Determination of Soil Metabolites of Alachlor in Aqueous Environmental Specimens by LC/MS/MS

AMENDMENTS: ❑❑❑❑❑

PURPOSE & SCOPE

This multiresidue analytical method has been developed for the determination of alachlor soil metabolites in ground and surface waters. Specifically, the degradates: alachlor tertiary-oxanilic acid (AlOX), [2-(2,6-diethylphenyl)(methoxymethyl)amino]-2-oxoacetic acid; alachlor sulfonic acid (AlESA), [2-(2,6-diethylphenyl) (methoxymethyl)amino]-2-oxoethanesulfonic acid; alachlor sulfinylacetic acid (AlSAA), N-(2,6-diethylphenyl)-N-(methoxymethyl)-2-amino-2oxo-ethyl)sulfinylacetic acid; and alachlor secondary oxanilic acid (AlsOX), (2,6-diethylphenyl)amino-2-oxoacetic acid are determined in ground water, raw surface water, and finished surface water. The structures are shown in Figure 1.

SUMMARY OF THE METHOD

This analytical method describes the analysis of the multiresidue analytes in ground water and surface water. The method consists of analysis of environmental samples by direct aqueous injection reversed-phase liquid chromatography tandem mass spectrometry (LC/MS/MS). The accuracy of the analytical method is estimated based on the recovery of known concentrations of each degradate fortified into control water samples which are then carried through the analytical procedure. The method was validated over the range 0.25 ppb to 20 ppb for all components.

ABBREVIATIONS

The following abbreviations are used in this SOP:

Abbreviation	Definition
Al	alachlor
DI	deionized
ESA	ethanesulfonic acid
ESI	electron spray ionization
LC	liquid chromatography
LOQ	limit of quantitation
M	molar
MRM	multiple reaction monitoring
MS	mass spectrometry
sOX	secondary oxanilic acid
OX	tertiary oxanilic acid

MATERIALS & REAGENTS

The following materials, equipment, and reagents are required to perform the analysis. Appropriate substitution for items is left to the discretion of the analyst unless otherwise noted and should be recorded in the raw data. Glassware and other equipment must be thoroughly cleaned to minimize contamination. Control water samples are analyzed routinely to confirm a minimum contribution of interferences to actual specimens from the reagents, solvents and glassware.

Equipment and Example Part Numbers

- Electronic analytical balance: Mettler AE 163
- Volumetric flask (100 mL, 1000 mL)
- Volumetric glass pipette: Various sizes
- Pasteur pipette (5.75 and 9 inch lengths): VWR No's. 53283-911 and 53283-915
- Refrigerated centrifuge (DuPont/Sorvall RC-5B): No. 50253
- Nalgene centrifuge bottle (250 mL): Fisher No. 05-564-1
- Disposable syringes (5 cc): Beckton-Dickenson No. BD30160302
- Disposable syringe filters (25 mm x 0.45 µm): Pall No. 4184
- Glass screw cap autosampler vial (2 mL) with teflon lined septum: National Scientific No. C4000-82W
- PE Sciex API 3000 MS/MS using Sciex Analyst software
- Hewlett-Packard 1100 HPLC system, including: G1312A binary pump, G1322A degasser, G1313A autosampler, and G1316A column heater
- Analytical column, Betasil C_{18} (100 x 2 mm x 5 µ): Keystone Scientific No. 105-701-2
- Guard column, Betasil C_{18} (10 x 2 mm): Keystone Scientific No. 842015-701-P
- Guard column holder: Keystone Scientific No. 842-00
- Eppendorf fixed volume pipettors (0.50 mL): Brinkmann No. 2247145-7
- Eppendorf fixed volume pipettors (1.0 mL): Brinkmann No. 2247165-1
- Eppendorf pipette tips (1.0 mL): Brinkmann No. 2235090-1

Reagents

- Alachlor oxanilic acid, [2-(2,6-diethylphenyl)(methoxymethyl)amino]-2-oxoacetic acid, sodium salt: analytical grade, ≥95% purity
- Alachlor sulfonic acid, [2-(2,6-diethylphenyl)(methoxymethyl)amino]-2-oxoethanesulfonic acid, sodium salt: analytical grade, ≥90% purity
- Alachlor s-oxanilic acid, (2,6-diethylphenyl)amino-2-oxoacetic acid, sodium salt: analytical grade, ≥ 95% purity
- Alachlor sulfinylacetic acid, (N-(2,6-diethylphenyl)-N-(methoxymethyl)-2-amino-2-oxo-ethyl)sulfinylacetic acid, sodium salt: analytical grade, ≥95% purity
- Methanol, CH_3OH (Optima grade): Fisher No. A454
- Reagent Water (Optima grade): Fisher No. W7-4
- Ammonium acetate (ACS reagent grade): J.T. Baker No. JT0598-8
- Acetonitrile: EM Omnisolve HPLC grade or equivalent
- Formic acid: EM reagent EM-FX0440-11 or equivalent
- Ethanol, C_2H_5OH (absolute-200 proof): Midwest Grain Products No. 6810-00-242-3645
- Deionized water: Milli-Q water purification system (Millipore Company). This system consists of an activated carbon cartridge for the removal of organics in series with two mixed-bed ion-exchange cartridges for the removal of ionic species.

Reagent Preparation

Prepare an adequate quantity of the following solutions and reagents.

- 1 M ammonium acetate (NH_4OAc): Dissolve 77.09 g NH_4OAc in 1 liter of DI H_2O.
- 50 mM NH_4OAc: Combine 50 mL of 1 M NH_4OAc and 950 mL of DI H_2O.
- Mobile phase A (5 mM NH_4OAc with approximately 0.1% formic acid): Combine 100 mL of 50 mM NH_4OAc, 900 mL DI H_2O, and 1 mL of formic acid.
- Mobile phase B (90:10 ACN:50 mM NH_4OAc, approximately 0.1% formic acid): Combine 900 mL of ACN, 100 mL of 50 mM NH_4OAc, and 1 mL of formic acid.

Analytical Standards

Analytical standards are prepared for two purposes: for fortifying control matrices to determine analytical accuracy and for calibrating the response of the analyte in the mass spectrometer.

The purity of all standards must be verified before preparation of the stock solutions. All standard solutions (stock, fortified and calibration) should be stored refrigerated (2-10°C) in clean amber glass bottles with foil/teflon lined screw caps. The absolute volume of the standard solutions may be varied at the discretion of the analyst, as long as the correct proportions of the solute and solvent are maintained and the changes are documented. The stock solutions below are adequate to prepare fortification and calibration standards in the range of 0.10 to 20.0 ppb of each analyte. Calibrate the analytical balance prior to weighing any neat analytical standard for this method.

Standard Stock Solutions

- 1000 µg/mL Individual Degradate Solution: Weigh 0.1000 ± 0.0010 g (weight adjusted for purity of free acid) of each analytical grade degradate into individual 100 mL volumetric flasks. Dilute to volume with absolute ethanol and mix to insure complete dissolution. This solution contains 1000 ± 10 µg/mL of degradate. Repeat for all degradates.
- 1.0 µg/mL Mixed Degradate Solution: Pipet 1.0 mL of each of the six degradates, from their individual 1000 µg/mL solutions, into a single 1 liter volumetric flask. Dilute to volume with reagent water and mix. This solution contains 1.0 µg/mL each of the six degradates.
- 100.0 µg/L Mixed Degradate Solution: Pipet 10.0 mL of the 1.0 µg/mL mixed degradate solution into a 100 mL volumetric flask. Dilute to volume with reagent water and mix. This solution contains 100.0 µg/L each of the six degradates. (Note, this solution is now specified as µg/Liter.)
- 10.0 µg/L Mixed Degradate Solution: Pipet 1.0 mL of the 1.0 µg/mL mixed degradate solution into a 100 mL volumetric flask. Dilute to volume with reagent water and mix. This solution contains 10.0 µg/L each of the six degradates. (Note, this solution is now specified as µg/Liter.)

Fortification Solutions

In order to estimate the analytical accuracy of the method within a given set of water specimens, it is necessary to fortify a certain number of control water samples with a known amount of each degradate. Control water samples are fortified at different analyte levels across the range of anticipated

concentrations. The solutions used to fortify control water samples may be prepared in the following manner.

- 0.10 ppb Fortification Solution: Pipet 1.00 mL of the 10.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 0.25 ppb Fortification Solution: Pipet 2.50 mL of the 10.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 0.50 ppb Fortification Solution: Pipet 5.0 mL of the 10.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 1.00 ppb Fortification Solution: Pipet 1.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 2.00 ppb Fortification Solution: Pipet 2.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 5.00 ppb Fortification Solution: Pipet 5.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 10.0 ppb Fortification Solution: Pipet 10.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 20.0 ppb Fortification Solution: Pipet 20.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.

Calibration Standards

The detector calibration standards are made at convenient concentrations of each analyte. Experience has shown a linear range from 0.25 to 20.0 µg/L with our instrumentation. Concentrations other than the ones shown below also may be prepared and used if necessary. The LC/MS/MS calibration standards may be prepared in the following manner.

- 0.10 ppb Analytical Standard: Pipet 1.00 mL of the 10.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 0.25 ppb Analytical Standard: Pipet 2.50 mL of the 10.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 0.50 ppb Analytical Standard: Pipet 5.0 mL of the 10.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.
- 1.00 ppb Analytical Standard: Pipet 1.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.

- 2.00 ppb Analytical Standard: Pipet 2.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.

- 5.00 ppb Analytical Standard: Pipet 5.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.

- 10.0 ppb Analytical Standard: Pipet 10.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.

- 20.0 ppb Analytical Standard: Pipet 20.0 mL of the 100.0 µg/L mixed stock solution into a 100 mL volumetric flask and dilute to volume with reagent water.

ANALYTICAL PROCEDURE

The following is a general method for ground and surface water specimens. Unique interferences in particular specimens may require modification of this method. If modifications are necessary, they should be fully documented in the raw data.

Aqueous Specimen Preparation

Specimens are generally prepared and analyzed in (but not limited to) sets of 33, which includes at least one control and one fortified control water sample. Depending on the appearance of the specimens, filtration maybe required.

Aliquots for analysis maybe taken directly into autosampler vials or if convenient a larger aliquot may be taken for intermediate storage. If this is the case, transfer approximately 5 mL of specimen to a small vial or other suitable container and return to chilled storage.

Ground water and raw surface water are typically filtered through a 0.45 µ filter prior to analysis. This is not generally required of finished surface water.

Optima grade bottled water may be used as the control and laboratory fortified matrix for all water types.

Analytical Sample Preparation

Transfer 1-2 mL of the sample directly into an autosampler vial for LC/MS/MS analysis.

INSTRUMENTATION

Detector Calibration

A calibration curve is generated for every set of samples with a minimum of 5 standard levels. The standards are placed among the analytical samples for each set. The first and last sample in each analytical sample set must be a standard. Typically, the standards are selected by concentration and placed in the set with several samples between the standard levels.

The calibration curve is generated by plotting the peak area of each analyte in a calibration standard against its concentration. Least squares estimates of the data points are used to define the calibration

curve. Linear, exponential, or quadratic calibration curves may be used, but the analyte levels for all the samples from the same protocol must be analyzed with the same curve fit. In the event analyte responses exceed the upper range of the standard calibration curve by more than approximately 20%, the samples must be reanalyzed with the extended standards or diluted into the existing calibration range. Only those analytes out of range will normally be quantitated during reanalysis. This reanalysis may necessitate a reduction in instrument sensitivity or sample dilution with control water.

Liquid Chromatography/Mass Spectrometry/Mass Spectrometry

A LC/MS/MS is used for separation and quantitation of the degradates. Using MRM in the negative ion ESI mode, the LC/MS/MS gives superior specificity and sensitivity compared to conventional LC/MS techniques. The improved specificity eliminates interferences typically found in LC/MS or LC/UV analyses. Data acquisition is with a data system which provides complete instrument control of the mass spectrometer.

The instrument is tuned and mass calibrated in the negative ion ESI mode. Typically, two ions are monitored for each analyte, 1 transition (parent) ion for each degradate and 1 quantitation (fragment) ion for each degradate. The typical transition and quantitation ions for the analytes are shown below. Alternate ions may be used if they provide better data.

Analyte	MRM Transition Ion (Daltons)	Quantitation Ion (Daltons)
alachlor-s-oxanilic acid	220	148
alachlor-t-oxanilic acid	264	160
alachlor sulfinylacetic acid	340	160
alachlor ethanesulfonic acid	314	176

The following are suggested instrument conditions. The conditions may require modification to achieve satisfactory sensitivity and resolution.

- LC pump: HP 1100 HPLC binary pump (or equivalent)
- Autoinjector: HP 1100 (or equivalent)
- Column: Betasil C_{18} (5 µ), 100 x 2 mm
- Guard: Betasil C_{18} (5 µ), 10 x 2 mm
- Mobile phase A: 5 mM NH_4OAc with approximately 0.1% formic acid
- Mobile phase B: 90:10 ACN:50 mM NH_4OAc with approximately 0.1% formic acid
- Flow rate: 0.3 mL/minute (post-column split @ approximately 2:1, [0.2 mL/min to ion source])
- Gradient (A/B): Initial conditions: 90/10
  Linear gradient to 30/70 in 7 minutes.
  Re-equilibrate for approximately 3 minutes.
- Divert: Divert flow to waste for approximately 3 minutes after injection.
- Injection Volume: 100 µL

(The MRM experiments do not require chromatographic separation of the degradates. Therefore, other LC conditions, columns, gradient, and injection volumes may be used provided there is adequate sensitivity and the chromatographic quality is not compromised.)

- ESI conditions: Typical instrument parameters are shown below. The actual conditions used for sample analysis should be recorded in the raw data.

Ionization mode:	Negative
Ionspray voltage:	4.2 kV
Curtain gas:	8 at approximately 80 psi (nitrogen)
Nebulizer pressure:	12 at approximately 80 psi (nitrogen)
Turbo temperature:	350 °C
Turbo gas flow:	6 L/minute (nitrogen)

- Total run time: About 10 minutes, injection to injection

These conditions may be changed to obtain optimal instrument performance and maximize sensitivity

Column/Injector Maintenance

The use of a guard column has been found to provide significantly improved LC performance. Loss of performance manifests itself in poor peak shape and reduced sensitivity. Plugging of the guard column results in increased back pressure on the pumping system. The frequency of this maintenance is at the discretion of the analyst and is based on the quality and number of samples analyzed and the historical performance of the method. The maintenance should be based on the peak performance and back pressure rather than a fixed time interval. With the method as described, at least 1000 environmental specimens have been analyzed on our system without significant negative effects on performance.

Quantitation Criteria

Requirements for sample analyses include, but are not limited to:

Each individual control and environmental sample will be analyzed at least once. Analyte calibration must be performed for each chromatographic set using a multi-point calibration curve with a minimum of five (5) calibration levels. The range of calibration standards will be defined in each analytical set.

The complete chromatographic set containing calibration standards, control, fortified control and treated samples should be arranged such that the set begins and ends with a calibration standard (i.e. control, fortified and treated samples are bracketed by calibration standards).

The minimum correlation coefficient for the linearity of the analyte calibration curve is 0.98. The analyte detector response of all samples must be within 20% of the upper range of the standard calibration curve. When the analyte detector response of any sample is greater than this range, the sample must be diluted and reanalyzed. The diluted sample may be added to any chromatographic set, provided that the set contains control and fortified samples and meets the other requirements. Alternatively, the original sample extract may be reanalyzed relative to standards of higher concentrations while concomitantly reducing instrument sensitivity.

The average analytical recovery of fortified samples for each analyte in each set should range between 70 and 130% of the amount fortified. Each chromatographic set shall contain at least one (1) untreated control sample and one (1) fortified untreated control sample. Fortification samples for each analyte must cover the range of concentrations in treated samples within a study.

Multiple chromatographic peaks are detected for the ESA and OX degradates. These multiple peaks are thought to be rotomers of the specific degradates and are present in both analytical standards and environmental samples. For ESA quantitation the rotomers are combined due to their poor chromatographic resolution as well as the need to enhance detectability. For OX the rotomers are well separated with good sensitivity resulting in only the major peak being used for quantitation. A previous validation[1] has demonstrated that comparable results are obtained for the OX when either the major peak is quantitated or both peaks are summed for quantitation.

INTERFERENCES

Specimen Matrix

At least one control water sample must be run concurrently with the water specimens to determine the presence of matrix interferences and/or background levels of the degradates. Optima grade bottled water is used for the control and fortified matrices for all water types because it is difficult to obtain ground and surface water specimens that are completely free of the degradates. Our experience with ground and surface waters has demonstrated the presence of low level interferences in these matrices.

Other Pesticides

Interferences from other pesticides are unknown because none have been examined. However, none are expected due to the high level of specificity of the LC/MS/MS analysis.

Solvents

A solvent blank may be injected with the samples as part of an analytical set to confirm the cleanliness of a solvent used.

Labware

Disposable labware should be utilized where possible.

CONFIRMATORY TECHNIQUES

The samples are analyzed by MS/MS. A confirmatory technique is not needed because the analysis identifies the analyte by its retention time and specific molecular weight and fragment ion.

ANALYSIS TIME

A set of 33 specimens may be taken from preparation through chromatography in about 7 hours. Analysis of samples on the LC/MS/MS is about 10 minutes per injection.

POTENTIAL PROBLEMS

Safety Concerns

The method requires general lab safety awareness.

Chromatographic Interferences

Chromatographic interferences may be a problem in some matrices. Use of the MS with MRM analysis greatly reduces the interferences for these samples.

CALCULATIONS

Linear, exponential or quadratic calibration curves may be used to quantitate the amount of analyte in each sample. Quantitation of each analyte is made independently.

Quantitation Using a Linear Calibration Curve

The concentration of the analyte in the injected sample is determined based on the height or area of the analyte peak and interpolation of the internal or external standard linear calibration curve according to the following equation:

$$(\mu g/L \text{ analyte})\ m + b = PKR_{analyte}$$

where,

- $PKR_{analyte}$ is the detector response (as peak height or area) of the analyte
- m is the slope of the linear least squares fit of the calibration curve
- b is the Y-intercept of the linear least squares fit of the calibration curve

Generally no sample concentration or dilution is involved and the resulting µg/L value is taken directly from the regression curve and represents the concentration of the analyte in the injected sample. If dilution is necessary then the ratio of the original and final volumes are included in the calculation as shown in the equation below.

$$\text{ppb (degradate)} = \frac{(\mu g/L \text{ degradate found})(\text{final volume in mL})}{\text{water specimen volume in mL}}$$

Weighting of the calibration curve, $1/x$ or $1/x^2$, is expected to provide better curve fit at the lower concentration levels. Calculations using alternative calculations, exponential or quadratic curve fits are acceptable if they provide improved precision and/or accuracy.

Analytical Accuracy

The estimated analytical accuracy of the method can be obtained from the mean of the accuracys of each individual fortification using the following equation:

$$\text{Estimated Accuracy} = \frac{\sum C_f \,/\, A_f (100\%)}{N_i}$$

where,

- C_f is the concentration of degradate found in the fortified control sample
- A_f is the concentration fortified into the control sample
- N_i is the number of fortified control samples

There should be near equal numbers of fortifications at each level so the estimated analytical accuracy will not be disproportionately weighted. If a control water sample to be fortified is found to contain a significant concentration of any of the six degradates, then this concentration is subtracted from the amount found in the fortified control sample in order to calculate the accuracy for the sample. This is done for those samples that have been found to contain low concentrations with respect to the fortification level.

As a general rule, the amount fortified should be at least twice that concentration found in the identical sample that is not fortified. In this case, the variable C_f in the above equation should be replaced by the expression $(C_f - C_b)$, where C_b is the concentration found in the identical sample that was not fortified.

RESULTS & DISCUSSION

This multiresidue analytical method provides very good precision and accuracy for the analytes over a 0.25 to 20.0 ppb range. Based on preliminary validation data, the LODs are estimated to range from 0.1 ppb and 0.25 ppb. The common LOQ is estimated to be 0.5 ppb for all analytes.

REFERENCES

"Method for the Determination of Soil Metabolites of Acetochlor, Alachlor and Metolachlor in Aqueous Environmental Specimens by Direct Aqueous Injection LC-API/MS/MS", ALTA Analytical Laboratory, AMMON1, Revison 2, February 2000.

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