APPLICATION NOTE

Food Safety - ELISA

Author:

Swapna Gone, Ph.D.

Clay Knight

PerkinElmer, Inc.
Austin, TX

High Throughput Screening Solutions for Nitrofurans and Chloramphenicol in Shrimp Samples

Introduction

Nitrofurans and phenicols are a class of broad-spectrum antibiotics that are widely used to kill or slow down the growth of bacteria in the aquaculture industry. The use of nitrofurans and their metabolites has been banned by several countries and organizations within the European Union, United States and China, due to their harmful side effects to human health. Nitrofurans have been defined as Class A prohibited drugs in many countries, and a Minimum Required Performance Limit (MRPL) of 1.0 µg/kg has been set for food, animal and aquaculture products. U.S. Food and Drug Administration (FDA) has set a zero tolerance level for the use of Chloramphenicol in animals as a result of its potential side effects in humans, e.g., hematological abnormalities, aplastic anemia etc., which are known to be caused in humans exposed to it upon ingestion of food products that contain residues of the drug. In previous studies, nitrofurans were shown to transform rapidly to metabolites which readily bind to protein tissues. The bound metabolites are very stable and are used as an indicator of nitrofuran residues in various food, animal and aquatic products. The most widely used nitrofurans and their metabolites are furazolidone as 3-amino-2-oxazolidinone (AOZ), nitrofurazone as semicarbazide (SEM), furaltadone as 3-amino-5-morpholinomethyl-2- oxazolidinone (AMOZ) and nitrofurantoin as 1-aminohydantoin (AHD).

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In developing countries, the use of a veterinary drugs is prevalent in intensive marine shrimp farming to achieve sustainable production. Rejections of consignments by the importing countries have been recurrent in recent years due to detection of these banned antibiotics. The increasingly complex requirements for food safety assurance and traceability set by major export markets mandates screening for drug residues in the aquaculture industry.

ELISA assays are widely used for the detection of nitrofuran metabolites and chloramphenicol for regulatory conformance owing to the high sensitivity, selectivity and ease of use of the method. In the following study, we demonstrate the accuracy and precision (CCβ validation study) of the simultaneous 5-in-1 sample extraction method by manual ELISA and DS2 automation methods. Sensitivity of the assay kits (LOD) was also demonstrated using manual and DS2 method. Finally, sample variability testing was performed to characterize the effects of matrix from various shrimp sources.

Experimental

Materials and Methods

5-in-1 sample extraction method was performed using organic extraction reagents along with the reagents supplied from MaxSignal® HTS ELISA Kits (AOZ, AMOZ, AHD, SEM and CAP). A special procedure was used to extract all five analytes from single shrimp sample. The extracts were then used along with enzyme immunoassay components from the MaxSignal® HTS ELISA Kits to determine the concentration of all four nitrofurans and CAP. DS2 Automated Laboratory ELISA from Dynex Technologies, a two-plate automation platform with automatic data reduction capabilities was used for analysis of samples.

Shrimp samples (*Vannemei species*) and gulf coast varieties were sourced from local markets and screened prior by LCMS-MS methods for endogenous contamination. Metabolite spikes and derivatized standards were sourced from SIGMA and are VETRENAL grade.

Results and Discussion

Sensitivity (LOD)

LOD was established using blank sample matrices. A mean of ten replicates of blank samples were used for the LOD measurement. The LOD of a method is defined as the lowest concentration that can be reliably measured. The LOD is defined as the mean+3*StDev reported for the negative/blank samples. The results of LOD studies using the manual ELISA and DS2 methods are represented in Table 1.

It was observed that 5-in-1 method slightly elevates the background in CAP samples. This was an artifact created due to the higher dilution factor (2) compared to the previous single analyte extraction method in CAP (0.5). This will not be a risk to customers with respect to exporting since the reported LOD is still under the regulatory limit for CAP (0.3 ppb).

Table 1. Table 1 represents the results from LOD determinations.

Analyte	Manual Method (ppb)	RSD(%)	DS2 Method (ppb)	RSD(%)
	(n=10)		(n=10)	
AOZ	0.053	9.1	0.043	8.1
AMOZ	0.023	13.1	0.016	12.5
SEM	0.07	12.5	0.091	10.9
AHD	0.078	22.8	0.079	11.7
CAP	0.1	9.8	0.091	10.4

Accuracy and Precision

CCβ validation was performed for demonstrating the accuracy and precision of MaxSignal® HTS ELISA Kits in the following manner

a) 20 sample replicates of blank and spike at half the MRL for nitrofurans (0.5 ppb) and 0.15 ppb for Cap were processed following the 5-in-1 extraction method

b) Sample extracts were used for ELISA assays by manual and DS2 automation methods

c) Sample recoveries were checked for accuracy (60-140% recovery range)

d) 95% confidence limit (19 out of 20 samples) was used for precision measurements or the samples are checked for overlap between the lowest spike recovery and highest blank value.

The results from CCβ validations are summarized in Table 2. The results demonstrated good accuracy and precision for the 5-in-1 method by both manual and the DS2 method. There was no overlap between the lowest spike and highest blank samples indicating a successful CCβ.

Table 2. Table 2 summarizes the accuracy and precision results.

AOZ	Manual Method	DS2 Method	Target
Recovery	92-148%	91-154%	60-140%
Mean	122%	117%	
Lowest Spike	0.458 ppb	0.454 ppb	No Overlap
Highest Blank	0.059 ppb	0.042 ppb	
RSD % (Spike)	14%	16%	< 25 %
AMOZ	**Manual Method**	**DS2 Method**	**Target**
Recovery	61-85%	63-106%	60-140%
Mean	75%	84%	
Lowest Spike	0.305 ppb	0.314 ppb	No Overlap
Highest Blank	0.018 ppb	0.014 ppb	
RSD % (Spike)	14%	16%	< 25 %
SEM	**Manual Method**	**DS2 Method**	**Target**
Recovery	58-125%	61-152%	60-140%
Mean	81%	86%	
Lowest Spike	0.285 ppb	0.303 ppb	No Overlap
Highest Blank	0.084 ppb	0.124 ppb	
RSD % (Spike)	21%	24%	< 25 %
AHD	**Manual Method**	**DS2 Method**	**Target**
Recovery	59-89%	56-101%	60-140%
Mean	69%	67%	
Lowest Spike	0.296 ppb	0.284 ppb	No Overlap
Highest Blank	0.101 ppb	0.07 ppb	
RSD % (Spike)	15%	16%	< 25 %
CAP	**Manual Method**	**DS2 Method**	**Target**
Recovery	66-128%	67-125%	60-140%
Mean	89%	94%	
Lowest Spike	0.197 ppb	0.201 ppb	No Overlap
Highest Blank	0.125 ppb	0.124 ppb	
RSD % (Spike)	22%	16%	< 25 %

Method Comparisons

The performance of the manual ELISA method and DS2 automation method was compared to establish the performance of DS2 analysis. The validation design outlined in accuracy and precision studies was adopted for this purpose. A variation of < 20 % was established as good correlation between the manual method and DS2 automation method. The variation was calculated as the difference between mean spike recovery between the methods compared to the manual method.

The results indicate a good correlation between the established manual method of ELISA and DS2 automation method. The results from correlation are summarized in Table 3.

Table 3. Table 3 demonstrates a good correlation between manual ELISA and DS2 methods.

Analyte	Variation	Target
AOZ	4.26%	<20%
AMOZ	10.98%	<20%
SEM	4.50%	<20%
AHD	0.16%	<20%
CAP	5.96%	<20%

Sample Variability/Matrix Interference

Two matrix types: gulf coast (wild caught in USA) and *Litopenaeus vannamei* (commercially farmed in India and APAC regions) were used for testing to check for matrix variability and interference. Five replicates of shrimp samples from the two sources were processed and sample extraction was done following the 5-in-1 method. The sample extracts were analyzed by DS2 automation method.

The results showed no significant difference (<10%) between the two sources of shrimp samples proving that there is no effect of matrix on the method. The results are summarized in Table 4.

Table 4. Table 4 summarizes the results of sample variability testing.

	Analyte	Vannemei Shrimp (Farm Raised)	Gulf Shrimp (Wild Caught)	Delta %
Average Spike (PPB)	AOZ	0.609	0.552	5.7
	AMOZ	0.363	0.308	5.2
	AHD	0.321	0.309	1.2
	SEM	0.410	0.408	1.7
	CAP	0.266	0.210	5.6
Average Blank (PPB)	AOZ	0.030	0.023	0.71
	AMOZ	0.015	0.025	1.01
	AHD	0.064	0.064	3.2
	SEM	0.057	0.077	2.3
	CAP	0.082	0.084	1.7

Conclusion

Materials and Methods

MaxSignal HTS Nitrofurans and Chloramphenicol ELISA Kits assays are designed and developed specifically for the aquaculture industry, delivering a simple, simultaneous, 5-in-1 sample preparation method for AOZ, AMOZ, SEM and AHD Nitrofurans as well as Chloramphenicol. This speeds testing and reduces cross-contamination risks while requiring less reagents and hands-on technician time.

When used with the DS2 Automated Laboratory ELISA, analysis is then automated and provides highly accurate and consistent results that enable faster, more informed decisions for incoming seafood lots. Finally, the integrated bar-code scanner provides excellent sample traceability and data can be easily linked to LIMS for seamless results recording and sharing.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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