Importance of Azoxystrobin Determination

Azoxystrobin is a broad-spectrum fungicide which is effective against the four major groups of fungal diseases: *Ascomycetes*, *Basidiomycetes*, *Deutoromycetes*, and *Oomycetes*. It is used worldwide in both agricultural (cereal grains, fruits, vegetables) and non-agricultural settings (flowers, grass).

Humans may be exposed to Azoxystrobin through direct physical contact with treated plants or ingestion of contaminated foods or drinking water. Drinking water sources can be contaminated through run-off (surface water) or leaching (ground water); a groundwater advisory appears on Azoxystrobin products due to this potential for leaching. The U.S. EPA lists Azoxystrobin as a compound which is moderately persistent in soil. In humans, Azoxystrobin is an irritant to skin. Azoxystrobin is very toxic to aquatic organisms and to some plant species including Macintosh apple trees and other apple trees which are derived from Macintosh. Crabapple trees are also damaged by exposure to Azoxystrobin.

Due to the widespread use of Azoxystrobin on plants meant for human consumption, many countries have established Acceptable Daily Intake (ADI) levels. The European Union has established an ADI of 0.2 mg/kg of body weight. Australia has established an ADI of 0.1 mg/kg of body weight. Many countries have also established regulatory limits on the levels of chemical residues on many individual foods. These levels vary depending on country and food type.* For example, the European Union (EU) has established a maximum residue limit (MRL) of Azoxystrobin for dried beans and peas at 0.1 mg/kg, while the United States (US) has placed the limit at 0.5 mg/kg. Many foods and drinks have no established MRL. For example, although grapes have an Azoxystrobin MRL of 2.0 mg/kg in both the EU and the US, no MRL has been established for products made from grapes such as wine. Pesticide content in wine has become an area of concern, as grapes are one of the most highly pesticide treated foods. A study by the French Ministry of Agriculture showed the transfer of pesticides from contaminated grapes to the wine produced from it; contamination with Azoxystrobin was found in approximately 90% of wines produced from grapes containing measurable levels of Azoxystrobin.[Δ] A study released in 2008 evaluating the levels of pesticides in wine purchased in the European Union found that all conventional (non-organic) wines tested contained pesticides (a total of 24 different pesticides and as many as 10 different pesticides in one bottle); Azoxystrobin was found in 18% of the conventional wines tested.[‡]

*For listings of foods for which MRLs have been established: for the European Union, search "Official Journal of the European Union Commission Regulation (EU) No 270/2012 of 26 March 2012" and for the United States, go to www.federalregister.gov then search "Azoxystrobin; Pesticide Tolerances."
ΔFor full text of study, see Cugier et al. "Plan de surveillance résidus en Viticulture (Campagnes viticoles 1990-2003)", Direction Génerale de l'Alimentation, Ministère de l'Agriculture, de l'Alimentation, de la Pêche et de la Ruralité (2005).
‡For full text of study, search "Pesticide Action Network Europe Message in a Bottle."

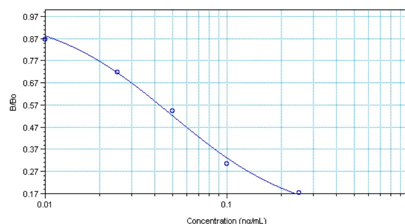
The Azoxystrobin ELISA allows for the analysis of 42 samples in duplicate determination. Less than 1 mL of sample is required. The test can be performed in less than 2 hours.

Performance Data

Test sensitivity: The limit of quantitation for Azoxystrobin (90% B/B_0 calculated from the average of 23 calibration curves) is approximately 0.009 ng/mL. The middle of the test (50% B/B_0 calculated from the average of 23 calibration curves) is approximately 0.054 ng/mL. Determinations closer to the middle of the calibration curve give the most accurate results.

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

Standard Curve:



For demonstration purposes only. Not for use in sample interpretation.

General Limited Warranty: Abraxis, Inc. 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.**

The monoclonal antibody and HRP conjugate included in this kit has been licensed from the Spanish National Research Council (CSIC) and the University of Valencia.

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R051017

Azoxystrobin ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Determination of Azoxystrobin in Water and Wine Samples

Product No. 500701

1. General Description

The Abraxis Azoxystrobin ELISA is an immunoassay for the quantitative and sensitive screening of Azoxystrobin. This test is suitable for the quantitative and/or qualitative screening of Azoxystrobin in water or wine samples. Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

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

3. Storage and Stability

The Azoxystrobin ELISA should 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 test is a direct competitive ELISA based on the recognition of Azoxystrobin by specific antibodies. Azoxystrobin, when present in a sample, and an Azoxystrobin-HRP analogue compete for the binding sites of the mouse anti-Azoxystrobin antibodies in solution. The Azoxystrobin antibodies are then bound by a second antibody (goat anti-mouse) immobilized on the wells of the microtiter plate. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Azoxystrobin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Azoxystrobin ELISA, Possible Test Interference

Although many organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects cannot be completely excluded.

The presence of the following substances were found to have no significant effect on the Azoxystrobin assay results: calcium sulfate, magnesium sulfate, sodium chloride, magnesium chloride, sodium nitrate, potassium phosphate, sodium thiosulfate, and aluminum oxide up to 10,000 ppm; calcium chloride, copper chloride, manganese sulfate, sodium fluoride, ferric sulfate, and zinc sulfate up to 1,000 ppm; humic acid up to 100 ppm.

Samples containing methanol must be diluted to a concentration ≤ 10% methanol to avoid matrix effects.

Mistakes in handling the test can cause errors. Possible sources for such errors include: 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 (lower than 10°C or higher than 30°C) during the test performance.

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

A. Reagents and Materials Provided

1. Microtiter plate (12 X 8 strips) coated with a secondary antibody, in a resealable aluminum pouch
2. Azoxystrobin Standards (6): 0, 0.01, 0.025, 0.05, 0.10, 0.25 ng/mL (ppb), 1 mL each
3. Antibody Solution (mouse anti-Azoxystrobin), 6 mL
4. Azoxystrobin-HRP Conjugate Solution, 6 mL
5. Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section C)
6. Sample Diluent, 25 mL
7. Substrate (Color) Solution (TMB), 16 mL
8. Stop Solution, 12 mL (handle with care)

B. Additional Materials (not delivered with the test kit)

1. Micro-pipettes with disposable plastic tips (50-200 µL)
2. Multi-channel pipette (50-250 µL) or stepper pipette with disposable plastic tips (50-250 µL)
3. Microtiter plate reader (wave length 450 nm)
4. Container with 500 mL capacity (for 1X diluted Wash Buffer, see Test Preparation, Section C)
5. Deionized or distilled water
6. Methanol
7. Glass vials with Teflon lined caps
8. Paper towels or equivalent absorbent material
9. Timer
10. Tape or parafilm

C. Test Preparation

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

1. Allow the microtiter plate, reagents, and samples to reach room temperature before use.
2. Remove the number of microtiter plate strips required from the aluminum pouch. The remaining strips are stored in the aluminum pouch with the desiccant (tightly sealed) in the refrigerator (4-8°C).
3. The standard solutions, conjugate, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the Wash Buffer (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water and mix thoroughly.
5. The stop solution must be handled with care as it contains diluted H_2SO_4.

D. Sample Preparation

Water Samples

Water samples should be collected in glass sample containers. Immediately upon collection, water samples should be preserved with methanol at a ratio of 1 mL of methanol per 9 mL of water. For example, a 90 mL sample of water added to a sample container should have 10 mL of methanol added.

The Azoxystrobin concentration contained in water samples is determined by multiplying the ELISA result by the dilution factor of 1.1. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further in sample diluent and re-analyzed.

Wine Samples

In order to eliminate matrix interferences, wine samples must be diluted in deionized or distilled water prior to analysis:

1. Add 10 mL of deionized or distilled water to an appropriately labeled glass vial.
2. Add 25 µL of wine sample to the vial.
3. Vortex thoroughly.
4. Diluted sample is ready to analyze (Assay Procedure, step 1).

The Azoxystrobin concentration contained in wine samples is determined by multiplying the ELISA result by the dilution factor of 400. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further in deionized or distilled water and re-analyzed.

E. 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 5: Standards
(0; 0.01; 0.025; 0.05; 0.1; 0.25 ppb)

Samp1, Samp2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	etc.									
B	Std 0	Std 4	etc.									
C	Std 1	Std 5										
D	Std 1	Std 5										
E	Std 2	Samp1										
F	Std 2	Samp1										
G	Std 3	Samp2										
H	Std 3	Samp2										

F. Assay Procedure

1. Add **50 µL of the standards or samples** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add **50 µL of antibody 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 30 seconds. Be careful not to spill the contents. Incubate the strips for 30 minutes at room temperature.
3. Add **50 µL of 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 30 seconds. Be careful not to spill the contents. Incubate the strips for 30 minutes at room temperature.
4. Remove the covering and decant the contents of the wells into a sink. Wash the strips **three times** using the diluted wash buffer. Please use at least a volume of **250 µL of 1X wash buffer** for each well in each washing step. Remaining buffer in the wells should be removed by patting the inverted plate dry on a stack of paper towels.
5. Add **150 µL of substrate (color) solution** to the 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 30 seconds. Be careful not to spill the contents. 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 stopping solution.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 4-Parameter (preferred) or Logit/Log. For a 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 Azoxystrobin concentration on the horizontal logarithmic (x) axis on graph paper. %B/B_0 for samples will then yield levels in ppb (or ng/mL) of Azoxystrobin by interpolation using the standard curve; results for water samples are determined by multiplying the concentration determined from the curve by the dilution factor of 1.1, results for wine samples are determined by multiplying the concentration determined from the curve by the dilution factor of 400. Samples showing lower concentrations of Azoxystrobin than standard 1 (0.01 ppb) should be reported as containing < 0.011 ppb of Azoxystrobin for water samples or < 4 ppb for wine samples. Samples showing a higher concentration than standard 5 (0.25 ppb) should be reported as containing > 0.275 ppb for water samples or > 100 ppb for wine samples. If a quantitative result is necessary, samples must be diluted further with the appropriate sample diluent and re-analyzed.