Laboratory of the Government Chemist

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Referee Analysis for evidence of administration of nitrofuran drugs – shellfish and other crustaceans

Summary

Following a number of referee cases in relation to nitrofuran veterinary residues the Government Chemist considered the following note on such casework may be useful to the wider analytical community. The note draws on the work of other institutions, see references for details.

Referee analysis of shellfish and other crustaceans such as crab for nitrofuran marker metabolites in general is dealt with as follows:

- For semicarbazide, SEM, bound residues are determined from the core of the animal
- For products in ice the sample is thawed and the water drained off
- Measurement Uncertainty is subtracted from the mean result to yield a 'not less than' figure used for reporting purposes 'beyond reasonable doubt'.

Referee analysis typically involves three replicates on each of three days along with blanks and spiked blank material with all key steps witnessed by a second scientist.

Introduction

Nitrofuran veterinary antibiotics were widely used prior to being banned owing to carcinogenicity. The parent drugs have a very short half life in the animal and so metabolite markers were selected to monitor for their illicit use.

The following compounds are used as markers[1]

Parent drug	Marker metabolite	Abbreviation
Furazolidone	3-amino-oxazolidinone	AOZ
Furaltadone	3-amino-5-morpholinomethyl-1,3-oxazolidinone	AMOZ
Nitrofurantoine	1-aminohydantoin	AHD
Nitrofurazone	Semicarbazide	SEM

Law

European law[2] provides that food of animal origin containing a nitrofuran metabolite such as one of the above fails to comply with Community legislation and is prohibited from entering the food supply chain unless the concentration of such a metabolite does not equal or exceed a reference point for action pursuant to Regulation 470/2009. Such a reference point for action for nitrofuran metabolites has been established[3] as a minimum required performance limit, MRPL, of 1.0 micrograms per kilogram, μg kg^{-1}. Where the results of analysis confirm the presence of a nitrofuran metabolite below the reference point for action but above a decision limit, CCα determined as part of the analytical procedure,[4] an investigation is required on the part of the competent authority, a topic that is beyond the scope of referee analysis.

Analysis

The analysis of food for the above marker metabolites involves acid hydrolysis, the formation of nitrophenyl derivatives and LC-MS/MS, with isotopically labelled standards.

[1] Methods were developed by e.g. Kennedy et al., AFBI
[2] Regulation **(**EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin read with Table 2 of Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin
[3] Commission decision 2003/181/EC of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin.
[4] Commission 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.

SEM from sources other than nitrofurazone

The possibility that one of the marker metabolites, SEM, may not arise simply as a metabolite of administered nitrofurazone was advanced in 2002. Kennedy *et al.* reported that SEM could be detected in a range of materials intended to coat chicken meat during the production of cooked chicken products. Most positive findings were associated with the use of breadcrumbs and other bread products. Subsequent investigations revealed that azodicarbonamide, a flour treatment agent that was commonly used in the production of certain breaded chicken products produced in Thailand, was the cause of the problem. This emphasized the importance of removing the coatings from chicken and shrimp products and of analysing the meat for the presence of bound residues of SEM.[5] The use of azodicarbonamide as a food additive is not permitted in the EU.

The EU Community Reference Laboratory in December 2002 issued the following advice on the confirmation of nitrofuran residues by SEM analysis.[6]

1 When testing composite food, only analyze the part of the product which is of animal origin, for example only the meat part of breaded products.
2 The detection of total (free+ bound) residues of metabolites of nitrofurans can be maintained at the screening level.
3 In case of a non compliant sample for total SEM, a sample must be reanalyzed for the bound residues of SEM only. To this end, free SEM should be extracted/washed out prior to this confirmation test.

Azodicarbonamide was also known to be used as a blowing agent for plastic gaskets, and SEM was also thought to arise from carrageenan and through hypochlorite treatment of nitrogen containing foods.[7],[8]

Further, it has been shown that SEM may occur naturally in the shell of crustaceans including crabs, langoustines and shrimps. Although the metabolic route for its production in the animals is not confirmed a role in protein synthesis is thought to be one of the possibilities.[9], [10] A solution is to analyse the inner core of the tested animal's meat, as SEM detected in wild-caught shrimp, presumably untreated, seems to be surface-associated.

A recent meeting of the Government Chemist Working Group called for research into a better marker of nitrofurazone use in products of animal origin.

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[5] AFBI –FoodBRAND Project Report: http://www.afbini.gov.uk/index/services/services-diagnostic-and-analytical/veterinary-drugs-marine-biotoxins/foodbrand-introduction/foodbrand-the-nitrofuran-crisis.htm
[6] Advice given by the CRL at http://crl.fougeres.anses.fr/publicdoc/noteCRL281103.pdf
[7] See for example Bartolo, I., Seafish: http://www.seafish.org/international-trade--distributors/legislation/legislation-news/sem-may-occur-naturally
[8] Hoenicke K, Gatermann R, Hartig L, Mandix M, Otte S., 2004, Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse? Food Addit Contam. 21(6):526-37.
[9] Kennedy, G. see references in ref.7 and ASSET Conference QUB March 2011.
[10] Van Poucke C, *et al.*,Investigation into the possible natural occurrence of semicarbazide in *Macrobrachium rosenbergii* prawns. J Agric Food Chem. 2011 Mar 9;59(5):2107-12. Epub 2011 Feb 7.

Ice

Many shellfish samples are received with a protective ice glaze, amounting sometimes to almost as much again by weight as the shellfish themselves. It is well accepted that water associated with a food but not intended to be consumed with it (e.g. the brine in a can of frankfurters in brine) is not part of an analysis. The definition of 'food' in Regulation (EC) No 178/2002 laying down the general principles of food law includes "any substance or product, whether processed, partially processed or unprocessed, *intended to be, or reasonably expected to be ingested* (my emphasis) by humans". 'Food' includes drink, chewing gum and any substance, including water, intentionally incorporated into the food during its manufacture, preparation or treatment. On the basis that ice in the sample was not intended to be ingested samples are thawed, drained and the drained liquid not included in the analysed sample.

Measurement Uncertainty

It is good analytical practice to report a mean result along with its associated measurement uncertainty. In referee analysis the default analytical strategy of three replicates on each of three days yields a case-specific expanded measurement uncertainty (U) expressed as a 95% confidence interval. The certificate issued in any individual case typically reports the CCα and CCβ, the mean result, x and U along with the resulting range of possible values expressed as 'not less than' (x – U) and 'not more than' (x + U) rounded outwards[11] to appropriate significant figures. In order to inform a court of our findings 'beyond reasonable doubt' the opinion expressed in the certificate appraises the datum 'not less than' (x – U) against the MRPL (now a *de facto* limit) as is common practice in forensic science – e.g. in blood alcohol analysis. It is interesting to note in this context that Commission has given a recommendation[12] in the context of Community legislation concerning contaminants in food and undesirable substances in feed as follows:

"In practice, when considering a maximum value in legislation, the analyst will determine the analytical level and estimate the measurement uncertainty at that level. The value obtained by subtracting the uncertainty from the reported concentration, is used to assess compliance. Only if that value is greater than the maximum level in the legislation is it certain "beyond reasonable doubt" that the sample concentration of the analyte is greater than that required by the legislation."

Conclusions

Referee analysis of shellfish and other crustaceans such as crab for nitrofuran marker metabolites in general is dealt with as follows:

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[11] That is to say, x - U of 1.23 µg kg^{-1} would be rounded outwards to not less than 1.2 µgkg^{-1} and x + U of 1.23 would be rounded outwards to not more than 1.3 µg kg^{-1}

[12] Report on the Relationship Between Analytical Results, Measurement Uncertainty, Recovery Factors and the Provisions of EU Food and Feed Legislation, with Particular Reference to Community Legislation… http://ec.europa.eu/food/food/chemicalsafety/contaminants/report-sampling_analysis_2004_en.pdf (accessed 22.09.11).

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