9th Workshop on (Bio)sensors and bioanalytical microtechniques in environmental and clinical analysis, Université de Montréal, Montréal, CANADA, June 14th to June 17th 2009

Validation of 4 ELISA kits for the screening of nitrofuran metabolites (AOZ, AMOZ, SEM, AHD) in porcine muscle

Valérie GAUDIN, Céline HEDOU, Eric VERDON

***AFSSA** (French Food Safety Agency), **LERMVD** (Laboratory for Studies and Research on Veterinary Drugs and Disinfectants), Community Reference Laboratory for Antimicrobial Residue Control in Food Producing Animals, La Haute Marche-Javené, 35302 Fougères, France*
e-mail : v.gaudin@fougeres.afssa.fr

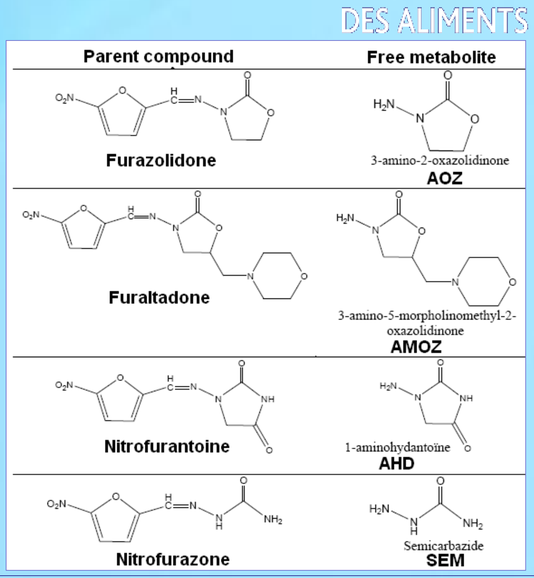
AGENCE FRANÇAISE DE SÉCURITÉ SANITAIRE DES ALIMENTS

SUMMARY

The use of nitrofurans (furaltadone, nitrofurantoin and nitrofurazone) has been banned for food producing animals since 1993 and since 1995 for furazolidone. Nitrofuran parent drugs are very quickly metabolised. The main protein-bound metabolites are 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), semicarbazide (SEM). and3-amino-2-oxazolidinone (AOZ), respectively.

Since beginning of 2000's, the analysis of residues of nitrofuran drugs is based on the detection of the protein-bound metabolites. In 2003, a definitive MRPL (Minimum Required Performance Limit) was set at 1.0 µg/kg in the European Union for nitrofuran metabolites in poultry and aquaculture products. Since that date, costly LC/MS-MS methods have been developed for the screening and the confirmation of nitrofuran metabolites in line with EU regulations.

Until last year, only two ELISA kits were commercialized for the screening of AOZ and AMOZ. However, 4 ELISA kits have been recently developed and commercialized for the detection of the 4 nitrofuran metabolites (AOZ, AMOZ, SEM and AHD). These kits have been evaluated and validated in our laboratory according to European Decision 2002/657/EC [1] which concerns the performance of analytical methods. Different performance characteristics (specificity, detection capabilities and cross-reactions) have been determined for each kit.



Material and methods

Preparation of porcine muscle:

1g ± 0.05g of the homogenized sample
↓
4 ml deionized water, 0.5 ml 1 M HCl, 100 µl 10 mM 2-Nitrobenzaldehyde solution, shake
↓
Incubate at 37°C over night
↓
5ml 0.1 M K2HPO4, 0.4ml 1M NaOH, 5ml ethyl acetate, shake 30s
↓
Centrifuge at 3500 g at room temperature for 10 min
↓
Transfer 2.5ml ethyl acetate layer (upper layer)
Evaporate to dryness by nitrogen at 50°C
↓
Dissolve dry residue in 1ml N-hexane, Add 1ml of diluted redissolving solution, mix
↓
Centrifuge at 3500 g at room temperature for 10min
↓
Take 50µl of the lower aqueous phase per well in the assay

Fold of dilution of the sample:2

ELISA procedures:

Add 50 µl of extract or 50 µl of standard solution into each well,
↓
Add 50 µl of the antibody solution, seal the microplate
↓
Incubate at 37°C for 30 min
↓
Add 250 µl/well of washing bufferfor 15 sec
Take out and flap to dry with absorbent paper
Repeat 5 times
↓
Add 100 µl enzyme conjugate
Incubate at 37°C for 30 min
↓
Add 250 µl/well of washing bufferfor 15 sec
Take out and flap to dry with absorbent paper
Repeat 5 times
↓
Add 50 µl of the substrate A solution and 50 µl of the B solution
↓
Mix gently
Incubate at 37°C for 15 min at dark
↓
Add 50 µl of the stop solution. Mix gently
↓
Read at 450 nm (Recommended dual-wavelength 450/630 nm)

Validation protocol:

The analyses have been performed blindly (random number given to samples).

- Specificity, false positive rate: Capacity of a method to distinguish the measured analyte from other substances.

Analyse in duplicate n> or = 60 representative blank samples.

- Detection capability CCβ: **CCβ = the concentration level, where only ≤ 5 % false compliant results remain (maximum 3 false compliant out of 60 fortified samples).**

Analyse in duplicate n> or = 60 blank samples spiked at the MRPL (1 µg/kg) or lower with the target molecule of each kit.

- Cross-reactions: Molecules at 10000 ng/g in duplicate: 4 parent nitrofuran molecules (furazolidone, furaltadone, nitrofurazone, nitrofurantoin), the 3 other metabolites depending on the kit + 6 major categories: beta-lactams, sulfonamides, tetracyclines, macrolides, aminoglycosides, quinolones.

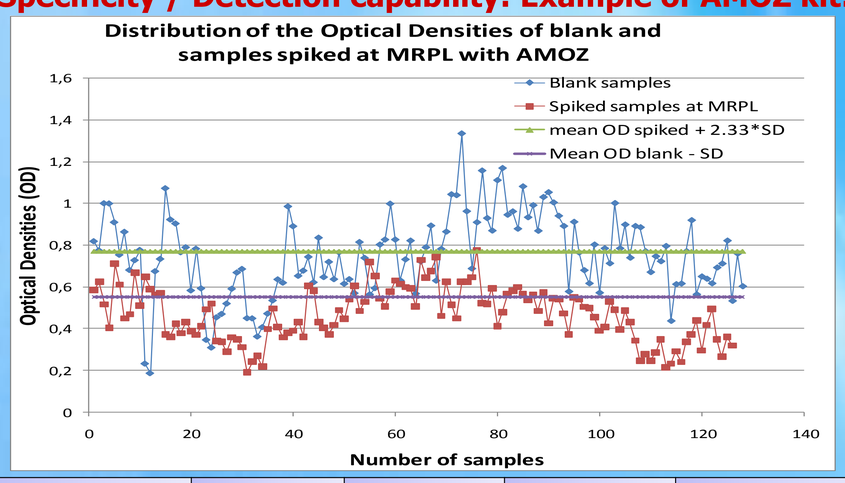
- Precision (intra and inter-day repeatability): Spiked samples at 1, 1.5 and 2 times the MRPL. Analyse at least 6 replicate (3 days) .

Participation to an international proficiency test (IPT)

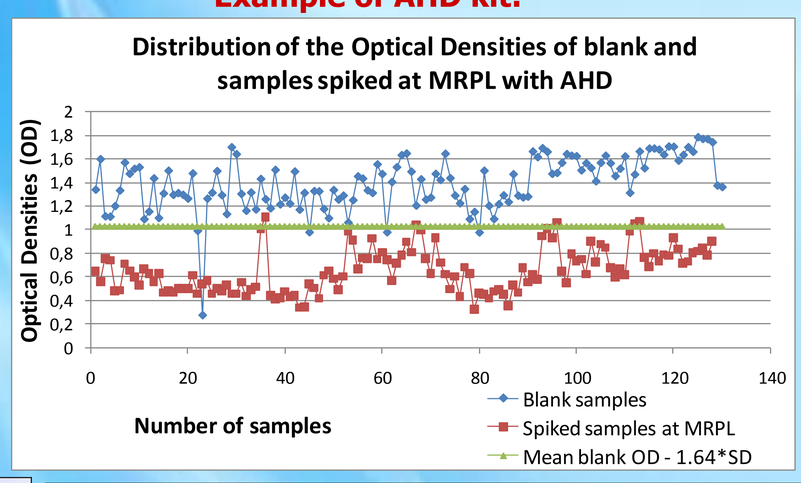
An international proficiency test for the screening and the confirmation of nitrofuran metabolites in porcine muscle was held during March-April 2008 by the Community Reference Laboratory (CRL). We have participated with the 4 ELISA kits for nitrofuran metabolites, to complete the validation study.

Results

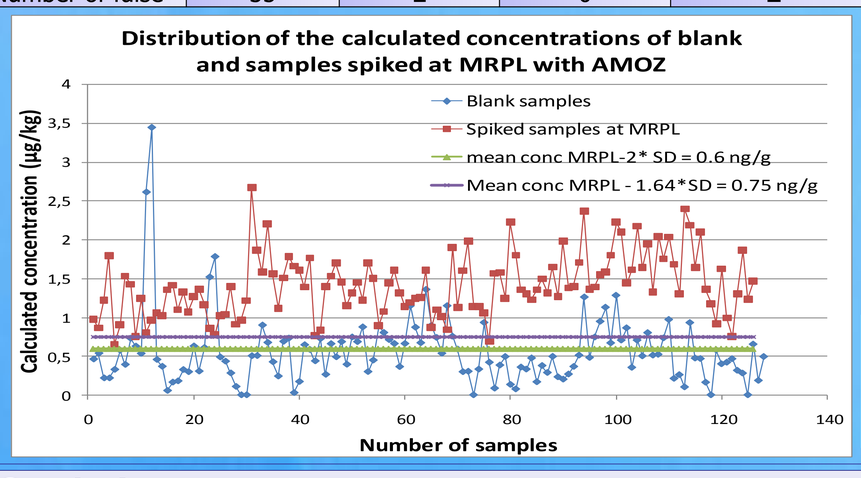
Specificity / Detection capability: Example of AMOZ kit:



Example of AHD kit:



Cut-off AMOZ	cut-off=0,55 / sample + if OD<0,55	**cut-off=0,77 / sample + if OD<0,77**	cut-off=0,6/ sample + if conc>0,6 ng/g	**cut-off=0,75/ sample + if conc>0,75 ng/g**
Number of false +	15	**70**	47	**23**
Number of false -	35	**1**	0	**2**



Cut-off AHD	cut-off = 1.03 OD	**cut-off = 1,1 OD**	cut-off = 0,6 conc	**cut-off = 0,34 conc**
Number of false +	5	**11**	2	**11**
Number of false -	5	**1**	19	**1**

The better cut-off value is the one where the false negative rate is lower than 5 % and the false positive rate as low as possible.
For the screening of AMOZ, the best choice was to set the cut-off at 0.75 ng/g for the calculated concentration or at 0.77 OD unit (mean OD spiked + 2.33*SD).
For the screening of AHD, the best choice was to set the cut-off at 0.34 ng/g for the calculated concentration or at 1.1 OD unit (mean OD blank -1.64*SD).

International Proficiency Test

The results were satisfactory for AHD. The results for AOZ were satisfactory if the cut-off was set in concentration. The results for AMOZ and SEM were unsatisfactory.

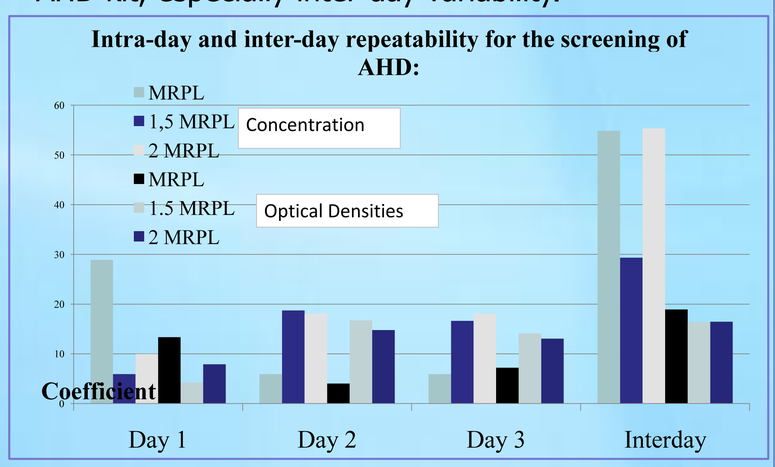
Cross-reactions

The 4 ELISA kits showed no cross-reactions (< 0.01 %) with the other nitrofuran metabolites and with other families of families. However, the 4 ELISA kit for each metabolite showed variable cross-reactions with the parent drug.

ELISA kit for	Parent Drug	Cross-reactions (%)
AOZ	Furazolidone	1.5
AMOZ	Furaltadone	42
AHD	Nitrofurantoine	0.1
SEM	Nitrofurazone	13

Precision

The variability of the response in OD was smaller than the variability of the calculated concentrations for the AHD kit, especially inter-day variability.



Conclusions

The overall conclusion of this validation study is that the ELISA kit for the screening of AHD is very interesting, regarding its performance and knowing that it is the first commercial kit available for the screening of AHD in food matrices. It should be interesting to test this kit on other matrices (milk, shrimp, fish, honey). The ELISA kit for the screening of SEM is also a new offer. Unfortunately, its performance was unsatisfactory. It was impossible to set a reasonable cut-off value which should be a compromise between the false negative and the false positive rate. Moreover, the participation to the IPT gave many false positive results. The results of the 2 ELISA kits for the screening for AMOZ and AOZ kit were unsatisfactory with high false positive rates.

References

[1] Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Off. J. European Union L221 (2002) 8–36.
[2] V. Gaudin, C. Hedou, P. Sanders, Validation of a Biacore method for screening eight sulphonamides in milk and porcine muscle tissues according to European decision 2002/657/EC. Journal of AOAC, 90 (6) (2007) 1706-1715.