1 INTRODUCTION

1.1 Scope of the method

For monitoring purposes a residue analytical method for the active ingredient in water with a limit of determination of 0.05 µg/kg is needed.
Method No. 534 allows the determination of E- and Z-isomer of BAS 320I and the metabolites M320I04 and M320I23 with the required limit of determination in water.

The purpose of this study was to demonstrate the validity of the method by performing recovery trials with spiked water samples using tapered flasks or non-returnable culture tubes. Both possibilities are validated.
The recovery trials were carried out with two types of water, tap water and surface water (physical-chemical properties see attachment 1 and 2 on page 33).

The spiking levels were 0, 0.05, 0.5 and 5.0 µg/kg. For E- and Z-isomer of a.i. the spiking levels were 0, 0.025, 0.25 and 2.5 µg/kg each. 5 replicates of the respective fortification level were investigated. The validation was performed by the same person, with the same equipment, in the same laboratory, within a short interval of time.

In the following the design and the results of the study is reported.

1.2 Principle of BASF method 534/0

BASF method No. 534/0 allows the determination of BAS 320 I (E and Z isomer) and its metabolites M320I04 and M320I23 in water samples.
BAS 320 I and its metabolites M320I04 and M320I23 are extracted from water samples using dichloromethane. To reduce volume of the aliquot culture tubes or tapered flasks can be used. Both possibilities are validated and the results are shown in chapter 3
The final determination of BAS 320 I and its metabolites M320I04 and M320I23 is performed by HPLC-MS/MS. Parameters are given in attachment 7 on page 46.
No confirmatory method was developed because of the high specificity of MS/MS detection.
The method has a limit of quantitation of 0.05 µg / kg water.

1 Introduction

BAS 320 I is an insecticide used in various crops. The analytical method 534/0 allows the determination of BAS 320 I and its metabolites M320I04 and M320I23 in tap and surface water using returnable and non-returnable glassware.

Other compounds of similar physico-chemical properties may also be analysed by this methodology.

This method was developed at BASF Aktiengesellschaft, Limburgerhof, Germany.

2 Materials

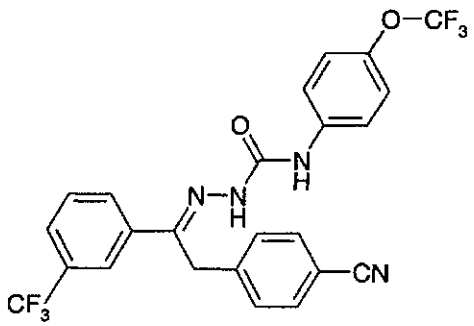
2.1 List of Abbreviations

LOQ	Limit of Quantitation
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry

2.2 Test and Reference Items

BAS 320 I (E-Isomer)

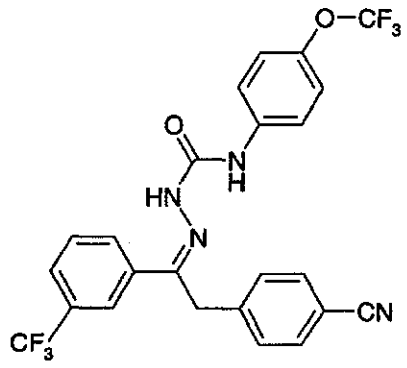
Chemical Name	4-{(2E)-2-({[4-(trifluoromethoxy)anilino]carbonyl}hydrazono)-2-[3-(trifluoromethyl)phenyl]ethyl}benzonitrile
BASF – Code:	E-Isomer of BAS 320 I
CAS Registry No.:	139968-49-3
BASF Reg. No.:	4102472
Structure:	



Molecular Formula:	$C_{24}H_{16}F_6N_4O_2$
Molecular Weight:	506.40

BAS 320 I (Z-Isomer)

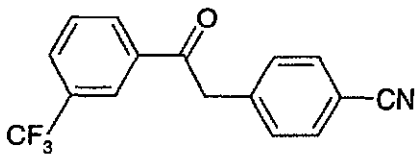
Chemical Name	4-{(2Z)-2-({[4-(trifluoromethoxy)anilino]carbonyl}hydrazono)-2-[3-(trifluoromethyl)phenyl]ethyl}benzonitrile
BASF – Code:	Z-Isomer of BAS 320 I
CAS Registry No.:	139968-49-3
BASF Reg. No.:	4102572
Structure:	



Molecular Formula:	$C_{24}H_{16}F_6N_4O_2$
Molecular Weight:	506.40

M320I04

Chemical Name	4-{2-oxo-2-[3-(trifluoromethyl)phenyl]ethly}benzonitrile
ISO Common Name:	-
CAS Registry No.:	146653-56-7
BASF Reg. No.:	4096485
Structure:	

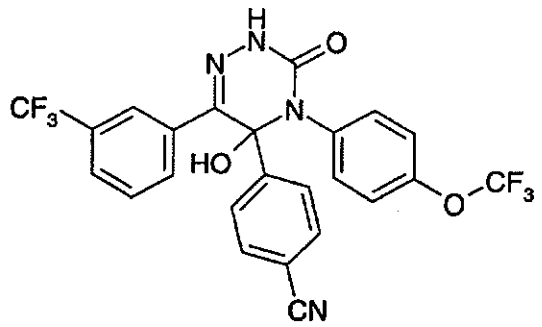


Molecular Formula:	$C_{16}H_{10}F_3NO$
Molecular Weight:	289.26

Method 534/0 Page 7 of 18

M320I23

Chemical Name	4-{5-hydroxy-3-oxo-4-[4-(trifluoromethoxy)phenyl]-6-3-(trifluoromethyl)phenyl]-2,3,4,5-tetrahydro-1,2,4-triazin-5-yl}benzonitrile
BASF – Code:	Parent ketone
CAS Registry No.:	-
BASF Reg. No.:	4984051
Structure:	



Molecular Formula:	$C_{24}H_{14}F_6N_4O_3$
Molecular Weight:	520.39

2.3 Equipment for Extraction and Sample Clean-up

Equipment	Size, Description	Manufacturer	Catalog No.
Balance , Topload		Sartorius	
Wide neck powder bottle Screw caps with PTFE seals	150 ml GL 45	Schott Glaswerke, Mainz Schott Glaswerke, Mainz	
Graduated cylinders	50 ml, 100 ml	Schott Glaswerke, Mainz	
Centrifuge tubes (culture tubes)	10 ml GL 18	Schott Glaswerke, Mainz	
Screw caps with PTFE seals	GL 18	Schott Glaswerke, Mainz	
Tapered flask	25 ml		
Volumetric flasks, stopper	various volumes		
Mechanical shaker	Bühler-Schüttler		
Volumetric pipettes	5 ml, various volumes	Schott Glaswerke, Mainz	
Rotary evaporator equipped with heating water bath, Vacuum and thermo controller		Janke&Kungel	
Vacuum pump incl. Vacuum controller		Janke&Kungel	
Modified evaporator adapter (to connect culture tube) see figure 2	GL 14 -> GL18		
Autosampler vials with snap caps	1.8 ml, N 11-1	Macherey-Nagel GmbH, D 52313 Düren	702 714 702717

Note: The equipment and instrumentation listed above may be substituted by those of similar specifications. If the use of materials of specifications other than those stated is intended, applicability of the new equipment for this method must be confirmed.

BAS 320 I and its metabolites M320I04 and M320I23 have a strong affinitiy to glass, and particularly to PE and steel surfaces. Therefore it is recommended to use only non-returnable glassware throughout the methodology.

2.4 Reagents

2.4.1 Chemicals

Chemical	Manufacturer/Supplier	Catalog No.
Dichloromethane	Merck, Darmstadt, FRG	No. 106054
Methanol	Merck, Darmstadt, FRG	No. 1.06011
Formic acid p.a.	Merck, Darmstadt, FRG	No. 100264
Water: Baker® or Millipore®	J.T.Baker / Millipore/Waters	

Note: The chemicals listed above may be substituted by that of similar specifications. If the use of chemicals of specifications other than those stated is intended, applicability of the new chemicals for this method must be confirmed.

2.4.2 Solutions and Solvent Mixtures

Solvent for final volume 1	Millipore® water / Methanol	50/50, v/v
HPLC mobile phase A	Millipore® water / Formic acid	100/0.1, v/v
HPLC mobile phase B	Methanol / Formic acid	100/0.1, v/v

2.4.3 Standard Solutions

Prepare a stock solution containing 1.0 mg/ml of BAS 320 I (E- and Z-Isomer) and its metabolites M320I04 and M320I23 in methanol. This can be done by weighing in e.g. 10 mg each of BAS 320 I (E- and Z-Isomer) and the metabolites M320I04 and M320I23 and diluting with 10 ml of methanol.
A stock solution containing BAS 320 I (E- and Z-Isomer) and its metabolites M320I04 and M320I23 at a concentration of 100 µg/ml is prepared by mixing 5 ml of the 1 mg/ml solution in a 50 ml volumetric flask.

Examples for Preparation of Fortification Solutions

Take solution (µg/ml)	Volume (ml)	Dilute with Methanol to a final volume of (ml)	Concentration (µg/ml)
100	1	100	1
1	10	20	0.5
0.5	1	10	0.05

Standard solutions and solutions used for fortification have been proved to be stable for at least four weeks (cited in BASF method 531/0).

Preparation of Standard Solutions for calibration
Examples for the preparation of standard solutions are shown in following table:

Take solution	Volume (ml)	Dilution with solvent to final volume (Methanol/Water 1+1) of [ml]	Concentration
100 µg/ml stock solution	1	100	1.0 ug/ml
1.0 ug/ml	1	100	10 ng/ml
1.0 ug/ml	0,5	100	5 ng/ml
10 ng/ml	5	100	0.5 ng/ml
10 ng/ml	2,5	100	0.25 ng/ml
10 ng/ml	1	100	0.1 ng/ml
5 ng/ml	1	100	0.05 ng/ml
5 ng/ml	0,5	100	0.025 ng/ml

Note: It is recommended to compare a matrix-matched standard (e.g. 100 % LOQ) with the same standard without matrix to see the instrument recovery in every sample queue.

Analytical Procedure

2.5 Spiking of Samples for Recovery Experiments

50 g of water sample are weighed into a 150 ml wide neck powder bottle. The spiking solution containing BAS 320 I (E- and Z-Isomer) and the metabolites M320I04 and M320I23 is added according to the following table:

Sample weight	Concentration of spiking solution	Volume of spiking solution	Level of fortification
50 g	0.005 µg/ml	0.5 ml	0.05 µg/kg*
50 g	0.05 µg/ml	0.5 ml	0.5 µg/kg
50 g	0.5 µg/ml	0.5 ml	5.0 µg/kg

* Limit of Quantitation (LOQ)

2.6 Analysis of water samples

2.6.1 Extraction of the Sample Material

2.6.1.1 Extraction conditions for water samples

Weigh 50 g of water into a 150 ml wide neck powder bottle, add exactly 25ml of dichloromethane and shake using a lab shaker for about 15 minutes. Wait for phase separation.

Notes: Dichloromethane is the lower phase. To take the aliquot, carefully insert a pipette into the lower phase and draw off the required amount.

Take exactly a 5 ml aliquot of the organic phase into a 1g Millipore® water containing tarred tapered flask and strip off the organic solvent at approx. 30°C using a rotary evaporator. Check the weight of the tapered flask and adjust to 1 g with Millipore® water if necessary.

Notes:.

Because of the high volatility of M320I04 during the evaporation step, water is added as a kind of keeper to avoid losses of the M320I04. Avoid evaporation to dryness of the waterphase.

Instead of using tapered flasks for evaporation of the organic phase, culture tubes as non-returnable glassware can also be used:
Take exactly a 5 ml aliquot of the organic phase into a 0.7 g Millipore® water containing tarred culture tube. Attach culture tube with the aid of a modified adapter (see page 16) and strip off the organic solvent at approx. 30°C using a rotary evaporator. Check the weight of the tapered flask and adjust to 1 g with Millipore® water.

2.6.2 Preparation of the Final Volume

Add exactly 1 ml Methanol to the residue (from section 2.6.1.1) to get a final volume of 2 ml of Methanol/Millipore® water 50/50 (v/v). In case of high residues, an appropriate dilution with Methanol/Millipore® water 50/50 (v/v) may be necessary.

2.7 Final Determination by HPLC-MS/MS

The following HPLC-MS/MS parameter sets for the final determination can be used for water samples.
The equipment listed in section 2.7.1 may be substituted by instruments with similar specifications. If the use of material with specifications other than those stated is intended, applicability of the new equipment for this method must by confirmed.
The instrument conditions, injection volume, column and gradient steps may be modified, but any changes must be recorded in the raw data.

2.7.1 Instrumentation and Conditions

(final volume: methanol/millipore® water, 50/50; v/v)

Instrument:	PE API 4000 Mass Spectrometer			
Inlet [HPLC System]	Agilent 1100 LC Binary Pump			
Column:	Betasil C18 100*2 mm 5µ			
Injection:	50 µl (or higher)			
Mobile Phase A: Mobile Phase B:	Millipore ® water / Formic acid Methanol / Formic acid		100/0.1, v/v 100/0.1, v/v	
Flow Rate:	600 µl/minute			
Steps	Time (min)	Dura. (min)	Phase A	Phase B
	0 6.0 7.5 7.6 10.0		35 0 0 35 35	65 100 100 65 65
Expected Retention Times	E-Isomer of BAS 320 I Z-Isomer of BAS 320 I M320I04 M320I23		Approx. 5.36 min. Approx. 4.76 min. Approx. 2.36 min. Approx. 3.97 min.	
Ionisation Mode:	E-Isomer of BAS 320 I Z-Isomer of BAS 320 I M320I04 M320I23		Positive Positive Negative Positive	
Transitions:	E-Isomer of BAS 320 I Z-Isomer of BAS 320 I M320I04 M320I23		507→ 287* and 507→ 178 507→ 287* and 507→ 178 288→ 142* and 288→ 114 521→ 130 and 521→ 318*	

- used for quantification

2.7.2 Calibration Procedures

Calculation of results is based on peak intensity measurements (peak area or peak height) using a calibration curve. The standard curve is obtained by direct injection of a standard mix of BAS 320 I,M320I04 and M320I23 into the HPLC-MS/MS in the range of 0.025 ng/ml to 0.5 ng/ml. In a given injection run, the same volume is used for all samples and standards.
The calibration curves are obtained by plotting peak area or height (monitoring transitions listed above) versus the concentration of parent and metabolites.
Establish the stability of the detection response by injecting several concentrations of standards. Each injection set should begin and end with injections of standards, and each standard concentration should be injected at least in duplicate.

2.7.3 Limit of Quantitation

The limit of quantitation is defined as the lowest fortification level successfully tested. For both matrices, the limit of quantitation is 0.05 µg/kg for BAS 320 I (sum of E- and Z-Isomer) and 0.05 µg/kg for M320I04 and M320I23.

3 Calculation of Results

3.1 Principle

Calculation of results is based on peak area (or height) measurements. The residues of BAS 320 I and /or the metabolites are calculated from the respective calibration curve.

3.2 Calculation

The individual concentrations of BAS 320 I and / or the metabolites M320I04 and M320I23 in µg/kg are calculated as shown in equation **I**:

I. $$\textbf{Residue [µg/kg]} = \frac{V_{end} \times C_A}{G \times A_F \times 1000}$$

V_{end}	=	Final volume of the extract after all dilution steps [ml]
C_A	=	Concentration of analyte as read from the calibration curve [ng/ml]
G	=	Weight of the sample extracted [g]
A_F	=	Aliquot factor
1000	=	Factor remaining after all unit conversions

The recoveries of spiked compounds are calculated according to equation II:

II. Recovery % $$= \frac{\text{Residue in fortified sample} - \text{Residue in control} \times 100}{\text{Amount of analyte fortified}}$$

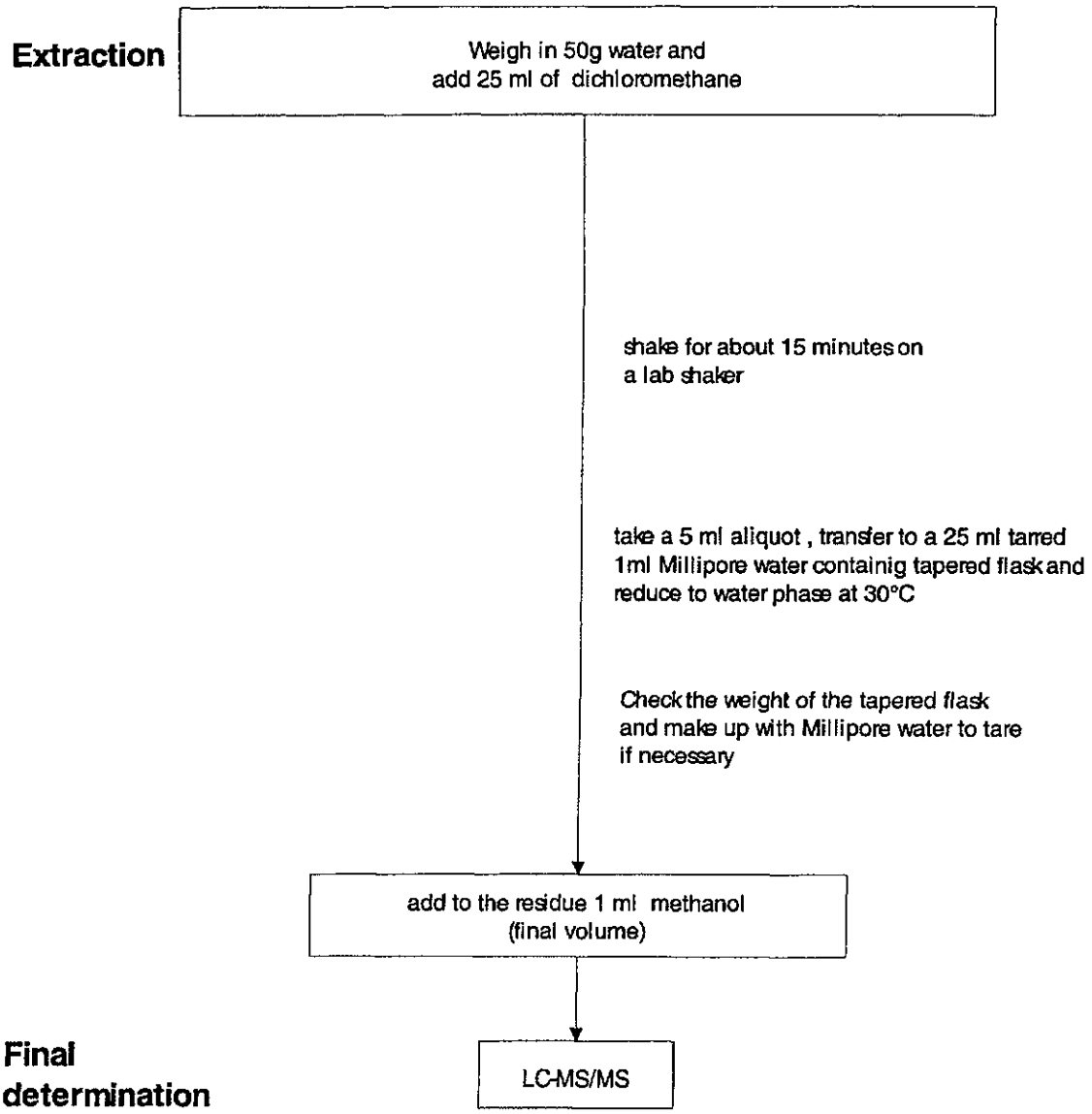
4 Method Management and Time Requirement

The analysis of one series of samples (= 20 unknown samples, 2 fortified samples for recovery experiments, 2 blank samples) requires 8 working hours. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

5 Confirmatory Technique

The HPLC-MS/MS final determination for BAS 320 I and its metabolites M320I04 and M320I23 is a highly selective analytical technique. For each of these compounds the quantitation is possible using at least two different transitions. Therefore, no confirmatory technique is required.

Figure 1: Flow chart of method 534/0 (using tapered flasks)



6 Appendix

6.1 Calculation of Residues (Example)

Tap water sample fortified at 0.05 µg/kg:

BAS 320 I (E-isomer),transition 507 -> 287

Concentration in the final volume [ng/ml]

$$\textbf{Concentration [ng/ml]} = \frac{\text{Response} - Intercept}{Slope} = C$$

Residue in the sample [µg/kg]

$$\textbf{Residue [µg/kg]} = \frac{V_{end} \times C_B}{G \times A_F \times 1000}$$

Recovery [%]

$$\textbf{Recovery \%} = \frac{\text{Residue in fortified sample} - \text{Residue in control} \times 100}{\text{Amount of analyte fortified}}$$

The following values were used for calculation of BAS 320 I (E-isomer) concentration:

Response of fortified sample	3640
Response of control sample	0
Slope:	25836
Intercept:	-136
Sample Weight (G):	50 g
Final volume (V_{end}):	2 ml
Aliquot factor A_F:	0.1 (= 10%)
Conversion factor ng → µg:	1000

Concentration (ng/ml)

$$= \frac{3640 - (-136)}{25836} = 0.146\ ng\ /\ ml$$

Residue (µg/kg)

$$= \frac{2\,ml \times 0.146\,ng/ml}{50\,g \times 0.1 \times 1000} = 0.0000584\,\mu g/g = 0.0584\,\mu g/kg$$

Recovery %

$$= \frac{(0.0584\ \mu g/kg - 0.00\ \mu g/kg) \times 100}{0.05\ \mu g/kg} = 116{,}8\,\%$$