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IN-HOUSE VALIDATION AND QUALITY CONTROL OF COMMERCIAL ENZYME-LINKED IMMUNOSORBNET ASSAYS FOR SCREENING OF NITROFURAN METABOLITES IN FOOD OF ANIMAL ORIGIN

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ABSTRACT

Application of nitrofuran antimicrobials at food production animals was prohibited by Commission Regulation 2003/181/EC because of their potential carcinogenic and mutagenic effects on humans. Main protein-bound metabolites of nitofurans are 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD), semicarbazide (SEM) and 3-amino-2-oxazolidinone (AOZ). Since then numerous costly liquid chromatography with tandem mass spectrometry (LC/MS/MS) methods have been developed for screening and confirmation of nitrofuran metabolites in line with the EU requirements for performing official controls. As an inexpensive and less time consuming alternative, enzyme-immunoassay methods were developed for screening of the respective compounds. In this study validation and evaluation of four commercial enzyme-linked immunosorbent assay (ELISA) has been performed. According to the requirements of Commission Decision 2002/657/EC, different performance characteristics (specificity, detection capability, precision) for various matrices (liver, eggs, honey) have been determined for each kit. The validation study has confirmed that the methods studied possess suitable characteristics: detection limits between 0.126 and 0.240 µg/kg, detection capabilities ≤1.0 µg/kg and the inter-day precision in the range from 16.20 to 22.11 %. The validation study was finalized by participation in FAPAS Proficiency testing scheme in 2011, and the obtained results have confirmed the capability of applied methods for unambiguous discrimination between negative and positive sample.

Key words: nitrofurans, metabolites, AOZ, AMOZ, SEM, AHD, ELISA, detection capability

INTRODUCTION

Nitrofurans (NFs), by their chemical structure, belong to a group of antimicrobial substances which all contain 5-nitrofuran ring and different substituents on the position 2 (Figure 1A). Use of the NF antibiotics in food-producing animals was prohibited within the European Union (1), because of their potentially carcinogenic and mutagenic effects on

human health. During the years, it was found that monitoring of parent NFs in animal tissues was not suitable for regulation enforcement due to the poor stability of the parent NFs. It has been confirmed that they metabolite rapidly, and the metabolites bind to cellular proteins where they persist for a considerable period after the treatment (2). Therefore, NF metabolites AOZ (3-amino-2-oxazolidone), AMOZ (3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one), AHD (1-aminohydantoin) and SEM (semicarbazide) (Figure 1B) have been established as markers for NF residue testing.

NFs are substances without established Maximum Residue Levels (MRLs) (3), and therefore

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European Commission has set the Minimum Required Performance Levels (MRPLs) for these substances at 1 μg/kg (1). The term of MRPL has been introduced with Commission Decision 2002/657/EC (4) for banned substances for which no MRLs have been established. With this document, the concept of routine or reference methods has been suppressed by criteria approach, whereas the criteria for performances and procedures for method validation have been established.

Since then, the chromatographic methods, predominantly LC/MS/MS methods, have been developed for detecting, quantifying and confirming NF metabolites at concentration levels lower than the established MRPL (5, 6). These methods are highly sensitive and specific; however, they are also time-consuming and expensive. Enzyme-linked immunosorbent assay (ELISA) is an effective alternative

to instrumental detection offering fast, reliable and relatively inexpensive detection and quantitative determination of low concentrations of target analytes. For screening purposes, a method offering efficient routine analysis, which enables the pre-selection of positive samples prior to confirmatory analysis, is required. ELISA technology provides a very convenient way to reach the sample frequency requirements with reduced cost in terms of both time and resources.

The first reported ELISA method has been for detection of the metabolite AMOZ in edible tissues (7). Later, few more papers were focused on development and optimization of ELISA methods for AMOZ in shrimps (8) and different food samples (9). Some of the published ELISA methods were designated for detection of AOZ in tissue samples (10-12), AHD in water and tissue samples (13, 14),
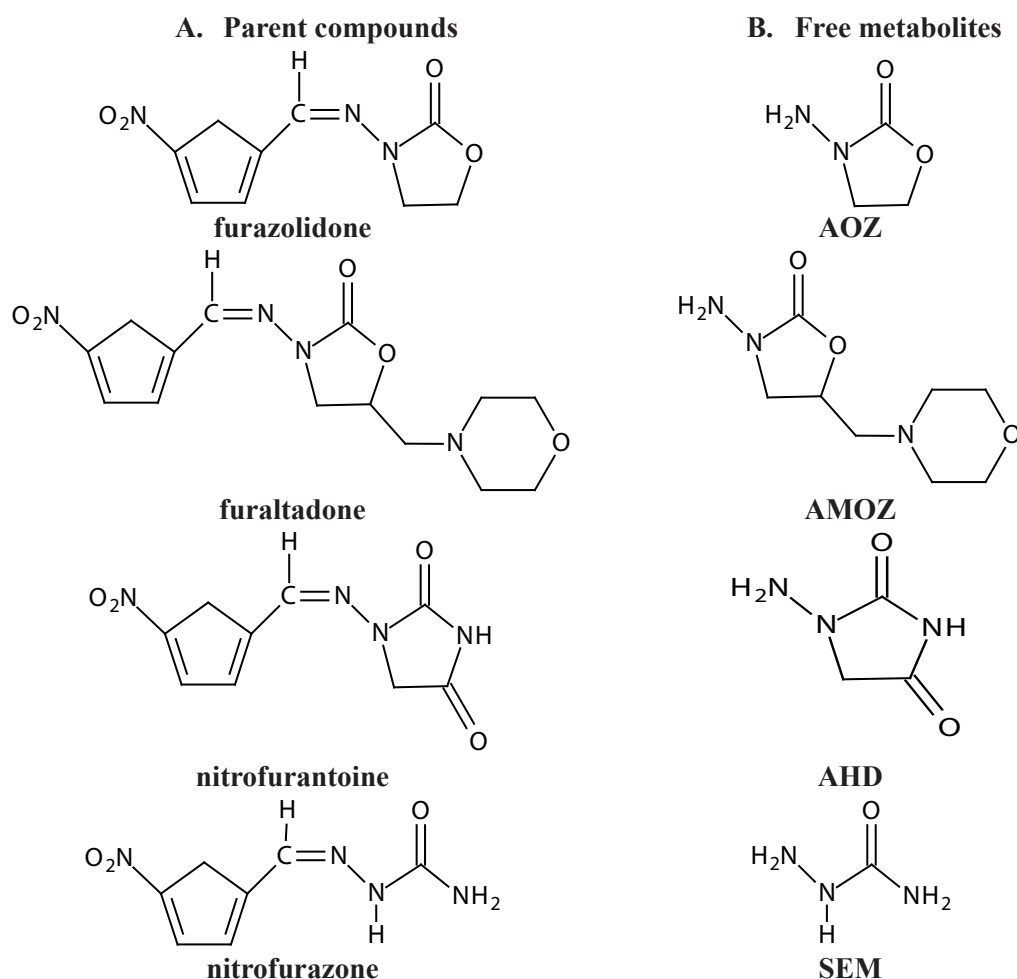
Figure 1. Chemical structures of nitrofuran parent compounds (A) and their correspondent metabolites (B)

and SEM in tissue samples and eggs (15, 16). All of these methods were developed employing monoclonal antibodies for single-component analysis. Usually, sample preparation involves acid-hydrolysis for the release of tissue-bound residues in a sample, followed by derivatisation with o-nitrobenzaldehyde, or alternatively benzaldehyde (12). The formed compounds are derivatised nitrophenyl (NP) analytes, enabling more sensitive detection of total NF metabolites (bound and free).

To the best of our knowledge, no data are available for validation of ELISA screening methods for detection of NFs in honey samples. The importance for application of sensitive, selective and precise methods arises from the fact that during the 2007 and 2008 on the EU Rapid Alert System there were 54 notifications related to the presence of NF substances in food, mainly imported to EU from third countries (17).

This paper describes the in-house validation with aim to evaluate the performances of four ELISA kits for the determination of AOZ, AMOZ, SEM and AHD in different matrices - liver, eggs and honey, in accordance with the EU performance criteria for qualitative screening methods (4). Validation was performed by analyzing fortified blank samples at different concentration levels of interest. The suitability of the methods was estimated by the obtained performances for the sensitivity, selectivity, detection capability and precision. Furthermore, the methods were tested for their validity through participation in FAPAS proficiency testing in incurred sample material - hen eggs.

MATERIALS AND METHODS

Blank sample materials

Liver samples from pigs and poultry, chicken eggs and floral honey were previously tested by ELISA to be confirmed as blanks. Because no measurable amounts of the tested compounds were found, the samples were considered to be free of NF metabolites.

Reagents and standard solutions

AOZ (type R3703) and AMOZ (R3711) ELISA kits were purchased by R-Biopharm (Darm-

stadt, Germany), while AHD (NF3463) and SEM (NF3461) were products of Randox (Crumlin, UK). 2-nitrobenxoic aldehyde, dimethylsulfoxide, ethylacetate, n-hexane dipotassiumhydrogen phosphate, hydrochloric acid (1 mol/dm^3) and sodiumhydroxide (1 mol/dm^3) were purchased from Merck (Darmstadt, Germany). The certified pure neat standards of semicarbazide hydrochloride, AOZ, AMOZ, 2-nitrophenyl AHD (VETRANAL) were supplied by Fluka (St. Gallen, Switzerland).

Preparation of standards and spiking of blank samples

Stock standard solutions with the concentration 1 mg/ml were prepared monthly by dissolving the neat NF metabolite compounds in methanol. Intermediate working solutions were prepared prior to application in sample dilution buffers, provided in the test kits. These solutions were used for spiking blank tissue, eggs and honey samples at two concentration levels: one half of MRPL and MRPL. Following fortification, samples were allowed to equilibrate for 15 min before extraction.

Instruments

For sample preparation vortex model Relax Top by Heidolph (Schwabach, Germany), centrifuge model 2K15 by Sigma (St. Louis, USA), evaporator model DriBlock DB-3D by TECHNE (Staffordshire, UK) were used. The optical density at 450 nm for the ELISA tests was measured by microplate reader Model 680 Bio-Rad (Philadelphia, USA).

Sample preparation for the screening methods

Sample preparation, as well as preparation of all reagents was done according to the manufacturer's instructions. All samples were derivatized with 10 mM solution of 2-nitrobenzoic aldehyde for 2 hours at 37oC. After extraction with ethylacetate, an aliquot was evaporated under a stream of nitrogen. The sample extracts were defatted with n-hexane, dissolved in phosphate sample dilution buffer and applied on the microtiter plates. Sample dilution factor was 2.

Validation of the screening methods

Performance characteristics of EIA methods were determined as prescribed for qualitative screening

methods in Commission Decision 2002/657/EC (4). The limits of detection (LOD) and limits of quantification (LOQ) were determined measuring the signal of twenty repetitive samples for each matrix. Ten pig and ten chicken liver samples were tested in one batch. Recovery was calculated from the repeated measurement of ten fortified blank sample's replicates, at 0.5, and 1.0 µg/kg. From the recovery experiments, the method precision, expressed trough the coefficient of variation (CV) was obtained, as well. The calculations for the detection capability (CCβ) were performed by the formula provided in the EU Commission Decision (4). Specificity of the assays towards nitrophenyl derivatives – NP-AOZ, NP-AMOZ, NP-SEM and NP-AHD and potential cross-reactants was described elsewhere (18, 19).

RESULTS AND DISCUSSION

Calibration curves

The ELISA curves for AOZ, AMOZ, AHD and SEM in assay buffer are logarithmic, and the responses of the concentrations are expressed as B/B_o ratio in %, where B is the absorbance at a given concentration of the analyte and B_o the absorbance of the zero standard. The calibration stability and repeatability was assessed over six month's period from eight repetitive calibration curves. The CVs calculated for individual calibration points of the standard curves ranged from 4.6 to 16.3 %, indicating good calibration stability. The mean IC_{50} value (corresponding to 50 % binding inhibition) for the eight repeated assays was 0.124±0.012 ng/mL for AOZ, 0.791±0.148 ng/mL for AMOZ, 0.106±0.021 ng/mL for SEM, and 0.131±0.038 ng/mL for AHD. IC_{50} value is a quantitative characteristic for the assay sensitivity – lower IC_{50} indicates higher sensitivity. In this study lower sensitivity could be attributed to AMOZ test, while the highest sensitivity was estimated for semicarbazide. These data are comparable to the ones obtained from other authors (9, 20). For the calibration curves used for the quantification of the NFs in different samples in this study, standard solutions were prepared in phosphate buffer. In a

previous study (7) matrix-matched standard curves were usually used to reduce potential matrix effects in the analytical procedures. Although in that study good detection capability (0.4 µg/kg in tissues) has been obtained, the preparation of matrix-matched standards from blank samples prior to analysis might be less favorable (12), since it is more time consuming when different matrices are tested in single batch. Furthermore, some investigations suggest that the preparation of matrix-matched calibration curves might negatively affect the method precision and accuracy (7). For convenient incorporation of the ELISA kits, the standard curves based on phosphate buffer solution (PBS) were performed in this work.

Limit of detection and detection capability

The LODs of the ELISA assays for AOZ, AMOZ, SEM and AHD in various matrices based on 20 blank samples, accepting no false positive rates (average+3SD), are below 0.3 µg/kg (Figure 2) and significantly lower than the required MRPL value (1 µg/kg). The obtained LODs are similar to those obtained in the proposed method of certain authors (7), but somewhat higher than the assessed LODs of other authors (15).

The screening procedures do not need to be fully quantitative, but they should measure the presence or absence of the target analyte at/or below regulatory levels. Thus, performance data, including the detection capabilities of the assays were assessed with respect to the established MRPL for nitrofuran metabolites in food of animal origin (4). Detection capabilities (CCβ) of the assays were determined using 20 different liver, eggs and honey samples fortified at a level lower than the MRPL. The investigations were performed by one technician in two days, with one week distance.

Figure 2 demonstrates the assessment of the CCβ for AHD, AMOZ, AOZ and SEM respectively. All estimated CCβ values for each NF metabolite/matrix combination were significantly below the MRPL value, indicating that the requirement for false negative rates (not to be more than 5 %) is fully satisfied (4).
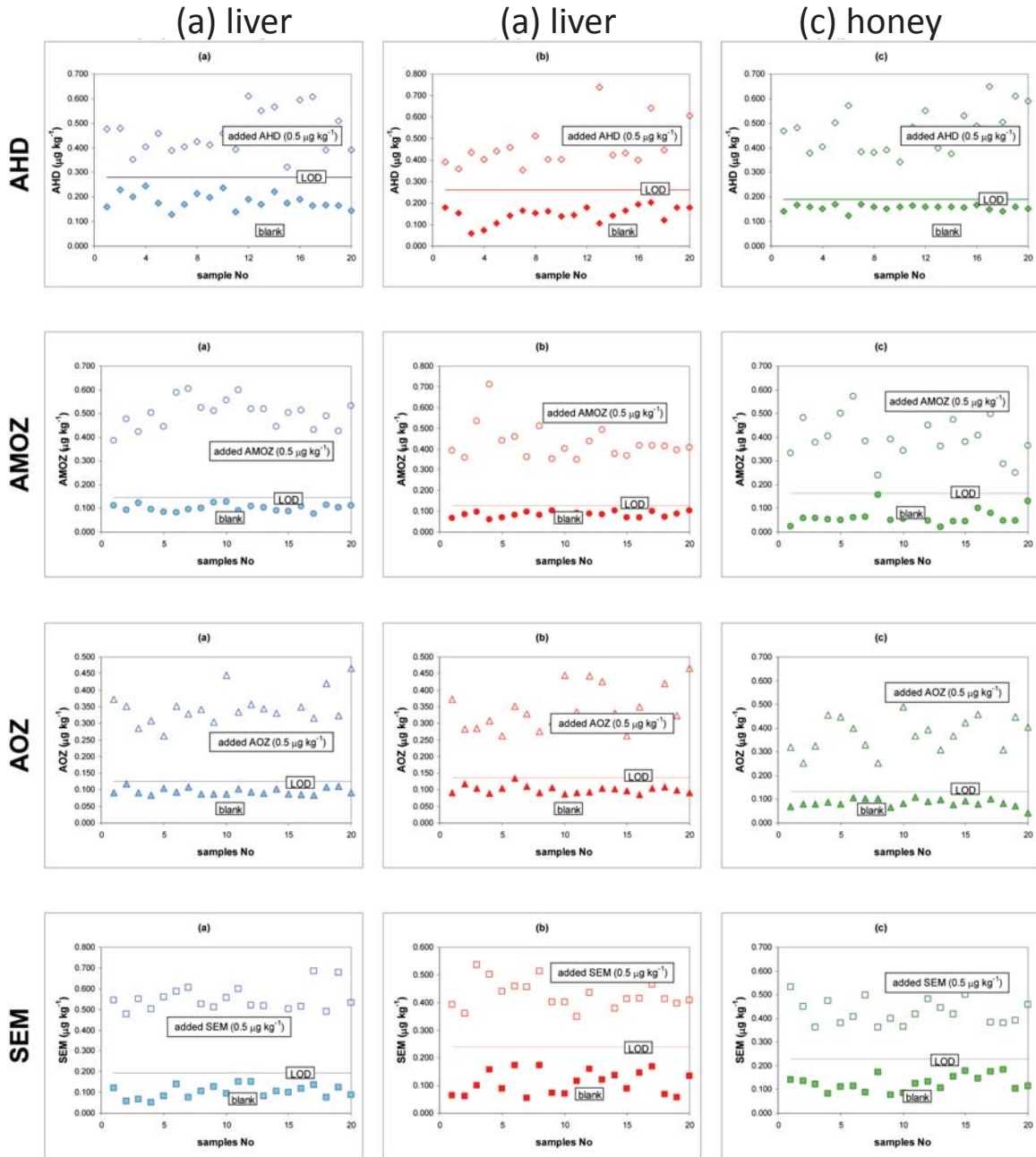
Figure 2. LODs and detection capabilities for AHD, AMOZ, AOZ and SEM in liver (a), eggs (b) and honey (c)

The estimated CCβ values for AHD, AMOZ, AOZ and SEM were in the range 0.567-0.617 μg/kg, 0.616-0.681 μg/kg, 0.584-0.671 μg/kg and 0.563-0.600 μg/kg, respectively.

For a quantitative screening test, Commission Decision 2002/657/EC (4) requires supplying the detection capability (CCβ), defined as the lowest concentration that can be determined with an error probability of β (≤5%). Theoretically, if 19 of the fortified samples were declared non-compliant, then CCβ is equal to the level of fortification. Additionally, if all of the fortified samples are declared non-compliant, then CCβ is lower than the level of fortification. In practice, the levels of fortification for each NF and for each matrix were chosen as to ensure that all of the fortified samples were declared non-compliant. This decision should avoid the problem of false negatives; we kept in mind, however, that the level of fortification must be as low as possible.

Assessment of assay recovery and precision

To ascertain the information on matrix interferences and assay variability, a set of blank samples were fortified with the analytes at concentrations of 0.5, and 1.0 μg/kg and carried through the ELISA procedure. The results of this study are shown in Tables 1-4. Mean recovery values were calculated from ten individual sample measurements at each concentration level for liver tissue, eggs and honey, applied in duplicate at the microtiter plate (N=20) The signals of the blanks were subtracted from the obtained signals of the fortified samples. In the most of the cases recovery values varied between 80 and 90 %. No significant variations in CVs between each compound and for different matrices have been observed. The recovery rates are fully in line with the recommendations laid down in Decision 657/2002 (4).

Intra-assay CV, calculated from ten recovery measurements, carried out using one microtitre plate simultaneously, ranged from 10.18 to 14.48 % for AOZ, from 10.52 to 15.16 % for AMOZ, from 8.52-16.62 % for AHD and from 8.59 to 12.94 % for SEM. The inter assay precision estimated on the basis of recovery experiments repeated after one week were within the range of 16.20 % to 22.11 %. The estimated intra- and inter-assay recovery and precision were satisfactory in terms on the requirement from the Commission Decision regarding the screening methods (4).

Table 1. AOZ recovery from fortified liver, eggs and honey blank samples

Matrix	Fortified concentrations (μg/kg)	Recovered concentrations (μg/kg) ±SD	Recovery (%)	CV (%)
Liver	0.5	0.436±0.039	87.16	10.94
	1.0	0.828±0.108	82.85	13.06
Eggs	0.5	0.421±0.061	84.30	14.48
	1.0	0.833±0.106	83.34	12.75
Honey	0.5	0.413±0.042	82.53	10.18
	1.0	0.816±0.093	81.57	11.38

Table 2. AMOZ recovery from fortified liver, eggs and honey blank samples

Matrix	Fortified concentrations (μg/kg)	Recovered concentrations (μg/kg) ±SD	Recovery (%)	CV (%)
Liver	0.5	0.446±0.049	88.45	10.98
	1.0	0.813±0.086	81.29	10.52
Eggs	0.5	0.436±0.048	87.24	11.10
	1.0	0.871±0.132	87.14	15.16
Honey	0.5	0.410±0.053	82.16	13.02
	1.0	0.828±0.104	82.84	12.56

Table 3. AHD recovery from fortified liver, eggs and honey blank samples

Matrix	Fortified concentrations (μg/kg)	Recovered concentrations (μg/kg) ±SD	Recovery (%)	CV (%)
Liver	0.5	0.425±0.041	85.04	9.73
	1.0	0.842±0.084	84.23	9.94
Eggs	0.5	0.416±0.048	83.18	11.47
	1.0	0.858±0.125	85.84	14.51
Honey	0.5	0.431±0.072	86.16	16.62
	1.0	0.888±0.109	88.80	12.26

Table 4. SEM recovery from fortified liver, eggs and honey blank samples

Matrix	Fortified concentrations (μg/kg)	Recovered concentrations (μg/kg) ±SD	Recovery (%)	CV (%)
Liver	0.5	0.412±0.043	82.40	10.37
	1.0	0.848±0.094	84.79	11.04
Eggs	0.5	0.446±0.058	89.23	12.94
	1.0	0.901±0.115	90.15	12.71
Honey	0.5	0.441±0.046	88.16	10.53
	1.0	0.890±0.109	88.98	12.26

Upon use of the PBS calibration standard curve, the good precision and accuracy of the applied ELISA methods were observed. There are some opinions that the use of a matrix-matched standard curves to reduce potential matrix effects in the analytical procedures was less feasible (12).

Specificity

Specificity of ELISA detection is determined by the binding properties of antibody incorporated into the assay system. High specificity of the antibodies towards NP-AOZ, NP-AMOZ, NP-AHD and NP-SEM allows good selectivity towards structurally related substances in the sample, without use of additional clean-up steps. Cross-reactivity values were calculated through 50 % binding inhibition by NP-NFs standards and 50 % binding inhibition of potentially interfering compound. Cross-reactivities of these assays for potential cross-reactants are presented elsewhere (18, 19). Additionally, beside the structurally related substances, it was determined that the assay revealed negligible response for o-nitrobenzaldehyde (<0.01 %), and thus eliminating the need for separation of this reagent from the reagent mixture after derivatization.

Test quality control in routine analysis

During the tests application, quality control (QC) procedures have been established for monitoring the tests validity. Following the suggested strategy in Chapter 7 of Guidelines (21), the internal QC was performed in a qualitative manner, i.e. controlling whether the day-to-day concentrations of negative and positive QC samples were, respectively lower and higher than the methods decision limits (0.65 μg/kg for AHD and SEM; 0.70 μg/kg for AOZ and AMOZ). This internal QC strategy is similar to that implemented in quantitative analysis at Shewhart control charts (22).

Except the internal QC, the methods validities have been tested by participation in FAPAS proficiency testing scheme 02179 (November-December 2011), with incurred hen eggs as the test material. AOZ was the only present compound in the sample and the only reported substance. Although the obtained Z-score was higher than ±2, the fact that neither false positive results nor false negative results were reported, confirms the applicability and reliability of the validated NF methods for screening purposes.

CONCLUSIONS

A practical example of validation of commercial screening immunochemical methods for the determination of nitrofuran metabolites AOZ, AMOZ, SEM and AHD in liver, eggs and honey have been reported describing possible strategies and their implications. This paper is an attempt to summarize the state of the art in the validation of quantitative screening test for banned substances according to Commission Decision 2002/657/EC (4). In 2010 the EURL's Guidelines (21) have indicated a practical and very useful approach for routine laboratories involved in Official control programs (23). Validation results considering sensitivity, precision, accuracy and detection capabilities are compatible with the EU requirements for qualitative and/or semi-quantitative assays. The detection capability of the assay for all matrices being tested was sufficiently lower than the MRPL of 1 μg/kg and still significantly different from the signals of the blanks. Internal quality control procedure was implemented building control charts for non-compliant day-to-day control samples. Practical applicability of the ELISA assay was demonstrated by participation in PT scheme for incurred hen eggs, demonstrating that the validated ELISA tests are suitable for application in screening

analysis for official controls. These results indicated that the method had the major advantages of being rapid, simple, and convenient for using in routine screening analysis, especially for the analysis of a large number of samples simultaneously.

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