# 1.0 INTRODUCTION / PURPOSE

EPA guidelines FIFRA, Subdivision E, 72 for ecological toxicity studies require an analytical method to be used for determination of the concentration of test solution in range-finding studies, preliminary trials and definitive studies. The measurement of the concentration of test substance represents the actual exposure of test substance to the test organisms and leads to correct LC/EC 50 values. It is especially useful for providing low realistic exposure for low rate-use pesticides. Moreover, the actual measurement confirms the operating test system thus avoiding errors due to chemical characteristics, binding, low solubility, short half-life, and mechanical problems (mixing etc:). The analytical method is also required to determine the purity of test substance and batch analysis. It also gives an indication of test substance mixing and dissolution in aquatic media.

The purpose of this study is to verify a method for measuring BAS 620 H in various aquatic media used for "wildlife and aquatic organisms" testing.

## 1.1 SCOPE OF THE METHOD

BASF method D9515 can be used for (i) the verification of the concentration of BAS 620 H in test solution prepared from technical material (TGAI) and EC-formulated material used in definitive studies, (ii) the determination of the solubility of BAS 620 H (AI) in aquatic media (test matrices), (iii) the determination of the stability of BAS 620 H (AI) during test conditions (room temperature) and (iv) the stability of BAS 620 H in sample storage prior to actual analysis (refrigerator conditions). It may also be useful to measure the concentration of BAS 620 H in standard or test solutions prepared using TGAI or EC-formulated material.

This method will be used for determining the level of BAS 620 H in aquatic media samples collected during actual ecological toxicity studies at 0-day, intermediate intervals and the end of the study.

## 1.2 SOURCE OF THE METHOD

Method D9515 which can be used to determine BAS 620 H residues in aquatic eco-tox media (pH ranging from 7.0 to 8.1) was developed and validated by BASF Corporation. The method is a modification of an earlier BASF AG method (**Reference 1**).

## 1.3 PRINCIPLE OF THE METHOD

An aliquot of aquatic media is diluted with an appropriate amount of pH-9 water to bring the concentration down to the level of optimum UV signal to be detected. The diluted

aliquot is injected onto HPLC/UVD. The total amount of BAS 620 H is determined by the comparison of the UV signal obtained from BAS 620 H in the aquatic media versus a standard curve constructed from various concentrations of BAS 620 H analytical standards.

Note: The dilution with pH-9 water is necessary to avoid degradation of BAS 620 H.

### 1.4 PROJECT HISTORY

The study protocol (see **Appendix 1**) was signed by the study director on, September 19, 1995. The experimental initiation date was, January 9, 1996. The fish and algal aquatic media were selected to validate this method. Initially this study was degined to use only "algal aquatic" media from Carolina Ecotox lab, Durham, North Carolina but later it was modified to use fresh water, salt water (esturine media) and Hogland's media (algal) [ see protocol change number 2]. The control samples selected for this study were actual aquatic media used in in-life eco-tox studies at Wildlife International, Inc., Easton, Maryland.

An analytical standard was used for spiking aquatic media instead of technical grade material (TGAI). Independent experiments were also conducted using EC-formulated material to explore the applicability of this method.

In general, this study was initiated to obtain the recommended preliminary information before starting definitive ecological toxicity studies and to determine the concentration of BAS 620 H in aquatic media samples from ecological tests (see **Appendix 2** for more information on the role of an analytical method in eco-tox studies).

## 2.0 MATERIALS

### 2.1 TEST SYSTEM

This method was validated using fresh water (pH 7), unfiltered salt water (pH 8.1), and Hoagland's media (pH 5). These media control samples were obtained from Wildlife International, Ltd, Easton, Maryland 21601.

### 2.2 TEST SUBSTANCE

Analytical standard (BAS 620 H) was characterized as required by 40 CFR part 160, FIFRA Good Laboratory Practices. The data on the synthesis and subsequent characterization of this substance are located at Landwirtschaftliche Versuchsstation der BASF Ag., Limburgerhof, Germany and are available to BASF Corporation, Agricultural Products Center, P. O. Box 13528, Research Triangle Park, NC 27709. The technical

data on the test substance which is identified by BASF number, scientific name, lot number, purity, and physical characteristics is presented here.

**Analytical reference standard:**

The active ingredient (a.i.) was used for all method development and validation work. The purity of analytical grade standard was about 99.9% (see **Appendix 3** for purity statement).

Note: The solid BAS 620 H standard was maintained frozen (<-5°C) until its use in this study.

**Common name:** Caloxydim

**BASF code:** BAS 620 H

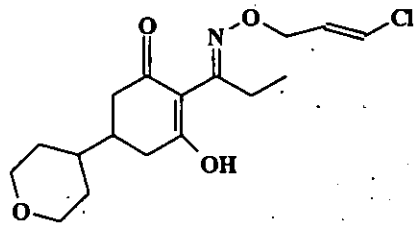
**Chemical name:** *2-[1-(3(E)-chloroprop-2-enyl) oxyiminopropyl]-3-hydroxy-5-tetrahydropyran-4-ylcyclohex-2-enone*

or

2-[1-(3-chloroprop-2-(E)-en-1-yl)oximinopropyl]-3-hydroxy-5-(terahydropyran-4-yl)cyclohex-2-en-1-one

**CAS Registry number**: 149979-41-9 for the molecule with stereochemistry defined at the C=C double bond

**Structure:**




**Empirical formula:** $C_{17}H_{24}ClNO_4$

**Molecular weight:** 341.84

**Melting point:** 72.5 to 74.4°C.

**Appearance:**White solid

**Odor:** Odorless

**Solubility (g/l solvent, 20°C) in water at pH = 9:** 7.25 g/l

(see **Appendix 4** for more general characteristics of BAS 620 H)

Note on **Isomerization** of BAS 620 H: The test substance BAS 620 H (E-isomer) isomerizes to a certain extent to the Z-isomer, at oxime-ether moiety of the molecule in a solution mainly in organic solvents where an equilibrium is established (**Reference 2**). The HPLC retention times are very different for E- and Z-isomers. Both the E-isomer and Z-isomer also may have corresponding cis isomers at the carbon-carbon double bond. But these cis isomers are not separated from their trans isomers by HPLC analysis. See **Figure 1** for structures of both E- and Z-isomers.

**BAS 620 H Formulated Product used for Spiking:**

The BASF 620 H -formulated product used in this method development and stability work, contained 19.2 ± 0.220% of Caloxydim % (see **Appendix 3** for GLP assay report).

| | |
|---|---|
| Type: | EC (Emulsion Concentrate) |
| Trade Name: | Undecided |
| Tested Formulation: | BAS 620 00 H (EC) |
| Lot Number: | 95-1 |
| GLP Certified Active Ingredient: | 19.2 ± 0.220% of Caloxydim |
| Analysis Report Number: | FR9546 (study no. 95141) |
| Date of Certification: | October 11, 1995 |
| Date of GLP Analysis Expiration: | October 11, 1997 |

## 2.2 EQUIPMENT

| Equipment | Suggested Sizes/Manufacturer |
|---|---|
| Volumetric Flasks | VWR Scientific Co. |
| Volumetric Pipettes | VWR Scientific Co. |

pH values were monitored with pH-paper only in this study, but a pH-meter can be used if desired.

## 2.3 REAGENTS AND CHEMICALS

Acetonitrile UV grade, CAS 75-05-8 (Baxter Health care Corporation)
B/J Brand Water, CAS 7732-18-5 (Baxter Health care Corporation)

B/J Brand Acetic Acid, Glacial, CAS 64-19-7(Fisher Scientific Corporation)

Note: Equivalent reagents and chemicals from other suppliers may be substituted.

## 3.0 ANALYTICAL METHOD

The general analytical procedure is described in the flow chart given in **Figure 2.** At the time of analysis, samples were removed from refrigerator storage and were warmed to room temperature in order to sub-sample for analysis. Because the water was relatively free of contaminants and had a high concentration of test material, samples did not have to be purified prior to analysis. Aliquots of the test samples were taken and diluted appropriately with water adjusted to pH 9 with 0.1 N Sodium Hydroxide prior to injection on a High Pressure Liquid Chromatograph (HPLC). Chromatography was achieved using a reverse phase C-18 column, acetonitrile/water mobile phase containing phosphoric acid, and an Ultraviolet detector.

### 3.1 STANDARD SOLUTIONS

### 3.1.1 Stock Solution of BAS 620 H (1.0 mg/ml):

Prepare a 1.0 mg/ml BAS 620 H stock solution by weighing an appropriate amount of solid BAS 620 H (analytical standard) into a volumetric flask. Dissolve with water adjusted to pH 9 and dilute to the mark by manual shaking. For example, to prepare a 25 ml stock solution, dissolve 25.0 mg of 620 H in a 25 ml volumetric flask with water adjusted to pH 9. Standard solution documentation can be found in the 1995 Standard Notebook and 1996 Standard Notebook located in BASF archives.

Notes: (1) Do not use sonication to avoid thermal decomposition of BAS 620 H. The use of sonication in preparation of BAS 620 H stock solution will generate heat and decompose the molecule. (2) Check pH during stock solution preparation and keep it around pH 9 by adjusting with 0.1 N NaOH solution. (3) Stock solutions (1 mg/ml) were made fresh every three months. Dilutions of the stock solution were made monthly.

### 3.1.2 Fortification Solutions

a. Spiking solution using analytical standard:

The fortification of analytical standard in aquatic media at three different concentrations was done at room temperature. They were made the same way as discussed in previous section (3.1.1). The spiking solutions at 2.5, 50 and 100 µg/ml were prepared by further dilution of stock solution with pH 9 water.

b. Spiking solution using formulated standard:

Appropriate amount of BAS 620 H-EC-formulated material was taken on the weight basis and diluted to appropriate concentration with pH 9 water.

Notes: (1) Standard solutions of BAS 620 H, were refrigerated (+3-6.2°C) during their use in this study. (2) Storage containers for standard solutions are amber bottles with Teflon-lined screw caps.

### 3.1.3 HPLC Injection Standard Solutions

The standard solutions at 0.02, 0.05, 0.1, 0.2 µg/ml were prepared in the same way as fortification standards (section 3.1.2).

### 3.1.4 Solubility of BAS 620 H in aquatic media

No problems were encountered when BAS 620 H was dissolved in aquatic media at 1 mg/ml.

## 3.2 CONTROL MATRICES

The matrices normally used for fish toxicity studies (subdivision-E) include filtered and unfiltered salt water (pH 8.0 to 8.2) and well-water or fresh water (pH 7). The aquatic media required for algal toxicity studies (subdivision-J) are normally prepared by dissolving nutrient components in salt-water (marine algal assay) or well-water (synthetic algal assay procedure) ranging between pH 7.5 to 8.1 except Hoagland's media. The pH for Hoagland's media is adjusted between 4.9 and 5.1.

The fresh water (pH 7), unfiltered salt water (pH 8.1), and Hoagland's media (pH 5) were used for initial method development studies. Once the method was developed using these three media, it was validated with the media used in ecological toxicity studies. These media samples used in this study were obtained from Wildlife International Ltd, Easton, Maryland 21601. (See attached information in **Appendix 7**).

## 3.3 SAMPLE WORK-UP

The aquatic media samples (50 ml for each sample) were diluted with distilled water adjusted to pH 9 using the following scheme to get an appropriate concentration within the calibration curve:

0.05 ppm spiked sample - Inject straight (The dilution is not required.)
1.0 ppm spiked sample - Dilute 1 to 10 [The original sample (1 ml) was diluted to 10 ml.]

100 ppm spiked sample — Dilute 1 to 1000 [The original sample (0.1 ml) was diluted to 100 ml.]
The samples from actual eco-toxicological studies will be adjusted according to expected concentrations.

### 3.4 EXPERIMENTAL DESIGN

This study was based on the following parameters:

1. Aquatic media: Fresh water (pH 7), Salt water (pH 8.1) and Hoagland's algal media (pH 5)

2. Spiking Material: Analytical standard BAS 620 H (99.9% pure) and EC-formulated BAS 620 H material (19.2% BAS 620 H equivalent).

Note: The analytical standard was added to aquatic media for this study. The technical grade can be used if required for eco-tox studies.

3. Test Concentrations or Levels to spike aquatic media: 0.05, 1.0 and 100 PPM

Normally the test concentrations are selected based on toxicity levels determined from a range finding study or data obtained from preliminary studies. General recommended levels for eco-tox studies are low (normally at LOQ), medium (in case 100 ppm is at saturation level) and high levels (usually at 100 ppm).

4. Temperatures for Test Compound StorageStability

The stability of the test compound was studied at room temperature and at 3-6.2°C with normal lab light as a part of the method development and validation studies. The storage at room temperature was done to mimic the conditions during testing period. The ecological toxicity studies are normally run at room temperature with normal lab light. The storage at 3-6.2°C was done to mimic samples being stored prior to analysis if needed. In general, samples are analyzed as soon as possible after in-life studies are completed. Sometimes the samples are stored temporarily at 3-6.2°C for short times before actual analysis. The samples were stored in refregerator no. 723 which is located in room no. C224 at BASF Corporation, Agricultural Products Center, Research Triangle Park, North Carolina 27709-3528.

5. Storage time period: Samples were analyzed after 4, 7 and 14 days of storage from initial spiking.

## 3.5 HPLC INSTRUMENTATION

The quantitation of BAS 620 H in aquatic media samples was determined by HPLC analysis using the following conditions:

**Description of Equipment:**

| | |
|---|---|
| Liquid Chromatograph: | Hewlett Packard 1050 |
| HPLC Column: | C8, 250 mm x 4.6 mm at ambient conditions |
| Detector: | Applied Biosystems 785A Programmable Absorbance Detector |
| Injector: | Hewlett Packard 1050 |
| Integrator: | Hewlett Packard 3396 Series II |

**Typical Operating Conditions:**

| | |
|---|---|
| Injection Volume: | 100 µl |
| Gradient Mobile Phase: | Initial Mobile Phase: 100% Water (0.2% Phosphoric Acid)<br>Final Mobile Phase: 60% Acetonitrile + 40% Water (0.2 % Phosphoric Acid)<br>Gradient %: 15% Acetonitrile/minute |
| Flow Rate: | 1.5 ml/min. |
| Wavelength: | 264 nm |
| 620 H Retention Time: | 9.0 min. |

Note: The preceding specifications are suggested and may be altered as needed. Actual use conditions and any changes must be documented in the raw data.

## 3.6 CALIBRATION PROCEDURES

Inject several standards (total amount of 2, 5, 10, 20 ng in 100 µl of BAS 620 H until stable responses are observed.

Calculation of results is based on peak height measurements using a standard calibration curve. The detector response (peak height) versus weight of standard injected is plotted. The standards should give a linear response. The calibration curve is obtained by direct injection of 100 µl aliquots of the mixed standards of concentration 0.02, 0.05, 0.1 and 0.2 ng/ml, concurrently with sample analysis.

Note: Different standard concentrations may be used as appropriate.

### 3.7 SAMPLE ANALYSIS

100 µl aliquots of an aquatic media samples are injected into the liquid chromatograph.

The peak heights of unknown samples are directly compared with the standard calibration curve to obtain ng of BAS 620 H.

Every 2-3 samples are bracketed with standards to check for shifts in sensitivity or retention time. To do this, an injection sequence including standards and samples must be planned.

If the peak height(s) of the unknown is (are) larger than the highest standard, dilute the unknown appropriately and re-inject.

### 3.8 COLLECTION OF DATA

The retention time and peak-height (or peak-area) are measured using computerized chromatography data reduction software (Multichrom2 from VG data systems/Fison instruments). The collected raw data are organized using a BASF "Master Sheet" format The examples of this "Master Sheet" format and pertinent information are given in **Appendix 5**. Statistical treatment of the data including determination of averages and standard deviations for the recovery data are given in the "Master Sheets" and the "Recovery Results Worksheets."

### 3.9 RECOVERY TEST

The validity of the analytical method is demonstrated by recovery tests before actual analysis of unknown samples is attempted. An untreated sample (control) and fortified (spiked control) samples will also be processed with each set of samples to be analyzed. Typically, one fortification sample is run at the limit of quantitation. For each fortified sample, an appropriate volume of BAS 620 H standard solution is added to a control aquatic media sample. The BAS 620 H recoveries are calculated using only E-isomer signals throughout this study. If isomerization is suspected, quantitation can be determined by adding both signals (HPLC).

### 3.10 CONFIRMATORY TECHNIQUES

The HPLC analysis using a different column was conducted during the method development work. A single peak was observed from a standard except in EC-formulated spiking at 100 ppm level. An additional peak for Z-isomer of BAS 620 H was seen in EC-formulated spiking at 100 ppm level.

### 3.11 TIME REQUIRED FOR ANALYSIS

Analysis of a set of 8 aquatic media samples requires 1 working day, including sample work-up and HPLC analysis. Automation, including use of autosampler and computerized data systems, would increase the analysis throughput.

### 3.12 INTERFERENCES

1. Sample Matrices

No problems with interferences from matrices have been encountered in this study. If interfering peaks from the matrix occur in the chromatogram, change the HPLC operating conditions or use an alternative HPLC column. It is desirable to clean the chromatographic system by flushing with acetonitrile, then re-equilibrating the conditions in initial mobile phase.

2. Other Sources

No problems with interferences or questionable peak identity from labware, solvent or other chemicals, have been encountered to date.

### 3.13 POTENTIAL PROBLEMS

pH 9 should be maintained for all standard solutions, stock solutions, aquatic media solutions in order to avoid decomposition.

Note: The concentration of stock solution using technical grade material or EC-formulated material should be verified before starting any in-life work.

## 4.0 METHOD OF CALCULATION

### 4.1 STANDARD CALIBRATION CURVE

Construct a linear least squares calibration curve in the form $y = bx + c$ from the standards by plotting peak height versus weight of standard injected for an analyte.

### 4.2 CALCULATION OF ANALYTE IN SAMPLES

The distinct peak of BAS 620 H seen by HPLC/UV from aquatic media is characterized by a retention time and a peak height. The retention time is used qualitatively to identify an

analyte and peak height is used to quantitatively determine the amount of BAS 620 H in aquatic media. The retention time and height of the peak (or peak-area) are measured using computerized chromatography data reduction software (Multichrom2 from VG data systems/Fison instruments).

The calculation of results is based on peak height measurements. A typical example is given in **Figure 3.** Using the peak height measurements for BAS 620 H in the samples, determine the amount of the analyte in ng from the appropriate least squares calibration curve. Use full computer/calculator precision in any intermediate calculations. Round only the final value.

Calculate ppm values by the equation below.

$$ppm = \frac{A}{B}$$

where: A = ng value interpolated from calibration curve
B = mg sample injected

$$= \frac{\text{Dry Sample Wt.(g) x ml injected}}{\text{Final dilution volume (ml)}}$$

The "final dilution volume" includes any dilution which have been made.

Note: The recoveries are calculated using only BAS 620 H E-isomer signals throughout this study.

### 4.3 CALCULATION OF PROCEDURAL RECOVERIES

Correct fortification results for residues found in the control sample as follows:

ppm (corrected) = ppm in fortified control - ppm in control

Determine percent recovery from the fortification experiments as follows:

$$\% \text{ Recovery} = \frac{\text{ppm (corrected) X 100}}{\text{ppm BAS 620H added}}$$

Note: Only results for procedural recovery samples should be corrected for residues in the controls. Do not correct treated sample results for either control residues or recoveries.

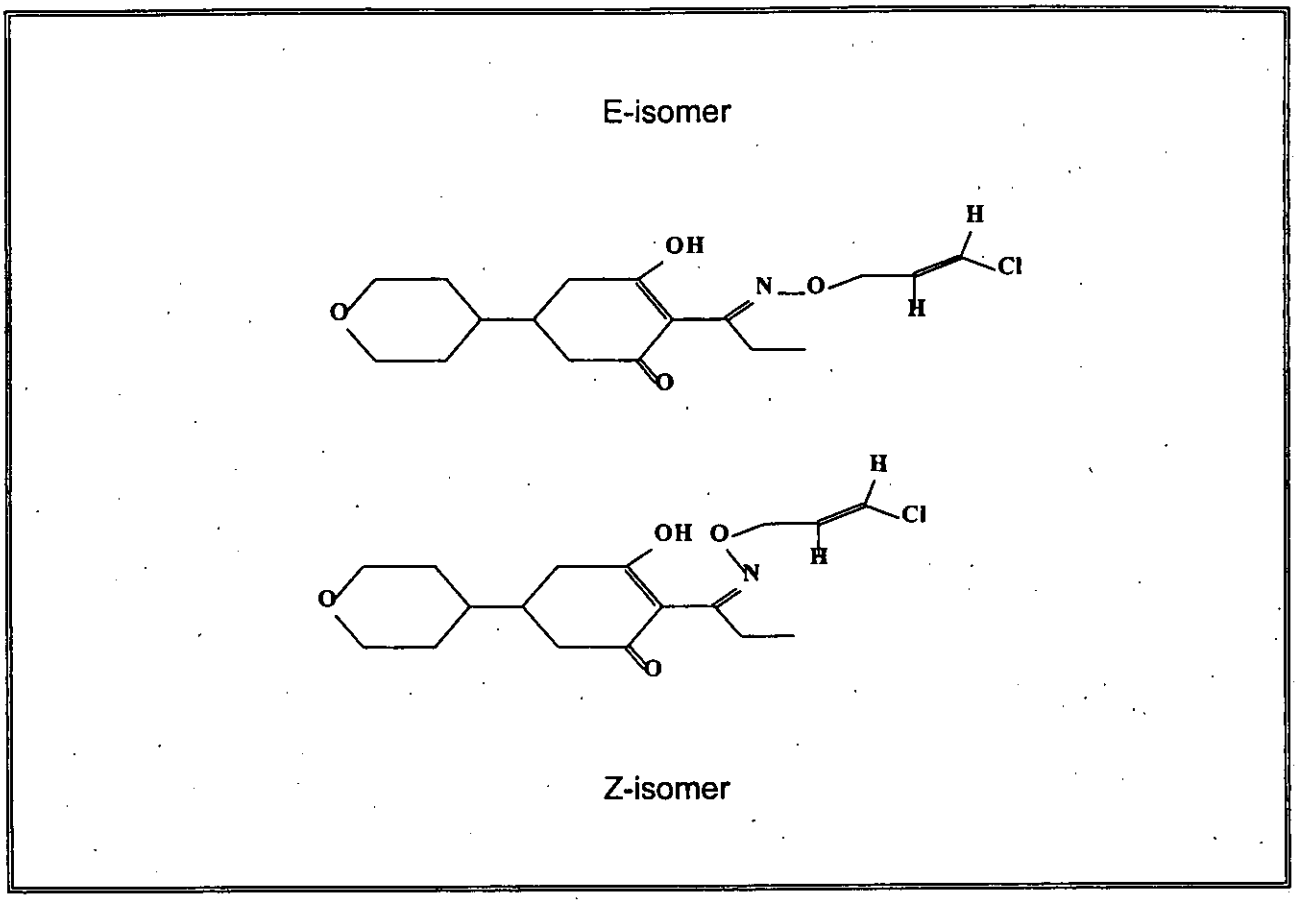



**Figure 1.** Structures for BAS 620 H (E-isomer) and its Z-isomer

C-7

C-12

Aquatic Media Sample

— Dilute the sample using pH 9 DI water to a final concentration range of 0.05 to 0.1 ppm

— Inject onto HPLC/UV

HPLC/UV analysis Conditions:

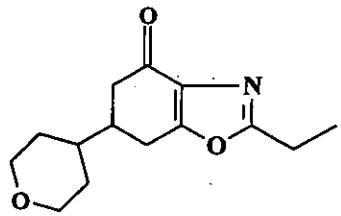
| | |
|---|---|
| HPLC Column: | C8, 250 mm x 4.6 mm at ambient conditions |
| Detector: | Applied Biosystems 785A Programmable Absorbance Detector |
| Gradient Mobile Phase: | Initial Mobile Phase: 100% Water (0.2% Phosporic Acid)<br>Final Mobile Phase: 60% Acetonitrile + 40% Water (0.2% Phosporic Acid)<br>Gradient %: 15% Acetonitrile / minute |
| Flow Rate: | 1.5 ml/min. |
| Wavelength: | 264 nm |
| 620H Retention Time: | 9.0 min. |

**Figure 2.** Flow chart of HPLC analytical procedure for analysis of BAS 620 H in Aquatic media

BASF Code: DP-2

Chemical name: 2-ethyl-6-(tetrahydropyran-4-yl)-4,5,6,7-tetrahydrobenzoxazol-4-one

Structural formula:




Empirical formula: $C_{14}H_{19}NO_3$

Molecular weight: 249.31

Appearance: White solid

**Figure 4.** Structures for "oxazole" DP-2 (a thermal product from BAS 620 H)