitative method as both trueness and precision are not within regulatory acceptable limits.

We have tested alternative solutions to solve the problem of trueness. The best solution found was to reduce the dosing range.

The method should be used as a qualitative method to detect the presence of nicarbazin in eggs with a cut-off of 20 μg/kg.

References

[1] L. Mortier, A.-C. Huet, C. Charlier, E. Daeseleire, P. Delahaut, C. van Peteghem, Food Addit. Contam. 22 (2005) 1120.
[2] Council Regulation (EC) No. 1831/2003, Off. J. Eur. Commun. L268 (2003) 29.
[3] Council Directive (EC) No. 70/524/EEC, Off. J. Eur. Commun. L270 (1970) 1.
[4] C.T. O'Keeffe, M. Capurro, E. Danaher, M. Campbell, K. Elliott, Food Addit. Contam. 24 (2007) 923.
[5] A. Cannavan, G. Ball, D.G. Kennedy, Food Addit. Contam. 17 (2000) 829.
[6] R. Young, A. Craig (Eds.), The Use and Misuse of Antibiotics in UK Agriculture—Part 3, Soil Association, Bristol, UK, 2001, p. 1.
[7] Council Regulation (EEC) No. 2377/90, Off. J. Eur. Commun. L224 (1990) 1.
[8] A.-C. Huet, L. Mortier, E. Daeseleire, T. Fodey, C. Elliott, P. Delahaut, Food Addit. Contam. Part A 22 (2005) 128.
[9] B. McCarney, I.M. Traynor, T.L. Fodey, S.R.H. Crooks, C.T. Elliott, Anal. Chim. Acta 483 (2003) 165.
[10] K. Campbell, T. Fodey, C. Flint Danks, M. Danaher, M. O'Keeffe, G. Kennedy, C.T. Elliott, J. Agric. Food Chem. 55 (2007) 2497.
[11] V. Hagren, S.R.H. Crooks, C.T. Elliott, T. Lovgren, M. Tuomola, J. Agric. Food Chem. 9 (2004) 2429.
[12] Commission Decision 2002/657/EC, Off. J. Eur. Commun. L221 (2002) 8.
[13] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (2) (1999) 135.
[14] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharma 13 (2003) 101.
[15] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (3) (2004) 57.
[16] E. Chéneau, J. Henri, Y. Pirotais, J.P. Abjena, B. Roudaut, P. Sanders, M. Laurentie, J. Chromatogr. B 850 (2007) 15.
[17] B. DeSilva, W. Smith, R. Weiner, M. Kelley, J.M. Smolec, B. Lee, M. Khan, R. Tracey, H. Hill, A. Celniker, Pharm. Res. 20 (11) (2003) 1885.
[18] E. Rozet, V. Wascotte, N. Lecouturier, V. Préat, W. Dewé, B. Boulanger, Ph. Hubert, Anal. Chim. Acta 591 (2007) 239.
[19] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, STP Pharma 16 (2) (2006) 87.