Importance of Nitrofurazone Determination

Antibiotic residues in foods pose a serious threat to public health. The nitrofuran class of broad spectrum antibiotics (Nitrofurazone, furazolidone, furaltadone and nitrofurantoin) are commonly used in food producing animals. Their potential for harmful effects on human health, specifically carcinogenicity, has led to bans on their use in food producing animals in many countries including the US, Canada, and the EU. These countries have also imposed bans on all imported foods containing nitrofuran residues. The monitoring of water sources and food products, such as meat, for antibiotic residues is necessary to ascertain that these compounds are not misused and do not present a danger to human and animal health.

The detection of nitrofurans themselves has proven challenging, as the drugs are rapidly metabolized after ingestion. The protein bound metabolites which are formed, however, persist in edible tissue for a considerable amount of time after treatment. SEM (semicarbazide), the metabolite moiety derived from Nitrofurazone, is not degraded by common cooking techniques and can be released from tissue under mildly acidic conditions, making it ideal for monitoring and detection in edible tissue.

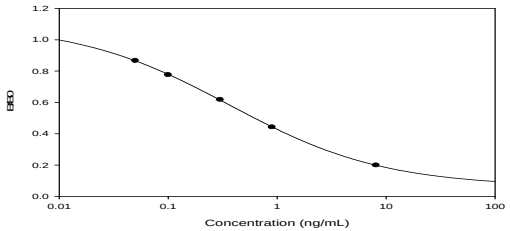
The Abraxis Nitrofurazone ELISA allows the determination of 41 samples in duplicate determination. Only a few grams or milliliters of sample are required. Hydrolysis and derivatization (overnight), and a subsequent solvent extraction step are necessary prior to assaying. The ELISA analysis can then be performed in less than 1 hour.

Performance Data

Test sensitivity: The limit of detection for Nitrofurazone, calculated as Xn +/- 3SD (n=20), in various matrices are as follows:
Fish, shrimp, 0.2 ppb

Standard Curve: Determinations closer to the middle of the calibration range of the test yield the most accurate results.

These values are used for demonstration purposes only; do not use these values for your determinations.



Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Selectivity: This ELISA recognizes Nitrofurazone and not related compounds.

Cross-reactivities:

Nitrofurazone (SEM)	100%
Furazolidone (AOZ)	<0.01%
Furaltadone (AMOZ)	<0.01%
1-aminohydantoin (AHD)	<0.01%

Samples: To eliminate matrix effects in fish, shrimp, and chicken, a sample clean-up is required. See Preparation of Samples, section H.

General Limited Warranty: Abraxis LLC warrants the products manufactured by the Company against defects and workmanship when used in accordance with the applicable instructions for a period not to extend beyond the product's printed expiration date. Abraxis makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose.

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ABRAXIS

Nitrofurazone (SEM) ELISA, Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Nitrofurazone in Contaminated Samples

Product No. 515667

1. General Description

The Nitrofurazone ELISA is an immunoassay for the detection of the Nitrofurazone metabolite (SEM). This test is suitable for the quantitative and/or qualitative detection of Nitrofurazone in contaminated samples. Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of SEM. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Nitrofurazone ELISA Kit should to be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The Abraxis Nitrofurazone (SEM) Plate Kit applies the principles of enzyme linked immunosorbent assay (ELISA) to the determination of Nitrofurazone. Calibrators (ready to use) and derivatized samples (please refer to reagent preparation section) are added, along with an SEM-HRP enzyme conjugate, to wells in a microtiter plate that contain immobilized antibodies specific for SEM. At this point, a competitive reaction occurs between the SEM which may be in the sample and the enzyme labeled SEM for the binding sites of the antibodies on the microtiter well. The reaction is allowed to continue for thirty (30) minutes. After a washing step, a substrate (Color Solution) is added.

The presence of SEM is detected by adding the "Color Solution," which contains the enzyme substrate (hydrogen peroxide) and the chromogen (3,3',5,5'-tetramethylbenzidine). The enzyme-labeled SEM bound to the SEM antibody catalyzes the conversion of the substrate/chromogen mixture to a colored product. After an incubation period, the reaction is stopped and stabilized by the addition of a diluted acid (Stopping Solution) and the color is evaluated using an ELISA plate reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run. Since the labeled SEM (conjugate) was in competition with the unlabeled SEM (sample) for the antibody sites, the color developed is inversely proportional to the concentration of SEM in the sample.

5. Limitations of the Nitrofurazone ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded. Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Nitrofurazone ELISA Kit provides screening results. As with any analytical technique (GC, HPLC, etc.), samples requiring regulatory action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated with antibodies against SEM (12 X 8 strips).
2. Nitrofurazone Standards/Calibrators (6): 0, 0.05, 0.1, 0.3, 0.9, and 8.0 ppb, 1.0 mL each. Standards are ready to use (already derivatized).
3. Nitrofurazone-HRP Conjugate Solution, 6 mL.
4. Sample Treatment Buffer (10X) Concentrate, 50 mL (2 x 25 mL bottle). Use to dilute samples.
5. Wash Solution/Sample Diluent (10X) Concentrate, 50 mL.
6. Derivatization Reagent, 12 mL.
7. Color (Substrate) Solution (TMB), 12 mL.
8. Stop Solution, 12 mL.

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the conjugate, antibody, substrate and stop solutions in order to equalize the incubation periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, conjugate, substrate and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the **Sample Treatment Buffer** concentrate at a ratio of 1:10. If using both bottles (50 mL), add to 450 mL of deionized or distilled water.
5. Dilute the **Wash Buffer/Sample Diluent** concentrate at a ratio of 1:10. If using the entire bottle (50 mL), add to 450 mL of deionized or distilled water.
6. The Derivatization Reagent is prepared in DMSO (Dimethylsulfoxide). It will freeze when stored at 4-8 ^{0}C. The reagent bottle may be placed in a warm water bath to accelerate thawing.
7. The stop solution should be handled with care as it contains diluted H_2SO_4.

C. Assay Procedure

1. Add 100 µL of the **standard solutions and derivatized samples** into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 50 µL of **enzyme conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for about 30 seconds. Be careful not to spill contents.
3. Incubate the strips for 30 minutes at room temperature.
4. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **four times** using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
5. Add 100 µL of **substrate (color) solution** to the wells. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for about 30 seconds. Incubate the strips for 20 minutes at room temperature. Protect the strips from direct sunlight.
6. Add 100 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs 4-Parameter (preferred) or Logit/Log. For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Nitrofurazone concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Nitrofurazone by interpolation using the standard curve. Samples showing lower concentrations of Nitrofurazone compared to Standard 1 (0.05 ng/mL) should be reported as containing < 0.03 ppb. Samples showing a higher concentration than Standard 6 (8 ng/mL) must be diluted further to obtain accurate results.

E. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (10-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate reader (wave length 450 nm)
4. Centrifuge, capable of spinning at 3,000 rpm
5. Vortex Mixer
6. 50 mL centrifuge tubes
7. 10 mL glass tubes or vials
8. Mixer (Stomacher, Ultraturrax)
9. Incubator (37 ºC)
10. Water bath (80-100 ºC)
11. Nitrogen (suggested but not essential)
12. Timer
13. Tape or Parafilm

F. Additional Reagents (not included with the test kit)

Ethyl Acetate; n-Hexane; 1N HCl; 1 N NaOH; Distilled or Deionized water

G. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 6: Standards
0; 0.05; 0.1; 0.3; 0.9; 8.0 ppb

Samp1, Samp2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	Samp 2									
B	Std 0	Std 4	Samp 2									
C	Std 1	Std 5	etc.									
D	Std 1	Std 5										
E	Std 2	Std 6										
F	Std 2	Std6										
G	Std 3	Samp 1										
H	Std 3	Samp 1										

H. Preparation of Samples

a) Fish/Shrimp Derivatization and Extraction

1. Weigh 1 g of homogenized fish or de-shelled shrimp (should have a paste-like consistency) into a 50 mL centrifuge tube.
2. Add 4 mL of distilled or deionized water, 0.5 mL 1N HCl, and 100 µL of Derivatization Reagent. Vortex thoroughly for 1 minute.
3. Incubate at 37ºC overnight (approximately 16 hours).
4. Add 5 mL of the diluted **Sample Treatment Buffer**, 0.4 mL of 1N NaOH and 5 mL of ethyl acetate and vortex thoroughly for 1 minute.
5. Centrifuge tube for 10 minutes at 3000 rpm.
6. Transfer 2 mL of the ethyl acetate layer (top layer) into a clean vial or glass tube.
7. Evaporate to dryness at 40-60ºC under a gentle stream of nitrogen.
8. Dissolve the residue with 1 mL of n-hexane, vortex. Add 1.6 mL of diluted **Wash Solution/Sample Diluent Solution**, and vortex thoroughly for 1 minute.
9. Boil the sample for approximately 3 minutes at 80-100ºC.
10. Centrifuge vial/tube for 10 minutes at 3000 rpm.
11. The aqueous layer (lower layer) will then be analyzed as sample (Assay Procedure, step 1).

The ELISA result will be multiplied by a factor of 4 to obtain the final SEM concentration in the sample (the factor is necessary to account for the sample dilution in the procedure). Samples showing lower concentrations than standard 1 (0.05 ppb) should not be multiplied by the factor (4) but should be reported as containing <0.05 ppb. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted and re-analyzed. Sensitivity = 0.2 ppb.

b) CHICKEN

1. Weigh 1.0 g of homogenized sample and mix with 4.0 mL of deionized water, 0.5 mL of 1M HCl, and 75 uL of Derivatization reagent. Vortex for 1 minute.
2. Follow steps H a. 3 to 11 of the **Fish/Shrimp Derivatization and Extraction** procedure. However, step H a. 8 needs to be changed to 3.2 of **Wash Solution/Sample Diluent Solution** from 1.6 mL

The ELISA result will be multiplied by a factor of 8 to obtain the final SEM concentration in the sample, sensitivity = 0.4 ppb

c) HONEY

1. Mix 1.0 g of sample with 4.0 mL of distilled water, 0.5 mL of 1M HCl, and 10 mL of hexane in a 50 mL centrifuge tube.
2. Vortex for 1 minute and centrifuge at 3000 RPM for 10 minutes.
3. Freeze at -20 °C for 60 minutes, remove the upper liquid layer.
4. Melt the frozen layer, add 75 uL of Derivatization reagent and vortex for 1 minute. Follow step H a. 4 to 11 of the **Fish/Shrimp Derivatization and Extraction** procedure, however the amount of 1N NaOH should be 0.5 mL.

The ELISA result will be multiplied by a factor of 4 to obtain the final SEM concentration in the sample, sensitivity = 0.2 ppb

d) MILK

1. Centrifuge 3-4 mL of milk at 3000 rpm for 10 minutes.
2. Carefully take 1.0 mL of the lower layer (**Must avoid taking up the upper fat layer**), then follow step H a. 2 to 11 of the **Fish/Shrimp Derivatization and Extraction** procedure.

The ELISA result will be multiplied by a factor of 2 to obtain the final SEM concentration in the sample, sensitivity = 0.2 ppb

NOTE:

An alternative derivatization (step 3 above) may be performed as follows:
Incubate samples at 60ºC for 3 hours. Proceed to step 4 above.

When using unknown samples of shrimp and fish with the incubation reduced from 16 hours at 37°C (section H, step 3) to 3 hours at 60°C, the recoveries were between 80-120% of those using the 16 hours at 37ºC derivatization procedure.

When using known **negative** samples of shrimp and fish spiked and derivatized with the incubation reduced from 16 hours at 37 ºC (section H, step 3) to 3 hours at 60ºC, the recoveries were between 70-110%.

For additional extraction procedures (mixed feed, chicken meat) please contact Abraxis LLC.