

**Time schedule:**

Study plan signed by study director: 17 NOV 1999  
Start of experimental phase: 24 JAN 2000  
End of experimental phase: 04 FEB 2000

**2 OBJECTIVES**

The objective of the study was to validate an analytical method for determination of AE F039866 and its main metabolites AE F061517 and AE F064619 in surface water. The limits of quantification (LOQ) to be validated for each analyte were 0.05 µg/l in water.

**3 RELEVANT RESIDUES**

Metabolism studies in soil show that the relevant residues in soil and water consist of the active ingredient Glufosinate-ammonium (AE F039866) and 2 metabolites AE F061517 and AE F064619 (Ref 1).

**4 ANALYTICAL METHOD****4.1 Principle of the Analytical Method**

AE F039866 (Glufosinate) forms strong complexes with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions which do not allow quantitative determination of Glufosinate residues. Removal of these cations before derivatisation is necessary and is achieved with a chelating resin (functional group: amino phosphonic acid) which also forms strong complexes with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and releases Glufosinate during this process.

AE F039866 and the two metabolites AE F061517 and AE F064619 are eluted under aqueous conditions from the chelating resin.

To concentrate the analytes in the water phase eluted from the cation exchange column, the aqueous eluate is transferred to a highly alkaline anion exchanger (hydroxide form). The analytes are retained on the resin under these conditions. They are subsequently eluted from the anion exchanger using formic acid.

The eluate is evaporated to complete dryness. The residues containing the active substance (AE F039866) and the metabolites (AE F061517 and AE F064706) are derivatised by refluxing the extract for 4.5 hours with a mixture of glacial acetic acid and trimethylorthoacetate. After derivatisation, the mixture is evaporated to dryness. The residue is transferred with a mixture of toluene/methylacetate on to a silicagel SPE cartridge. The substances are eluted from the cartridge with a mixture of methanol/methylacetate.

is performed by gas chromatography using a flame photometric detector (GC-FPD) with a 526 nm phosphorous mode filter.

The LOQ in water is 0.05 µg/l for each of the three analytes (calculated as Glufosinate-free acid, AE F035956).

#### 4.2 Test commodities, test and reference substances

##### 4.2.1 Water

10 L of water used for validation was taken on 24<sup>th</sup> of January, 2000 from a pond in front of the building G812/G813 in Höchst Industriepark and was analysed on the same day.

Characterisation of the surface water

mg/L Ca	mg/l Mg	Hardness (°dH)	p-H	TOC (organic C) mg/L
46. ± 1	17 ± 1	10.4	8.02 ± 0.02	6.8 ± 0.1

##### 4.2.2 AE F039866

Test and reference substance

AE F039866 in aqueous ammonia solution (0.015 mol/l) in various concentrations.

Chemical name (IUPAC): ammonium-DL-homoalanin-4-yl(methyl)phosphinate

Empirical formula: C<sub>5</sub>H<sub>15</sub>N<sub>2</sub>O<sub>4</sub>P

Certificate of analysis: AZ 07519

Drawn up by: Hoechst Schering AgrEvo GmbH

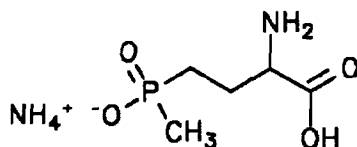
Produktanalytik

D-65926 Frankfurt am Main, Germany

Purity: 99.2 % (w/w)

Expiry date (d/m/y): 14-AUG-02

Chemical structure:



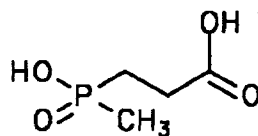
#### 4.2.3 AE F061517

Test and reference substance

For comments on dissolving the test substance refer to AE F039866

Chemical name (IUPAC):	3-methylphosphinico-propionic acid
Empirical formula:	C <sub>4</sub> H <sub>9</sub> O <sub>4</sub> P
Certificate of analysis:	AZ 06324
Drawn up by:	Hoechst Schering AgrEvo GmbH Produktanalytik D-65926 Frankfurt am Main, Germany
Purity:	97.9% (w/w)
Expiry date (d/m/y):	16-FEB-00

Chemical structure:



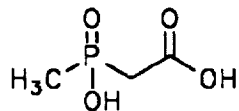
**4.2.4 AE F064619**

Test and reference substance

For comments on dissolving the test substance refer to AE F039866

Chemical name (IUPAC): 2-methylphosphinico-acetic acid  
Empirical formula:  $C_3H_7O_4P$   
Certificate of analysis: AZ 07471  
dated: 21.07.1998  
drawn up by: Hoechst Schering AgrEvo GmbH  
Entwicklung  
Produktanalytik, G 865a  
D-65926 Frankfurt am Main  
purity: 99.4 %  
expiry date: 13-Jul-01

Chemical structure:



Solutions of AE F039866, AE F061517 and AE F064619 in ammonia solution (0.015 mol/l) were used for fortifying the procedural recoveries.

**4.2.5 AE F064706**

Reference substance

AE F064706 is dissolved in methanol, while dilutions are made with methyl acetate to obtain various concentrations.

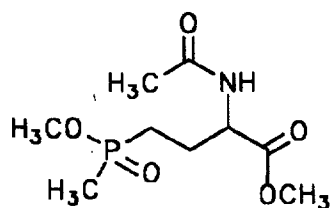
Chemical name (IUPAC): methyl-4-(methoxy)(methyl)phosphinoyl-2-acetamido butyrate  
Empirical formula:  $C_9 H_{18} N O_5 P$   
Certificate of analysis: AZ 07383

Drawn up by: Hoechst Schering AgrEvo GmbH  
Produktanalytik  
D-65926 Frankfurt am Main, Germany

Purity: 99.1 % (w/w)

Expiry date (d/m/y): 19-MAY-02

Chemical structure:



#### 4.2.6 AE F070951

Reference substance

For comments on dissolving the reference substance refer to AE F064706

Chemical name (IUPAC): methyl-3-(methoxy)(methyl)phosphinoylpropionate

Empirical formula: C<sub>6</sub> H<sub>13</sub> O<sub>4</sub> P

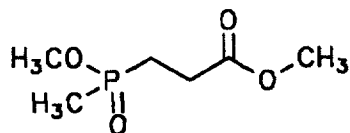
Certificate of analysis: AZ 08044

Drawn up by: Hoechst Schering AgrEvo GmbH  
Produktanalytik  
D-65926 Frankfurt am Main, Germany

Purity: 98.8% (w/w)

Expiry date (d/m/y): 12-AUG-03

Chemical structure:



#### 4.2.7 AE F096432

Reference substance

For comments on dissolving the reference substance refer to AE F064706

Chemical name (IUPAC): methyl-(methoxy)(methyl)phosphinoacetate

Empirical formula:  $C_5H_{11}O_4P$

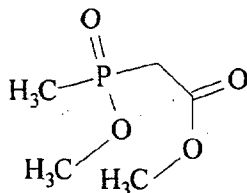
Certificate of analysis: AZ 05969

drawn up by: Hoechst Schering AgrEvo GmbH  
Entwicklung  
Produktanalytik, G 865a,  
D-65926 Frankfurt am Main

purity: 97.1 %

expiry date: 15-MAY-02

Chemical structure:



Solutions of AE F064706, AE F070951 and AE F096432 in methyl acetate were used for quantification of the derivatized analytes during gas chromatographic measurement.

#### 4.2.8 Standard Solutions

Stock solutions are prepared from the standard substances AE F039866, AE F061517 and AE F064619 (weighed out on an analytical balance) in 0.015 M ammonia solution at a concentration of exactly 1 mg AE F035956-equivalents/ml.

Dilutions are prepared from these stock solutions at the concentrations required for fortifications.

Stock solutions of exactly 1 mg AE F035956-equivalents/ml are prepared in **methanol** from the GC-derivatives AE F064706, AE F070951 and AE F096432. From these stock solutions, dilutions are prepared in **methyl acetate** at the concentrations required for quantification.

Chelating resin (e.g. Duolite C-467 from Supelco, Order No. 1-0354)

**Chelating resin:**

100 g Chelating resin is washed to a neutral stage with deionised water. A 4 fold quantity (ml) of 1 N hydrochloric acid is then added to the resin, the solution is stirred for 2 min and allowed to stand for 30 min. The hydrochloric acid is decanted and the resin is washed with deionised water until a neutral pH is reached. Subsequently, a 4 fold volume of 1 N sodium hydroxide solution is added to the resin, the solution stirred for 2 min and allowed to stand for another 30 min. The sodium hydroxide solution is decanted, drawn off via a frit and the resin is washed with deionised water until the pH is neutral.

Anion exchange resin AG 1X8, 50 - 100 dry mesh (e.g. Bio Rad or equivalent)

**Anion exchanger:**

100 g Anion exchanger is washed with deionised water until a neutral state is reached. The anion exchanger is decanted into a large vessel together with 1 litre 1 N sodium hydroxide solution, the solution is mixed thoroughly for approx. 2 min and then allowed to stand for 30 min. The sodium hydroxide solution is separated by filtering, and the resin is washed using deionised water until a neutral pH is reached. 3 - 5 l water are normally required for 100 g resin. The anion exchanger is now ready for use.

The following dilutions must be prepared:

Sodium hydroxide solution  $c(\text{NaOH}) = 1 \text{ mol/l} = 40 \text{ g sodium hydroxide - tablets in 1 l deionised water}$

Formic acid  $c(\text{CH}_2\text{O}_2) = 50 \% = \text{make up } 500 \text{ ml deionised water to } 1 \text{ l with } 100 \% \text{ formic acid.}$

Ammonia  $c(\text{NH}_3) = 0.015 \text{ mol/l} = \text{make up } 1 \text{ ml ammonia solution ca. } 28 - 30 \% \text{ NH}_3 \text{ to } 100 \text{ ml with deionised water.}$

Hydrochloric acid  $c(\text{HCl}) = 1 \text{ mol/l} = \text{make up } 100 \text{ ml (W=37\%)} \text{ to } 1000 \text{ ml with deionised water.}$

The following mixtures must be prepared:

Methanol HPLC grade / methyl acetate HPLC grade 1 / 1 (v / v)

Methanol HPLC grade / toluene HPLC grade 1 / 1 (v / v)

**4.3 Reagents and Materials**

Deionised water

formic acid 100 %

methyl acetate (GC)

glacial acetic acid 100 % (e.g. Baker)

trimethylorthoacetate 99 % (e.g. Aldrich)

methanol HPLC grade

toluene HPLC grade

hydrochloric acid 36 - 38%  
sodium hydroxide - pellets p.a.  
silica gel SPE cartridges, 500 mg with 10 ml reservoir

#### 4.4 Gas chromatographic conditions

Determination by gas chromatography is carried out using a flame-photometric detector (FPD) in the phosphorus mode. Typical GC conditions are described below.

Equipment: Carlo Erba 8000, FPD 800 (526 nm), micro PTV, splitless mode

Column: 15 m 0.5  $\mu$ m AT-Wax Alltech, i.d. 0.32mm

Injector SL time 90 sec, 60°C, 40 sec, 15°C /sec, 300°C, 2 min

Temperature: detector (base) 280 °C  
detector (body) 220°C  
column 80°C, 1.5/min,  
60°C/min 175°C, 2 min,  
10°C/min 185°C, 1 min  
79°C/min 280°C, 10 min

Gases: carrier gas He 4.0 ml/min (constant flow)  
detector gases air: 140 ml/min, H<sub>2</sub>: 87 ml/min

Injection volume: 5  $\mu$ l

Approximate retention time: AE F096432 4.5 min  
AE F070951 6 min  
AE F064706 10 min

#### 4.5 Laboratory Steps

##### 4.5.1 Preparation of chelating resin/anion exchange column

Cation exchange with chelating resin:

A chromatography column (type 1) with an internal diameter of 20 mm is used for the cation exchange clean-up with the chelating resin. The column is plugged with glass wool at the



lower end. 10 ml water is filled into the column and the top level of the water column is marked on the glass wall. The chelating resin is then poured into the column, slurry-packing with water to assist in packing the resin in the column up to the mark on the chromatography tube. Prior to further use, the resin in the column is washed with approx. 100 ml deionised water.

#### Anion exchange:

A chromatography column (type 2) with an internal diameter of approx. 10 mm is used in preference. The column is plugged with glass wool at the lower end. 12 ml water is filled into the column and the top level of the water column is marked on the glass wall. Before slurry-packing the resin as described above. Prior to further use, the column is washed to a neutral stage using approx. 100 ml deionised water.

#### 4.5.2 Remove of $\text{Ca}^{2+}$ , $\text{Mg}^{2+}$ from water samples

The chromatography tube (type 1) which is filled with the chelating resin (cation exchange) is installed above the anion exchange column. The water sample (ca. 500 ml) is transferred to the reservoir of the cation-exchange column so that the water flowing through the resin runs onto the anion exchange column. After the sample has passed through the column, the chelating resin is rinsed with 100 ml distilled water. The water flow should be regulated that 1-2 drops per second may pass through the resin.

#### 4.5.3 Further sample workup

##### Water samples

The total amount of water including the amount for rinsing the chelating resin is allowed to run onto the anion exchange column. The water then passes through the anion exchange column and the retained substances from the residues in water are washed with 100 ml of water. The analytes are eluted from the anion exchange column, using 100 ml 50 % formic acid. The eluate is collected in a 250 ml round-bottom flask and concentrated to dryness with a rotary evaporator (60°C). The rotary evaporator is operated at a moderately low speed which avoids excessive distribution of the sample over the walls of the flask. 10 ml water is added to the dried sample extract and concentrated to dryness as described above until no traces of formic acid are apparent. This procedure may be repeated if necessary. 5-10 ml methanol are added to the dried sample extract and concentrated as described above. This procedure is repeated once with 5-10 ml methanol.

#### 4.5.4 Derivatisation

3 ml glacial acetic acid are added to the sample residue in the round-bottom (from section 4.5.3). The solution is ultrasonicated at ambient temperature for approx. 1 min until all visible sample residues are dissolved or dislodged from the wall of the flask. 12 ml of trimethylorthoacetate and a few glass beads are added. The solution is then mixed by

swirling with ultrasonication. When mixing is complete, the reaction mixture is refluxed for 4.5 h.

The sample is then allowed to cool to room temperature. The procedure may be stopped at this point, e.g. derivatisation overnight, controlled by a time switch.

After derivatisation, the flask is removed from the reflux condenser and 15 ml toluene is added. The procedure may also be stopped at this point (the flask is closed and stored at room temperature).

The sample is evaporated to a final volume of approx. 1-2 ml using a rotary evaporator. Care should be taken to ensure that the water bath temperature does not exceed 40 °C, since in particular the derivatives AE F070951 and AE F096432 could evaporate. 2 portions of 15 ml toluene are each added successively and the evaporation procedure is repeated. The mixture is evaporated each time to an approximate volume of 1-2 ml to remove all traces of the derivatisation solution. It is important that the solution is not evaporated to dryness during the working-up procedure.

#### **4.5.5 Silica gel SPE cartridge clean-up**

Prior to analysis by GC/FPD, the derivatised sample is cleaned-up using commercially available silica gel solid phase cartridges.

Before use, the SPE cartridges are conditioned by passing ~10 ml degassed (ultrasonicated) methyl acetate/toluene (1:1 v/v) through each cartridge. The use of a degassed solvent mixture allows conditioning without causing air bubbles in the cartridges. The cartridges must not be allowed to become dry. If this happens, the cartridge must be conditioned again as described above.

To load the SPE cartridge, a 5 cm disposable syringe needle is attached to a 10 ml disposable syringe.

The remaining toluene in the round bottom flask is drawn into the syringe, dissolving the sample in the flask with aid of an ultrasonic bath.

The volume in the syringe is made up to 4 ml with toluene.

4 ml methyl acetate are added to the flask and the solution is again ultrasonicated to dislodge any residual sample material which may adhere to the flask.

The methyl acetate is drawn into the syringe. The final volume in the syringe is adjusted with methyl acetate to 8.0 ml, achieving a 1:1 ratio of methyl acetate/toluene. The empty flask is retained.

The syringe is inverted (plunger end down), the needle is removed and saved. A 0.5 µm disposable filter is attached to the tip of the syringe and the sample is passed through the disposable filter onto the cartridge.

The (retained) empty flask is washed with 5 ml methyl acetate and 5 ml toluene. The solution is ultrasonicated and also drawn into the disposable syringe (filter was removed and saved) via the re-attached syringe needle. After removal of the syringe needle, the filter is reconnected and the wash solution applied to the SPE cartridge. After the wash solution is eluted, the cartridge is dried using a slight vacuum suction. The total washings from cartridge clean-up are discarded.

The elution of the derivatives from the cartridge is done with 3 ml methanol/methylacetate (1:1) without applying vacuum to the SPE-box and collecting the sample in a volumetric flask

Normally, a final volume of 3 ml provides adequate sensitivity for determination. The solution is ready for quantification with GC/FPD

#### **4.5.6 Critical steps of the analytical method**

Care should be taken when solutions containing the GC-derivatives are evaporated using a rotary evaporator. The bath temperature should not exceed 40°C. The round bottom flasks should never run dry!

## **5 CALCULATION**

To establish a calibration curve or one-point calibration, test solutions with known amounts of AE F064706, AE F070951 or AE F096432 in methylacetate are injected as a mixture of all three derivatives into the GC-system.

### Calibration curve

Peaks heights measured by electronic integration are plotted against the amount of AE F039866, AE F061517 or AE F064619. Normally the calibration curve follows the general equation  $y = a + b \cdot x$ . However, a large range of concentrations can be described only by a function such as  $y = a + b \cdot x + c \cdot x^2$ . The amount of AE F039866, AE F061517 or AE F064619 in different matrices can be calculated directly from these calibration curves.

### One -point calibration

Peak heights are measured by electronic integration. The concentrations in the sample are calculated against the peak heights of a calibration sample.

Residue concentrations of AE F039866, AE F061517 and AE F064619 are calculated as follows:

### Determination of residues and apparent residues

$$R \text{ or } A = \frac{C_S [\text{pg}/\mu\text{L}] \cdot V_1 [\text{mL}] \cdot f}{W \cdot 1000}$$

$$\text{with } C_S [\text{pg}/\mu\text{L}] = \frac{A_S [\text{pg}]}{T_{\text{inj}} [\mu\text{L}]} \quad f = \frac{V_2 [\text{mL}] \cdot V_3 [\text{mL}] \cdot V_4 [\text{mL}]}{T_1 [\text{mL}] \cdot T_2 [\text{mL}] \cdot T_3 [\text{mL}]}$$

$C_S$  can be calculated from the calibration curve or one -point calibration with the following equation:

$$C_S [\text{pg}/\mu\text{L}] = \frac{C_T [\text{pg}/\mu\text{L}] \cdot F_S [\text{counts}]}{F_T [\text{counts}]} = \frac{A_T [\text{pg}] \cdot F_S [\text{counts}]}{T_{\text{inj}} [\mu\text{L}] \cdot F_T [\text{counts}]}$$

### Determination of the recovery efficiency

$$\text{Recovery } [\%] = \left( \frac{C_S [\text{pg}/\mu\text{L}] \cdot V_1 [\text{mL}] \cdot f}{W \cdot 1000} - A \right) \cdot \frac{100}{W_R}$$

#### List of symbols

- R residue in treated sample [ $\mu\text{g}/\text{l}$ ]
- A apparent residue in the control sample [ $\mu\text{g}/\text{l}$ ]
- $A_S$  amount of analytical target in the injection volume  $T_{\text{inj}}$  of the sample solution [pg]
- $A_T$  amount of analytical target in the injection volume  $T_{\text{inj}}$  of the test solution [pg]

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$C_S$	amount of AE F039866 obtained from the calibration curve or one- point calibration [pg/ $\mu$ L]
$C_S$	amount of AE F061517 obtained from the calibration curve or one- point calibration [pg/ $\mu$ L]
$C_S$	amount of AE F064619 obtained from the calibration curve or one- point calibration [pg/ $\mu$ L]
$C_T$	amount of AE F064706 in the test solution [pg/ $\mu$ L]
$C_T$	amount of AE F070951 in the test solution [pg/ $\mu$ L]
$C_T$	amount of AE F096432 in the test solution [pg/ $\mu$ L]
$F_S$	peak area or peak height of analytical target in the injection volume $T_{inj}$ of the sample solution [counts]
$F_T$	peak area or peak height of analytical target in the injection volume $T_{inj}$ of the test solution [counts]
$f$	dilution factor
$V_1$	volume of the final solution [mL]
$V_2$	volume of the final solution after refill of $T_1$ [mL]
$V_3$	volume of the final solution after refill of $T_2$ [mL]
$V_4$	volume of the final solution after refill of $T_3$ [mL]
$W$	weight of the analytical sample [g]
$W_R$	amount of AE F039866 added per sample weight [ $\mu$ g/g]
$W_R$	amount of AE F061517 added per sample weight [ $\mu$ g/g]
$W_R$	amount of AE F064619 added per sample weight [ $\mu$ g/g]
$T_{inj}$	injection volume [ $\mu$ L]
$T_1$	aliquot of $V_1$ [mL]
$T_2$	aliquot of $V_2$ [mL]
$T_3$	aliquot of $V_3$ [mL]

All masses and concentrations are calculated and expressed as Glufosinate free acid equivalents (AE F035956).

**Appendix I:**
**Flow diagram**

**Removal of cations**  
**AE F039866**  
**AE F061517**  
**AE F064619**

Pour 500 ml water  
 onto the chelating resin column and  
 collect water  
 flowing through resin.

**Enrichment and clean-up**

Let water run through anion exchange column.  
 Wash column with 100 ml water and discard washing.

**Isolation**

Elution with 100 ml formic acid (50 %).  
 Collect eluate.

Rotavap eluate to complete dryness (60°C)  
 until no traces of acid are left.  
 Add 10 ml water and rotavap.  
 2 \* 5-10 ml methanol.

**Derivatisation**

Dissolve residue with 3 ml glacial acetic acid (Ultrasonic).  
 Add 12 ml trimethylorthoacetate and some glass beads.  
 Mix (Ultrasonic).  
 Reflux for 4,5 h (Overnight and timer!).

**Attention: never let sample go dry!**

Add 1 x 15 ml Toluol.  
 Concentrate to 1-2 ml using rotavap (max 40°C).  
 Add 2 x 15 ml toluene and concentrate each time to 1-2 ml.

**Clean-up Silicagel cartridge (SPE)**

Conditioning of column with 10 ml (methylacetate/toluene; 1+1; V+V).  
 Draw up toluene in disposable syringe and fill up to 4 ml with toluene.  
 Dissolve residue in round bottom flask (Ultrasonic) in 4 ml methylacetate and draw up in syringe.  
 Filtration of solvent mixture (disposable filter) onto cartridge.  
 Dissolve residue with 5 ml methylacetate and 5 ml toluene (Ultrasonic).  
 Draw up in same syringe without filter.  
 Filtration (filter) onto cartridge.  
 Discard toluene/methylacetate.  
 Dry cartridge with vacuum.

Elution with minimum 3 ml  
(Methanol/methylacetate; 1+1; V+V)  
without applying vacuum to SPE-box  
collection in 3ml volumetric flask

**GC**

Final volume  
3 ml methanol/methylacetate  
GC-FPD (526 nm)