Research article

Drug Testing and Analysis

Published online in Wiley Online Library

(www.drugtestinganalysis.com) DOI 10.1002/dta.1357

Application of EU guidelines for the validation of screening methods for veterinary drugs

Alida A. M. (Linda) Stolker*

Commission Decision (CD) 2002/657/EC describes detailed rules for method validation within the framework of residue monitoring programmes. The approach described in this CD is based on criteria. For (qualitative) screening methods, the most important criteria is that the CCβ has to be below any regulatory limit. Especially when microbiological or immunochemical methods are involved, the approach described in the CD is not easily applied. For example, by those methods, a large number of analytes (all antibiotics) within several different matrices (meat, milk, fish, eggs, etc.) are detected. It is not completely clear whether all those analytes and all matrices have to be taken into account during method validation. To clarify this, a working group – from EU Reference Laboratories – came up with a practical approach to validate multi-analyte multi-matrix screening methods. It describes how many analyte/matrix combinations have to be tested and how these combinations are selected. Furthermore it describes how to determine CCβ for screening methods in relation to a large list of compounds and maximum residue limits (MRLs). First for each analyte/matrix combination the 'cut-off' level – i.e. the level at which the method separates blanks from contaminated samples – is established. The validation is preferably at the concentration of 50% of the regulatory limit. A minimum set of 20 different samples has to be tested. From the experiences with applying these guidelines it was concluded that the validation approach is very 'practical'; however, there are some remarks. One has to be careful with selecting 'representative' analytes and matrices and it is strongly recommended to collect additional validation data during the routine application of the method. © 2012 RIKILT-Wageningen University and Research. Drug Testing and Analysis © 2012 John Wiley & Sons, Ltd.

Keywords: single-lab validation; 2002/657/EC; qualitative; residues; CCβ

Introduction

In the EU, the use of veterinary drugs is regulated through Council Regulation EG/470/2009.[1] The prohibition of the use of growth-promoting agents is laid down in Council Directive 96/22/EC.[2] The European Commission also regulates the inspection of food-producing animals and animal products for the presence of residues of veterinary drugs, growth-promoting agents, and specific contaminants.[3] Technical guidelines, like guidelines for validation, and performance criteria for residue control in the framework of Council Directive 96/23/EC[3] are described in Commission Decision 2002/657/EC.[4]

Validation means nothing more than checking the validity of measurement results. Since the 'true' or 'correct' content can never be determined exactly, the question is, from what point onwards can measurement results be seen as valid? This is a matter of definition and is handled in different ways for the individual analytical areas. Two basic approaches have to be distinguished: the criteria approach like Commission Decision 2002/657/EC[4] and the approach of applying standardized methods e.g. Codex Alimentarius Commission or ISO methods.[5] The criteria approach means that each laboratory has to prove that it uses the method correctly with acceptable performance characteristics fulfilling the criteria laid down in CD 2002/657EC. For (qualitative) screening methods, the most important criteria is that the CCβ has to be below any maximum residue limit. Especially when multi-analyte multi-matrix methods are involved like microbiological, immunological, and full-scan mass spectrometry (MS) screening methods, the CD is not easily applied. It is, for example, not completely clear whether all possible analytes and all matrices have to be taken into account during method validation. To clarify this, a working group – from the EU Reference Laboratories – came up with a practical approach to validate multi-analyte, multi-matrix screening methods. This approach is described in the SANCO document 'Guidelines for validation of screening methods for residues of veterinary medicines (initial validation and transfer).'[6]

In this manuscript, the approach described in the SANCO document[6] for establishing the CCβ of the screening method will be described and discussed. Examples will be given regarding the application of the guidelines to different types of screening methods. First, the effect of different matrices on the characteristics of the method is demonstrated for an immunochemical detection of coccidiostats in eggs and animal feed. Second, the effect of the selection of representative analytes is demonstrated for a physical chemical screening method based on time of flight mass spectrometric (TOFMS) detection for the analysis of antibiotics in milk. The last example will demonstrate the need for ongoing collection of validation data during routine sample analysis. In this case the Enzyme Immunoassay procedure for the detection of chloramphenicol in urine is used as the test case. The selected examples demonstrate not only the practical applicability of the validation approach but also show the risks of the selection of representative analytes and/or matrices and the need for ongoing validation.

* *Correspondence to: Alida A. M. (Linda) Stolker, RIKILT –Wageningen University and Research Centre, Akkermaalsbos 2, 6708 WB Wageningen, the Netherlands. E-mail: linda.stolker@wur.nl*

RIKILT –Wageningen University and Research Centre, Wageningen, the Netherlands

Experimental

In this section, the EU guidelines regarding the validation of screening methods[6] are discussed especially with respect to the CCβ. Furthermore, three screening methods used to demonstrate the practical applicability of the guidelines are discussed.

CCβ of screening methods according to the EU guidelines

Detection capability, CCβ, is defined in[4] as the smallest content of the analyte that may be detected, identified, and/or quantified in a sample with an error probability of β. The β error (measure of false negatives) is the probability that the tested sample is truly non-compliant (violative) even though a compliant (non-violative) measurement has been obtained. For screening tests the β error (i.e. the false compliant rate) should be $< 5\%$ at the level of interest or so-called regulatory limit. The regulatory limit is for example the maximum residue limit (MRL), maximum limit (ML), reference point of action (RPA) or the minimum required performance level (MRPL). In other words the CCβ concentration with $\beta = 5\%$ should be $\leq$ regulatory level concentration. For prohibited compounds with no RPA or MRPL, the CCβ must be as low as possible.

During method validation it has to be demonstrated that the method is fit for purpose. For screening, this means that samples are classified as 'screening suspected' (presumptive positive) in case the analyte in question is present in the sample at or above the regulatory limit. Furthermore the validation must cover the entire matrix/species/analyte combinations.

First, it is necessary for validation to select the relevant/representative analytes and matrices. If the screening method cannot distinguish between different analytes within one chemical family validation should be carried out for each analyte which is considered relevant for the specific laboratory. Other criteria for analyte selection are one with the lowest inhibition (microbiological test), lowest cross-reactivity (immunological test), or lowest analytical recovery (physical chemical test).

Second, it has to be demonstrated that at the regulatory limit the β-error is ≤5%. Therefore, it is necessary to analyze 60 different blank samples of each matrix and 60 spiked samples. In case ≤3 spiked samples are screened negative the β-error at the spiking concentration is ≤5%. When the spiked concentration is far below the CCβ concentration (for example 50% of the regulatory limit) then less than 60 samples are adequate to test for the β-error at the level of interest. For example when the samples are spiked at 50% of regulatory limit, only 20 samples are adequate to test for the β error. When in this case ≤1 sample is found negative then it is concluded (statistically) that the CCβ (concentration level with a β-error of 5%) of the method is $<$ level of interest (criteria for screening method).

At the end of the validation process it is possible to set up a list with analyte/matrix combinations and CCβ levels. It is possible that a multi-analyte, multi-matrix method is not able to reliably detect all relevant target analytes at the regulatory limit in all matrices and species. If essential analytes or species are not covered, the additional tests, using an alternative method, must be added.

Flow cytometry-based immunoassay for the analysis of coccidiostats

Different coccidiostats are used as feed additive to control coccidiosis in poultry. To protect consumer health, MLs have been set by the European Union (regulation 124/2009 and directive 2009/8/EC) and monitoring has to be performed.

A multiplex flow cytometry-based immunoassay (FCIA) was developed within the EU-project CONffIDENCE[1] for the simultaneous detection of 6 frequently used coccidiostats. A new platform for robust multiplexed immunochemical detection is the MultiAnalyte Profiling (xMAP) technology from Luminex (Austin, TX, USA). It is an emerging technology that uses small carboxylated polystyrene microspheres, which are internally dyed with a red and an infrared fluorophore.[7] By varying the ratio of the two fluorophores, up to 100 different colour-coded microsphere sets can be distinguished, and each microsphere set can be coupled with a different biological probe.[8,9] Within the EU project, CONffIDENCE, this approach was applied to eggs and feed for the detection of the following coccidiostats: narasin, salinomycin, diclazuril, lasalocid, monensin, and nicarbazin in feed and egg extract. For validation purposes all coccidiostats had to be validated separately due to possible cross reactivity of the coccidiostat with the antibody of a second coccidiostats coated an a different microsphere. Full details of this method are described in Bienenmann-Ploum *et al.*[10]

Full-scan MS analysis of antibiotics

The EU has set MRLs for a variety of veterinary drugs in tissues, milk and eggs.[1] Ultra performance liquid chromatography combined with time of flight mass spectrometry (UPLC-TOFMS) has been used for screening and quantification of more than 100 veterinary drugs in milk, meat, fish, and egg. The veterinary drugs represent different classes including benzimidazoles, macrolides, penicillines, quinolones, sulphonamides, pyrimidines, tetracylines, nitroimidazoles, tranquillizers, ionophores, amphenicols, and non-steroidal anti-inflammatory agents (NSAIDs). After protein precipitation, centrifugation, and SPE clean-up, the extracts were analyzed by UPLC – ToF-MS. From the acquired full scan data the drug specific ions were extracted for construction of the chromatograms and evaluation of the results. The method is fully described in Stolker *et al.* and Peters *et al.*[11,12]

Enzyme immuno-assay for the analysis of choramphenicol

Chloramphenicol (CAP) is a broad-spectrum antibiotic with historical veterinary uses in all major food-producing animals. CAP is a suspected carcinogen, and for this reason the drug is banned for use in food-producing animals in the European Union (EU) and in many other countries, including the USA, Canada, Australia, Japan, and China. A quick and selective method to screen for residues of unauthorized antibiotics like CAP in samples of urine is by using an enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA).[13] For the detection of CAP in urine, several commercial EIA-based test kits are available. Before analysis, the urine samples are hydrolyzed and CAP is extracted with ethyl acetate. The ethyl acetate is evaporated and the residues are dissolved in buffer. The final extract is tested with an EIA. The EIA is based on the antigen-antibody reaction. The antibodies against chloramphenicol are raised in a rabbit. These antibodies are used for reaction with chloramphenicol. In the

[1]CONffIDENCE is the acronym of the EU FP7 project 'CONtaminants in Food and Feed: Inexpensive Detection for Control of Exposure'; Internet: www.conffidence.eu; Contact: coordination@conffidence.eu

final assay there is a competition between a peroxidase enzyme labelled chloramphenicol (which gives a colored reaction product after addition of a substrate and a chromogene) and the free chloramphenicol in the sample. Finally, the concentration is measured by the photometric adsorption of the extract at 450 nm. There are commercially available ELISA test kits available for the detection of chloramphenicol, for example, EuroPromixa B.V. (Arnhem, the Netherlands).[14]

Results and discussion

The EU guidelines for the validation of screening methods are applied to the three different methods viz. twee biological methods and one physical chemical method. It has to be emphasized that not all aspects of method validation will be discussed. This manuscript focuses on the determination of the CCβ because this is the most important parameter for screening methods. The specificity/selectivity of the method and the stability of analytes are also important parameters for validation but will not be discussed here.

Flow cytometry-based immunoassay for the analysis of coccidiostats

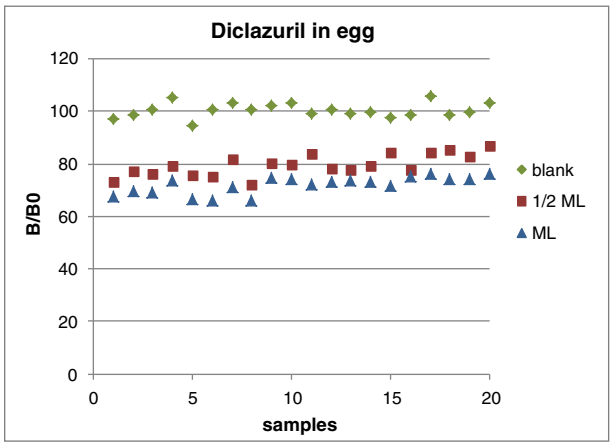
In this example, the focus is on the validation of diclazuril in eggs and animal feed.

EU legislation from 2009 regarding the unavoidable carry-over describes residue limits for coccidiostats in feed and food. For eggs the ML for diclazuril is set at 2 μg/kg[15] and the ML for chicken feed is set at 10 μg/kg.[16] Due to the low detection limits of the method, the spike level was set at 1 μg/kg for eggs and 5 μg/kg for animal feed (=50% of the regular limits). According to the guidelines for the validation of screening methods,[6] a set of 20 samples (blank and spiked) has to be analyzed to determine the CCβ (at 50% of regular limit). For comparison purposes also, 20 samples were spiked at the level of 2 μg/kg (=ML). The analyses were performed on three different days to introduce the variability in conditions.

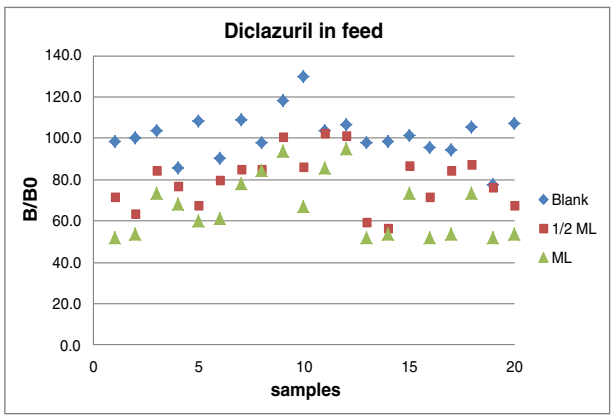
The responses obtained for the the 20 blank and spiked samples of eggs are presented in Figure 1. Please note the 'inverse' responses for blank and spiked samples; higher spike levels give lower responses.

From the results presented in Figure 1a, it is concluded that the whole set of spiked samples is separated (no overlap) from the blank samples. Since all the spiked samples at 50% ML (1/2 ML) elicited presumptive positive test responses, it is concluded that the CCβ of the method for the determination of diclazuril in eggs is ≤ 1 μg/kg. Consequently CCβ < regulatory limit of 2 μg/kg.

This conclusion can statistically be confirmed by the calculation of the mean and the standard deviation (sd) of the set of blank samples and the set of spiked samples at ML level. For the set of blank samples mean ± sd is 102 ± 4 and for the set of spiked samples at ML 72 ± 3. According to EU guidelines,[5,6] the cut-off value which is (mean −1.64*sd) of the spiked (ML) samples has to be higher than the mean of the blanks. In case of EIA, the response is inversely proportional to the concentration and so the cut-off value which is (mean + 1.64*sd) of the spiked samples has to be lower than the mean of the blanks. In this experiment, the cut-off value at ML is 72 + (1.64*3) = 77 and at 1/2ML 74 + (1.64 + 4) = 80; the mean of the blanks is 102. In conclusion, the method fulfils the criteria CCβ < regulatory limit. The same



(a)



(b)

Figure 1. Flow cytometry-based immunoassay responses for the analysis of the coccidiostat diclazuril in (a) Egg, ML=2 μg/kg and ½ML=1 μg/kg and (b) Feed, ML=10 μg/kg and ½ML=5 μg/kg.

validation approach used for eggs was applied to 20 samples of chicken feed spiked with 5 μg/kg of diclazuril. The results are presented in Figure 1b. From the results it is concluded that there is a significant overlap between the responses measured for the blank and spiked samples. Even at the ML concentration there is overlap for more than one sample with the set of blank samples. From these results it is not possible to draw conclusions regarding the CCβ. Due to the fact that even at 1 ML there is significant overlap between spiked and blank samples there is only one conclusion: CCβ > ML. Therefore, the method for diclaruzil in feed has to be optimized and the validation study has to be repeated for the optimized method.

This example for egg and feed clearly demonstrates that the validation has to be repeated for each new matrix.

Full-scan MS analysis of antibiotics

The applied UPLC-TOFMS method is able to analyze 100 different antibiotics belonging to different classes of veterinary drugs like sulfonamides, tetracyclines, macrolides, quinolones, and beta-lactams.[11,12] In this example, the focus is on the validation of tetracycline, oxytetracyline, and sulfamethoxazole in meat and eggs. Table 1 shows the MRLs for the registered drugs for meat and eggs. Since the method has low detection limits, the validation was performed at 50% of the regulator limits.

Table 1. Regulatory Limits (EU) for antibiotics in meat and egg[17]

µg/kg	Meat		Egg	
Analyte	MRL	Target Conc.	MRL	Target conc.
Oxytetracycline	**100**	**50**	**200**	**100**
Tetracycline	**100**	**50**	**200**	**100**
Sulfamethoxazole	**100**	**50**	**n.a.***	**50**

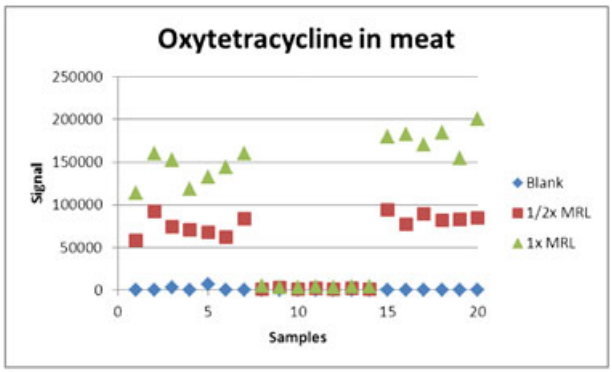
* not applicable.

For sulfamethoxazole in eggs there is no MRL so the target concentration was set at 50 µg/kg (based on MRLs for milk and meat). For all analyte/matrix combinations, 20 blank samples were analyzed and 20 spiked samples. After analysis the Extracted Ion Chromatograms are constructed from the Total Ion Chromatogram. The $[M+H]^+$-ions are for oxytetracycline $m/z = 461.1560$, for doxycycline $m/z = 445.1611$ and for sulfamethoxazole $m/z = 254.0599$.[11]

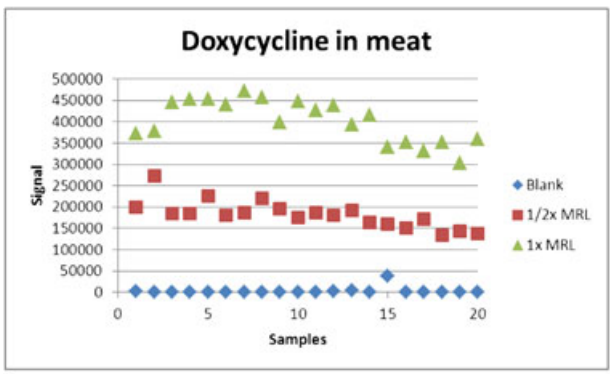
The results of the validation experiments are presented in Figure 2.

From the results it is concluded that for two very similar compounds tetracycline and oxytetracycline the validation results of the second day of validaton (samples 7 to 14) were significantly different. For tetracycline no signal is detected and for oxytetracycline there is no problem. A detailed review of the chromatograms (Figure 4) showed that the tetracyclines eluted around Rt 3.7–3.9 min together with a lot of other matrix components. It is possible that for the ionisation of tetracycline ion-suppression occurs and that this is not the case for oxytetracycline which elutes a little bit later. Looking at the drug sulfamethoxazole no problems were observed. This example demonstrates that even for a very selective technique like TOFMS (or probably especially for a TOFMS), all analytes have to be validated separately. Analytes eluting form the LC column at different retention times can observe different effects from the matrix and therefore show different ion-suppression (or enhancement). These matrix effects influence the CCβ level of the compound and so it is very difficult to select a 'representative analyte'.

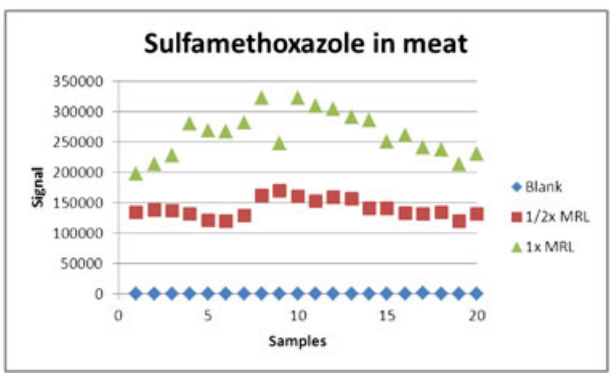
A second matrix viz. egg, was tested for the screening of antibiotics. The same validation approach was used that means that 20 blank samples of egg and 20 spiked samples were analyzed at the level of 50% of the Regular Limits and at the regular limit. Here again it is demonstrated (Figure 3) that it is



(a)

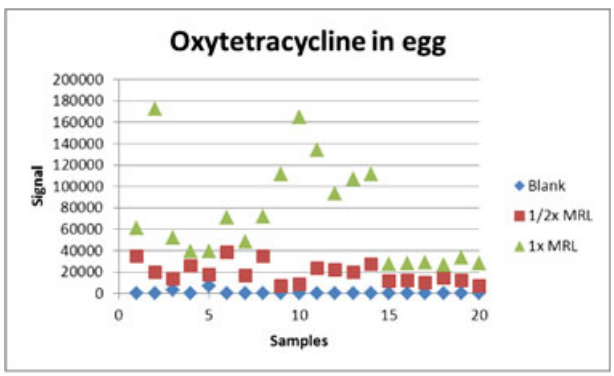


(b)

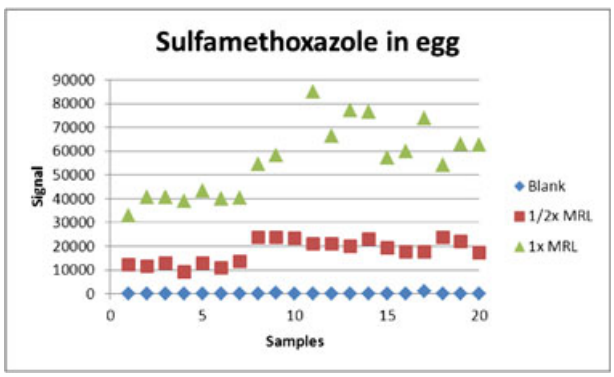


(c)

Figure 2. MS responses obtained for the analysis of antibiotics in meat (a) oxytetracycline, MRL=100 µg/kg and ½MRL=50 µg/kg (b) doxycycline, MRL=100 µg/kg and ½MRL=50 µg/kg and (c) sulfamethoxazole, MRL=100 g/kg and ½MRL=50 µg/kg.



(a)



(b)

Figure 3. MS responses obtained for the analysis of antibiotics in eggs (a) oxytetracycline, MRL =200 µg/kg and ½MRL=100 µg/kg and (b) sulfamethoxazole, target concentration (TC) = 50 µg/kg and ½TC = 25 µg/kg.

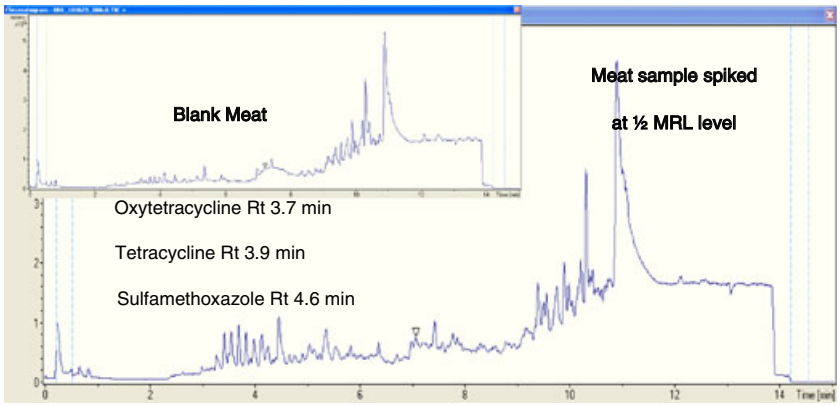
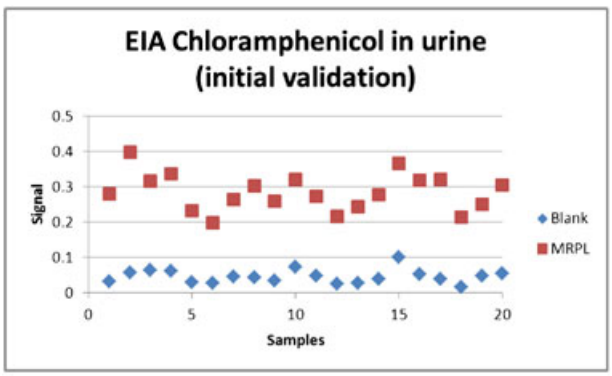
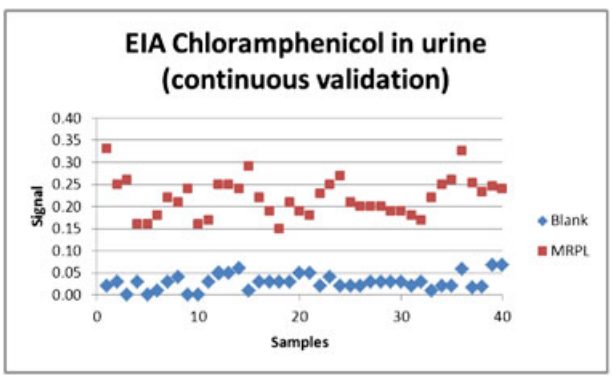


Figure 4. UPLC-ToF-MS total ion chromatogram for the analysis of antibiotics in meat for oxytetracycline, doxycycline and sulfametoxazole MRL= 100 μg/kg and ½MRL= 50 μg/kg.



(a)



(b)

Figure 5. Enzyme immuno assay responses for the determination of chloramphenicol in urine (a) initial validation and (b) continuous validation; MRPL = 0.3 μg/Lμg/kg.

necessary to validate each matrix separately. Although the oxytetracycline detection in meat showed some unexpected results for samples 7–14, fewer problems are observed for eggs. However for eggs it is observed that 2 samples (9 and 20) showed responses < highest response observed for blank samples. Therefore, the validation level was increased to 100% of the Regularoy Limit (instead of 50%). This has the consequence that not 20 but 60 samples had to be analyzed to establish if CCβ ≤ regulatory limit. For sulfametoxazole, all 20 spiked samples (at 50% of regular limit) showed responses higher than the highest blank so it was concluded CCβ ≤ regulatory limit.

Enzyme immuno-assay for the analysis of choramphenicol

In this example, the focus is on the unauthorized antibiotic chloramphenicol in samples of bovine urine. The applied EIA method is able to detect concentrations of 0.3 μg/L (MRPL level) or higher. Samples of urine, 20 blank samples and 20 spiked with chloramphenicol were screened for containing chloramphenicol. The results are presented in Figure 5 (initial validation). None of the spiked samples showed overlap with the blank samples. So, to determine if the CCβ ≤ 0.3 μg/L, the analysis of at least 40 additional samples is necessary. During routine analysis in each series of samples a blank sample spiked (so called quality control of QC sample) with chloramphenicol at the level of 0.3 μg/L was analyzed. The results obtained for 40 QC samples are presented in Figure 5 (continuous validation). From the results obtained for the initial validation it was concluded that samples are suspected for containing chloramphenicol (≥ 0.3 μg/L) if concentration measured by EIA are 0.18 μg/L or higher (cut-off level). However, it is obvious from the results obtained for the on-going validation that the cut-off level decreases from 0.18 to 0.15 μg/L. The QC results demonstrate that during the time the method is used the performance of the EIA changed a little so the method characteristics also changed. This example demonstrates the usefulness of continuous validation and evaluation of QC results after a specific time period in which the method is used. Only then, the real method characteristics like CCβ be established.

Conclusions

The guidelines described in the EU document for the validation of screening methods are a very practical and useful approach. The focus is on the determination of the CCβ (β=5%) which is the most important parameter for screening analysis. Furthermore this CCβ has to be ≤ Regulatory Level (MRL, MRPL, ML, RPA. etc). However, it is very important to realize that for multi-analyte multi-matrix methods (which are normally the character of a screening method), the selection of representative analytes and representative matrices are very important. From the examples presented in this manuscript, it is clear that each individual or unique matrix has to be validated when using a bioassay or TOFMS as the screening technique. Furthermore *even* or probably *especially* for TOFMS the validation of each individual analyte is necessary. Very similar compounds with slightly different Rt show different

method characteristics like CCβ. The differences in Rt and, as a consequence, the differences in co-eluting matrix compounds is probably the reason. In other words, the ion-suppression observed by the compounds of interest is different for each compound eluting at a (slightly) different Rt and different ion-suppression results in different method characteristics.

Finally, it is advisable not to stop the validation after the initial validation. Collecting additional validation data for example by analyzing QC samples during sample analysis and evaluate these results together with the initial validation results periodically helps to set realistic CCβ levels.

Acknowledgements

The results of the project were presented during the Saskatoon International Workshop on Validation and Regulatory Analysis which took place in Saskatoon, Saskatchewan, Canada, from 19 to 22 June 2011. A.A.M. Stolker participated in the conference and presented the results. The participation in the conference was financially supported by OECD under project contract number JA 64870.

Conflicts of interest

The author has no conflicts of interest to declare.

References

[1] Council Directive 470/2009/EC of 6 May 2009, *Off. J. Eur. Commun.* **2009**, 152, 11.
[2] Council Directive 96/22/EC of 29 April 1996, *Off. J. Eur. Commun.* **1996**, *125*, 3.
[3] Council Directive 96/23/EC of 29 April 1996, *Off. J. Eur. Commun.* **1996**, *125*, 10.
[4] Commission Decision 2002/657/EC of 12 Augustus 2002, *Off. J. Eur. Commun.* **2002**, *221*, 8.
[5] P. Gowik. The validation of methods for regulatory purposes in the control of residues. *J. Chromatogr. A* **2009**, *1216*, 8051.
[6] Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer) Community Reference Laboratories Residues (CRLs) 20/1/2010. http://ec.europa.eu/food/food/chemicalsafety/residues/Guideline_Validation_Screening_en.pdf (Accessed 30 April 2012)
[7] S.A. Dunbar, C.A. Vander Zee, K.G. Oliver, K.L. Karem, J.W. Jacobson. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAPTM system. *J. Microbiol. Meth.* **2003**, *53*, 245.
[8] J. Peters, M. Bienenmann-Ploum, R. de Rijk, W. Haasnoot. Development of a multiplex flow cytometric microsphere immunoassay for mycotoxins in feed. *Mycotoxin Research* **2011**, *27*, 63.
[9] P. Aqai, J. Peters, A. Gerssen, W. Haasnoot, M.W.F. Nielen. Immunomagnetic microbeads for screening with flow cytometry and identification with nano-liquid chromatography mass spectrometry of ochratoxins in wheat and cereal. *Anal. Bioanal. Chem.* **2011**, *400*, 3085.
[10] M. Bienenmann-Ploum, A.C. Huet, K. Campbell, T. Fodey, U. Vincent, W. Haasnoot, *et al*. Development of a five-plex flow cytometric immunoassay for the simultaneous detection of six coccidiostats in feed and egg. Accepted for publication in *Anal. Bioanal. Chem*, **2012**.
[11] A.A.M. Stolker, Pl. Rutgers, E. Oosterink, J.J.P. Lasaroms, R.J.B. Peters, J.A. van Rhijn, *et al*. Comprehensive screening and quantification of veterinary drugs in milk using UPLC–ToF-MS. *Anal. Bioanal. Chem.* **2008**, *391*, 2309.
[12] R.J.B. Peters, Y.J.C. Bolck, P. Rutgers, A.A.M. Stolker, M.W.F. Nielen. Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 8206.
[13] K.M. Cooper, J.V. Samsonova, L. Plumpton, C.T. Elliott, D.G. Kennedy. Ezyme immunoassay for semicarbazide – the nitrofuran metabolite and food contaminant. *Anal. Chim. Acta* **2007**, *592*, 64.
[14] EuroProxima. Chloramphenicol ELISA kit 5091CAP. Available at: hppt://www.europroxima.com
[15] Commission Regulation 124/2009/EC of 10 February 2009, *Off. J. Eur. Commun.* **2009**, *40*, 7.
[16] Commission Regulation 2009/8/EG of 10 February 2009, *Off. J. Eur. Commun.* **2009**, *40*, 19.
[17] Commission regulation 37/2010/EC of 22 December 2009, *Off. J. Eur. Commun.* **2010**, *15*, 1.