## 2 Objectives

The objective of this study is the validation of the analytical method EM F07/99 – 0 (1) for the determination of residues of AE F130360 in surface and drinking water by HPLC using UV - detection.

## 3 Test commodities

The drinking water used for the validation was "Vittel" and the surface water was taken from the pond "Kohlmannweiher" in Sossenheimer Unterfeld and from the pond "Angler See" at the Schwanheimer Düne.

The characteristics[1] of the surface water are:

| | | |
|---|---|---|
| "Kohlmannweiher" | pH | 7.9 |
| | DOC [mg/L][1] | 9.1 |
| | total hardness [° d][2] | 14.6 |
| "Angler See" | pH | 7.7 |
| | DOC [mg/L][1] | 5 |
| | total hardness [° d][2] | 17.4 |

[1] dissolved organic content
[2] degree german hardness [(mg CaO + mg MgO) / 100 mL water]

## 4 Relevant residue and reference substances

### 4.1 Relevant residue

The relevant residue for risk assessment consists of the parent compound AE F130360.

[1] Determination of the characteristics of the surface water was not done under GLP.

## 4.2 Test and reference substances

**AE F130360**

Chemical name (UPAC): N,N-dimethyl-2-[3-(4,6-dimethoxypyrimidin-2-yl) ureidosulfonyl]-4-formylaminobenzamide

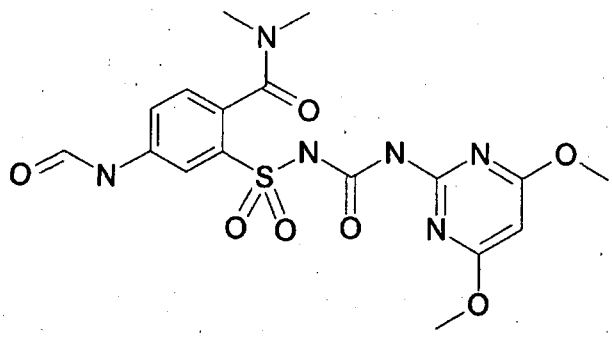
Empirical formula: $C_{17}H_{20}N_6O_7S$

Molecular weight: 452.5

Solubility (20 °C):

| Solvent | Solubility | | Source |
|---|---|---|---|
| Acetone | 1925 | mg/L | ref. 2 |
| Acetonitrile | 1111 | mg/L | ref. 2 |
| 1,2-Dichloroethane | 185 | mg/L | ref. 2 |
| Ethyl acetate | 362 | mg/L | ref. 2 |
| n-Heptan | <10 | mg/L | ref. 2 |
| Methanol | 1660 | mg/L | ref. 2 |
| p-Xylene | <10 | mg/L | ref. 2 |

Structural formula:



Certificate of analysis: AZ 07341
Drawn up by: Hoechst Schering AgrEvo GmbH
Produktanalytik
D-65926 Frankfurt am Main, Germany
Purity: 99.0 % (w/w)
Expiry date (d/m/y): 23 Apr 2001

# 5 Procedures

## 5.1 Principle of analytical method

The flow sheets of the analytical method EM F07/99 – 0 (1) are presented in Annex I.

The **drinking water** sample is adjust to pH 2.5 with phosphoric acid (2 N) and sucked through a C18-cartridge (conditioned with methanol and water). AE F130360 is eluated with methanol. The concentration of AE F130360 in the final solution of acetonitrile/water (1/1, v/v) is determined by HPLC/UV.

The **surface water** sample is adjust to pH 2.5 with phosphoric acid (2 N) and sucked over a glass microfiber filter, than over a membrane filter and through a C18-cartridge (conditioned with methanol and water). AE F130360 is eluated with methanol, reduced to dryness and dissolved in toluene, followed by a clean-up over a silicagel-cartridge. AE F130360 is eluated by toluene/methanol (95 : 5, v/v). The concentration of AE F130360 in the final solution of acetonitrile/water (1/1, v/v) is determined by HPLC/UV.

## 5.2 Reagents

- methanol Pestanal (Riedel-de Haën, Germany)
- methanol Chromasolv (Riedel-de Haën, Germany)
- acetonitrile Chromasolv p.A. (Riedel-de Haën, Germany)
- toluene Pestanal (Riedel-de Haën, Germany)
- deionized water
- water (e.g. prepared with Milli-Q-Plus, Millipore)
- phosphoric acid 2 N
- AE F130060, analytical standard (AgrEvo GmbH, Germany)
- C18 – cartridge, Cat. No. 730013 (Macherey-Nagel, Germany)
- silicagel – cartridge, Cat. No. 460-0050-H (Isolute IST)
- glass microfibre filters, 934-AH, 70 mm Ø, Cat. No. 1827 070 (Whatman)
- membrane filter, cellulose nitrat, 0.45 µm, Order No. 11306 50 N (Sartorius)

Stock solutions of the analytical standards were prepared by dissolving about 50 mg of analytical standard of AE F130360 in ca. 50 mL acetonitrile / methanol (1:1, v/v). Concentration of the stock solutions was 1.00 mg/mL. Working solutions were prepared from the stock solution by further dilution with acetonitrile / water, 1:1, v/v.

## 5.3 Apparatus

The following list contains the apparatus used in the laboratory of the author for validation. Suitable alternatives can be taken.

- standard laboratory glassware
- rotary vacuum evaporator with water bath
- HPLC system with UV-detector
- chromatography column, Prodigy ODS, 150 mm x 4.6 mm, 5 µm
- chromatography column, Waters Spherisorb Phenyl, 250 mm x 3 mm, 5 µm

## 5.4 Preparation of samples and storage

| Matrix | Name | Arrival at analytical laboratory |
|---|---|---|
| Drinking water | "Vittel" | 21.07.1999 (a) |
| Surface water | "Kohlmannweiher" | 28.07.1999 (b) |
| | "Angler See" | 11.08.1999 (b) |

a) purchased on 21.07.1999
b) sampling date

Samples of water were stored at room temperature.

## 5.5 Laboratory steps

### 5.5.1 Drinking water

#### 5.5.1.1 Extraction and C18-cartridge clean up

1000 mL of the water sample is adjust to pH 2.5 with phosphoric acid (2 N) and sucked through a C18-cartridge (conditioned with 5 mL methanol and 5 mL water) with a flow rate of ca. 10 – 20 mL/min. Wash used glassware with 200 mL Millipore water and suck the washing water through the cartridge. Suck the C18-cartridge to dryness within ca. 5 min. Eluate AE F130360 with 5 mL methanol into a test tube. Reduce the eluate to dryness using a vacuum rotary evaporator (bath temperature ca. 40 °C).

#### 5.5.1.2 Preparation of the final solution

Dissolve the residue in 1.0 mL acetonitrile/water (1/1, v/v).

### 5.5.2 Surface water

#### 5.5.2.1 Extraction and C18-cartridge clean up

1000 mL of the water sample is adjust to pH 2.5 with phosphoric acid (2 N) and sucked over a glass microfiber filter (Whatman), than over a membrane filter, 0.45 µm (Sartorius). Then the water sample is sucked through a C18-cartridge (conditioned with 5 mL methanol and 5 mL water) with a flow rate of ca. 10 – 20 mL/min. Wash used glassware with 200 mL Millipore water and suck the washing water through the cartridge. Suck the C18-cartridge to dryness within ca. 5 min. Eluate AE F130360 with 5 mL methanol into a test tube. Reduce the eluate to dryness using a vacuum rotary evaporator (bath temperature ca. 40 °C). Dissolve the residue in 5 mL toluene and reduce to dryness using a vacuum rotary evaporator (bath temperature ca. 40 °C).

#### 5.5.2.2 Silicagel-cartridge clean up

Dissolve the residue in the test tube with 20 mL toluene, transfer the solution onto a silicagel-cartridge (conditioned with 5 mL toluene). Discard the eluate. Suck the silicagel-cartridge to dryness. Wash the test tube with 30 mL toluene/methanol (95 : 5, v/v) (using an ultrasonic bath, if necessary) and elute AE F130360 with this solution. Reduce the eluate to dryness using a vacuum rotary evaporator (bath temperature ca. 40 °C).

#### 5.5.2.3 Preparation of the final solution

Dissolve the residue in 1.0 mL acetonitrile/water (1/1, v/v).

## 5.6 Determination of residues

The following conditions have been used successfully during validation of the analytical method. If different equipment and columns are used, modifications of the given conditions may be necessary.

### HPLC-conditions

| | |
|---|---|
| Instrument: | Beckmann |
| System | IBM-PC System 2 8570 Model 70386 |
| Pump: | 226 Beckmann |
| Detector: | Diode Array Detector 168 Beckmann |
| Injector: | Autosampler 507 Beckmann |
| Injection volume: | 100 µL |
| Column temperature: | 16 °C |
| Column | Prodigy ODS, 5 µm, 150 mm x 4.6 mm |
| Wavelength: | 233 nm |
| Flow rate: | 1.0 mL/min |
| Mobile phase: | |
| Eluent A | Acetonitrile Chromasolv |
| Eluent B | Phosphoric acid $c_{H3PO4}$= 0.01mol/L |

### Gradient program for the determination of AE F130360

| Time [min] | Total flow pump A + B [mL/min] | Pump A (eluent A) Acetonitrile Chromasolv [%] | Pump B (eluent B) phosphoric acid $c_{H3PO4}$= 0.01mol/L [%] |
|---|---|---|---|
| 0 | 1.0 | 20 | 80 |
| 10 | 1.0 | 50 | 50 |
| 20 | 1.0 | 50 | 50 |
| 25 | 1.0 | 80 | 20 |
| 27 | 1.0 | 80 | 20 |
| 32 | 1.0 | 20 | 80 |
| 34 | 1.0 | 20 | 80 |
| 40 | 1.0 | 20 | 80 |

Under these conditions the retention time for AE F130360 is about 20.0 min.

**Confirmatory method**

For confirmatory purposes a different stationary phase was used and adapted parameters were chosen:

**HPLC-conditions**

| | |
|---|---|
| Instrument: | Beckmann |
| System | IBM-PC System 2 8570 Model 70386 |
| Pump: | 226 Beckmann |
| Detector: | Diode Array Detector 168 Beckmann |
| Injector: | Autosampler 507 Beckmann |
| Injection volume: | 100 µL |
| Column temperature: | 16 °C |
| Column | Waters Spherisorb Phenyl, 5 µm, 250 mm x 3 mm |
| Wavelength: | 233 nm |
| Flow rate: | 1.0 mL/min |
| Mobile phase: | |
| Eluent A | Acetonitrile Chromasolv |
| Eluent B | Phosphoric acid $c_{H3PO4}$= 0.01mol/L |

**Gradient program for the determination of AE F130360**

| Time [min] | Total flow pump A + B [mL/min] | Pump A (eluent A) Acetonitrile Chromasolv [%] | Pump B (eluent B) phosphoric acid $c_{H3PO4}$= 0.01mol/L [%] |
|---|---|---|---|
| 0 | 1.0 | 20 | 80 |
| 10 | 1.0 | 50 | 50 |
| 20 | 1.0 | 50 | 50 |
| 25 | 1.0 | 80 | 20 |
| 27 | 1.0 | 80 | 20 |
| 32 | 1.0 | 20 | 80 |
| 34 | 1.0 | 20 | 80 |
| 40 | 1.0 | 20 | 80 |

Under these conditions the retention time for AE F 130360 is about 19.0 min.

The chromatography data were recorded and evaluated with TURBOCHROM© Client/Server system, PERKIN ELMER.

## 5.7 Calibration

The concentration of AE F130360 was calculated using external standards at up to 5 different concentrations over a range from 100 pg/µL up to 1000 or 2000 pg/µL. The recommended order of samples / test solutions for setting up a sequence for HPLC-determination is 'test solution – sample – test solution– sample '. If different equipment is used and /or more or less samples are worked up, modifications of this order may be necessary.

## 5.8 Calculations

**Determination of concentration of the analytical target in the final solution**
The concentrations of the analytes in control samples, fortified samples and treated samples were calculated using external standard procedures with multi level or single level calibration.

**Single level calibration (one point calibration):**

$$C_S = \frac{P_S}{P_R} \bullet C_R \bullet \frac{I_R}{T_4} \qquad \left[ pg/\mu L = \frac{counts}{counts} \bullet pg/\mu L \bullet \frac{\mu L}{\mu L} \right] \qquad (1)$$

| | | |
|---|---|---|
| $C_S$ | Concentration in final sample solution $V_{end}$ (identical with conc. in $T_4$) (treated, untreated and recovery) | [pg/µL] = [ng/mL] |
| $C_R$ | Concentration in reference solution | [pg/µL] = [ng/mL] |
| $P_S$ | Peak area or peak height of the sample solution | [counts] |
| $P_R$ | Peak area or peak height of the reference solution | [counts] |
| $T_4$ | Injection volume of the sample solution | [µL] |
| $I_R$ | Injection volume of the reference solutions | [µL] |

**Multi level calibration (calibration curve):**

For the calibration peak areas (heights) of the standards were plotted versus the corresponding concentrations. An optimized calibration curve of the following form

$$f(C_S) = P = a + bC_S + cC_S^2 \qquad (2)$$

is calculated, where $f(C_S)$ is the peak area (height), $C_S$ the concentration of the analyte in the final sample extract and a, b, c are constants.

**Determination of residues**

Calculation of residues was carried out by a data handling software according to the following procedure

$$Res = \frac{C_S \bullet V_{end} \bullet f}{W} \qquad \left[\mu g/L = \frac{(ng/mL) \bullet mL \bullet 1}{mL}\right] \qquad (3)$$

$$f = \frac{V_1 \bullet V_2 \bullet V_n}{T_1 \bullet T_2 \bullet T_n} \qquad \left[1 = \frac{mL \bullet mL \bullet mL}{mL \bullet mL \bullet mL}\right] \qquad (4)$$

| | | |
|---|---|---|
| ***Res*** | Residue | [µg/L] |
| $C_S$ | Concentration in final sample solution $V_{end}$ (treated, untreated and recovery) | [ng/mL] |
| ***W*** | Sample weight | [mL] |
| ***f*** | Dilution factor | without dimension |
| $V_1$ | Volume for primary extraction | [mL] |
| $V_2$ | Volume after making up of aliquot $T_1$ | [mL] |
| $V_n$ | Volume after making up of aliquot $T_{n-1}$ (n = 3, 4 and so on) | [mL] |
| $V_{end}$ | Final sample solution (identical with $V_2$ or $V_3$ or $V_n$ depending on the method) | [mL] |
| $T_1$ | Aliquot of $V_1$ | [mL] |
| $T_2$ | Aliquot of $V_2$ | [mL] |
| $T_n$ | Aliquot of $V_n$ (n = 3, 4 and so on) | [mL] |

**Determination of recovery rates**

Calculation of recovery rates were carried out by a data handling software according to the following procedure

$$Res_d = Res_{(Rec)} - Res_{(Unt)} \qquad \left[\frac{\mu g}{L} = \frac{\mu g}{L} - \frac{\mu g}{L}\right] \qquad (5)$$

$$Rec = \frac{Res_d}{Res_f} \bullet 100 \qquad \left[\% = \frac{\mu g/L}{\mu g/L} \bullet \%\right] \qquad (6)$$

| | | |
|---|---|---|
| $Res_{(Rec)}$ | Residue in the sample solution of the recovery test calculated with equation (3) and (4) | [µg/L] |
| $Res_{(Unt)}$ | Residue in the sample solution of the corresponding untreated control sample calculated with equation (3) and (4) | [µg/L] |
| ***Rec*** | Recovery rate | [%] |
| $Res_f$ | Concentration spiked for fortification | [µg/L] |
| $Res_d$ | Concentration detected by analytical method | [µg/L] |

## Annex I: Analytical method flow sheet

### Drinking water

*Extraction AE F130360 and C18-cartridge clean-up*

1000 mL water
is adjust to pH 2.5 with phosphoric acid (2 N)
and sucked through a C18-cartridge
(conditioned with 5 mL methanol and 5 mL water)
with a flow rate of ca. 10 – 20 mL/min

Wash used glassware with 200 mL Millipore water
and suck the washing water through the cartridge

Suck the C18-cartridge to dryness within ca. 5 min

Eluate AE F130360 with 5 mL methanol into a test tube

Reduce the eluate to dryness using a vacuum rotary evaporator
(bath temperature ca. 40 °C)

*HPLC*

Dissolve the residue in 1.0 mL water/acetonitrile (1/1, v/v)
quantification with UV/HPLC

### Surface water

*Extraction AE F130360*

1000 mL water
is adjust to pH 2.5 with phosphoric acid (2 N)
and sucked through a glass microfiber filter and a membrane filter

*C18-cartridge clean-up*

The solution is sucked through a C18-cartridge
(conditioned with 5 mL methanol and 5 mL water)
with a flow rate of ca. 10 – 20 mL/min

Wash used glassware with 200 mL Millipore water
and suck the washing water through the cartridge

Suck the C18-cartridge to dryness within ca. 5 min.

Eluate AE F130360 with 5 mL methanol into a test tube

Reduce the eluate to dryness using a vacuum rotary evaporator
(bath temperature ca. 40 °C)

*Silicalgel-cartridge clean-up*

Dissolve the residue in toulene
and sucked through an silicagel-cartridge
(conditioned with 5 mL toluene)
with a flow rate of ca. 10 – 20 mL/min

Wash used glassware with 30 mL toluene/methanol
and eluate AE F130360 with the washing solution

Reduce the eluate to dryness using a vacuum rotary evaporator
(bath temperature ca. 40 °C).

*HPLC*

Dissolve the residue in 1.0 mL water/acetonitrile (1/1, v/v)
quantification with UV/HPLC