

**Summary**

This method describes the determination of the active ingredient KWG 4168 and the metabolites Desethyl-KWG 4168 (KWG 4557), Despropyl-KWG 4168 (KWG 4669) and KWG 4168 N-Oxide (WAK 6301) in soil.

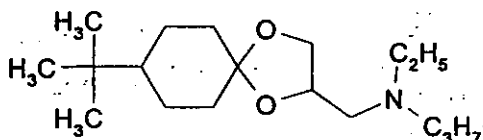
Soil samples of 30 g are extracted with 100 ml of methanol / water / ammonia (25%) (800 + 200 + 10 parts by volume) during 60 minutes on a mechanical shaker and filtered. From the filtrate an aliquot of 40.0 ml is concentrated in a Turbo Vap to the aqueous remainder of about 5 ml (do not evaporate extracts to dryness) and internal standard is added. The volume is adjusted to 10 ml and a part of the sample is centrifuged. Quantitative determination of the active ingredient and the metabolites is done by high performance liquid chromatography using MS/MS detection.

The limit of quantification of the method is 5 µg/kg for KWG 4168, Desethyl-KWG 4168, Despropyl KWG 4168 and KWG 4168 N-Oxide.

The limit of detection of the method is 2 µg/kg for KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168 and KWG 4168 N-Oxide.

## 1 Introduction

The active ingredient KWG 4168 is used as fungicide and has the following chemical structure:



KWG 4168

**Chemical Name:** 8-(1,1-Dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4.5]decane-2-methanamine

**CAS-No.:** 118134-30-8

**Total formula:** C<sub>18</sub>H<sub>35</sub>NO<sub>2</sub>

**Molar mass:** 297.5 g

**Appearance:** colorless liquid

**vapor pressure:** 4.0 \* 10<sup>-3</sup> Pa at 20°C (Isomer A)  
5.7 \* 10<sup>-3</sup> Pa at 20°C (Isomer B)

**solubility [g/l at 20°C]:**

water:	pH3	> 200	(Isomer A and B)
	pH7	0.47	(Isomer A)
		0.34	(Isomer B)
	pH9	0.014	(Isomer A)
		0.010	(Isomer B)

**solubility [g/l at 20°C]:**

n-Hexane	> 200
Toluene	> 200
Dichloromethane	> 200
2-Propanol	> 200
1-Octanol	> 200
Polyethyleneglycol	> 200
Polyethyleneglycol + Ethanol	> 200
Acetone	> 200
Dimethylformamide	> 200
Ethylacetate	> 200
Acetonitrile	> 200

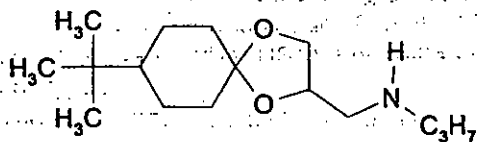
**Partition coefficient:**

(n-Octanol/Water)	log POW	2.79 (Isomer A)
		2.92 (Isomer B)

**Hydrolytic stability:**

half live period at 37°C pH 2.1:	6.4 h (Isomer A)
	15,4 h (Isomer B)
half live period at 50°C pH 4:	> 250 h
	pH 7: > 250 h
	pH 9: > 250 h

Technical KWG 4168 is a mixture of Diastereomers A and B at a ratio of 55 : 45.



Desethyl-KWG 4168 (KWG 4557)

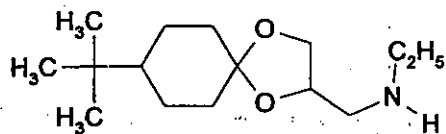
Chemical name: 8-(1,1-Dimethylethyl)-N-propyl-1,4-dioxaspiro[4.5]decane-2-methanamine

CAS-No.: 127504-73-8

Total formula:  $C_{16}H_{31}NO_2$

Molar Mass: 269.5 g

KWG 4557 (Desethyl-KWG 4168) is a mixture of Diastereomers A and B at a ratio of 42 : 56.



Despropyl-KWG 4168 (KWG 4669)

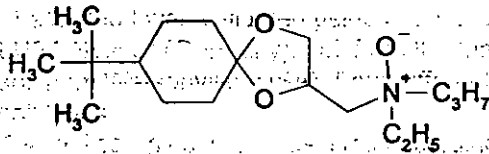
Chemical name: 8-(1,1-Dimethylethyl)-N-ethyl-1,4-dioxaspiro[4.5]decane-2-methanamine

CAS-No.: 148174-97-4

Total formula:  $C_{15}H_{29}NO_2$

Molar Mass: 255.5 g

KWG 4669 (Despropyl-KWG 4168) is a mixture of Diastereomers A and B at a ratio of 55 : 43.



KWG 4168 N-oxide (WAK 6301)

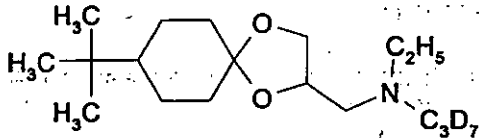
Chemical Name: 8-(1,1-Dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4.5]decane-2-methanamine-N-oxide

Total formula: C<sub>18</sub>H<sub>35</sub>NO<sub>3</sub>

Molar mass: 313.5 g

Appearance: colorless liquid

The compound KWG 4168-D7 (KWG 7498) is used as internal standard and has the following chemical structure:



KWG 4168-D7 (KWG 7498)

**Chemical Name:** 8-(1,1-Dimethylethyl)-N-ethyl-N-(D<sub>7</sub>-propyl)-1,4-dioxaspiro[4.5]decane-2-methanamine

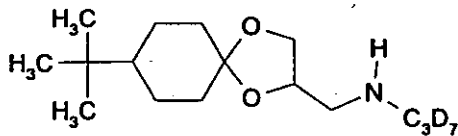
**Total formula:** C<sub>18</sub>H<sub>28</sub>D<sub>7</sub>NO<sub>2</sub>

**Molar mass:** 304.5 g

**Appearance:** yellowish liquid

KWG 4168-D7 is a mixture of Diastereomers A and B at a ratio of 51 : 47.

The compound KWG 4557-D7 (Desethyl-KWG 4168-D7, KWG 7566) is used as internal standard and has the following chemical structure:



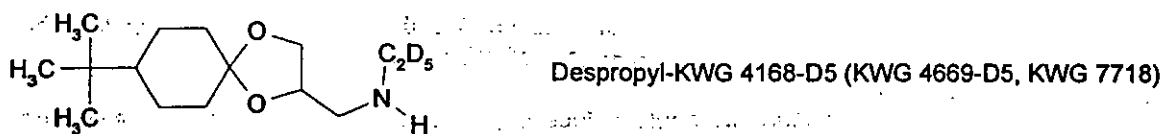
Desethyl-KWG 4168-D7 (KWG 4557-D7, KWG 7566)

**Chemical Name:** 8-(1,1-Dimethylethyl)-N-(D<sub>7</sub>-propyl)-1,4-dioxaspiro[4.5]decane-2-methanamine

**Total formula:** C<sub>16</sub>H<sub>24</sub>D<sub>7</sub>NO<sub>2</sub>

**Molar mass:** 276.5 g

The compound KWG 4669-D5 (Despropyl-KWG 4168-D5, KWG 7718) is used as internal standard and has the following chemical structure:

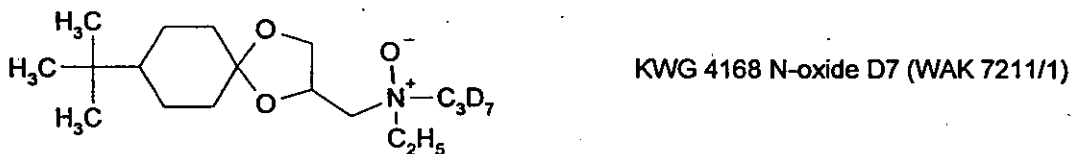


Chemical Name: 8-(1,1-Dimethylethyl)-N-(D<sub>5</sub>-ethyl)-1,4-dioxaspiro[4.5]decane-2-methanamine

Total formula: C<sub>15</sub>H<sub>24</sub>D<sub>5</sub>NO<sub>2</sub>

Molar mass: 260.4 g

Appearance: yellowish liquid



Chemical Name: 8-(1,1-Dimethylethyl)-N-ethyl-N-(D<sub>7</sub>-propyl)-1,4-dioxaspiro[4.5]decane-2-methanamine-N-oxide

Total formula: C<sub>18</sub>H<sub>28</sub>D<sub>7</sub>NO<sub>3</sub>

Molar mass: 320.5 g

## 2 Test System

The method was validated using a mixture of the standard soils 2.1, 2.2 and 2.3 (1+1+1 w/w/w) of LUFA:Speyer (Germany), soil from the trial station Höfchen (Germany), soil from Fresno (USA) and soil from Watsonville (USA). Four different soils were used to register a possible influence of different soil types.

The soil samples were classified according to DIN and USDA specifications.

Soil parameters as well as the textural classification are summarized in Table 1.

The data are reported in the appendix (Table 12-17).

Table 1

Soil	Type of Soil	Origin of Classification
LUFA 2.1*	sand	DIN
LUFA 2.2*	loamy sand	DIN
LUFA 2.3*	sandy loam	DIN
Höfchen	strong loamy silt	DIN
Fresno	loam	USDA
Watsonville	loam	USDA

\* Soils 2.1, 2.2, 2.3 were used as mixture

## 3 Principle of the Method

With the following method the active ingredient KWG 4168 and the metabolites Desethyl-KWG 4168 (KWG 4557), Despropyl-KWG 4168 (KWG 4669) and KWG 4168 N-Oxide (WAK 6301) in soil can be determined down to a limit of quantification of 5 µg/kg for each compound. The limit of detection is 2 µg/kg with the conditions described.

Soil samples of 30 g are extracted with 100 ml of methanol / water / ammonia solution (25%) (800 + 200 + 10 parts by volume) during 60 minutes on a mechanical shaker and filtered. From the filtrate an aliquot of 40.0 ml is concentrated in a Turbo Vap to the aqueous remainder of about 5 ml (do not evaporate extracts to dryness!) and internal standard is added. The volume is adjusted to 10 ml and a part of the sample is centrifuged. Quantitative determination of the active ingredient and the metabolites is done by high performance liquid chromatography using MS/MS detection.

#### 4 Instruments

**Balance:** PC 4400 and PM 4800  
Mettler Instruments GmbH  
D-35387 Giessen, FRG

**Mechanical Shaker:** SM - 25  
Edmund Buehler Laborgeraetebau  
D-72072 Tuebingen

**Evaporator:** Turbo.Vap LV, custom made for 50 ml centrifuge tubes  
Zymark GmbH  
D-65510 Idstein, FRG

**Centrifuge:** Biofuge A, Diameter at the bottom of the tubes: 15 cm  
Heraeus Christ  
D-63450 Hanau, FRG

**Centrifuge tubes:** 50 ml centrifuge tubes  
Zymark GmbH  
D-65510 Idstein, FRG

Eppendorf 1.5 ml  
Micro Test Tubes 3810  
Order-No.: 0030 102.002

**Liquid chromatograph:** HP 1090  
Hewlett-Packard  
D-40880 Ratingen, FRG

**Column:** PRP-1, length 25 cm, 10  $\mu$ m, i.d. 4 mm  
Hamilton Deutschland GmbH  
D-64293 Darmstadt, FRG

**Mass Spectrometer:** VG Quattro with electrospray interface  
Fisons Instruments  
D-55252 Mainz, FRG

Volumetric flasks, pipettes and other instruments commonly used in the laboratory



**5 Reagents**

**Methanol:**

Promochem, No. 3041, Lot MB002  
D-46485 Wesel, FRG

**Acetonitrile:**

Riedel de Haen, No. 34998  
D-30926 Seelze, FRG

**Ammonia solution:**

25 % NH<sub>3</sub> Riedel de Haen, No. 30501  
D-30926 Seelze, FRG

**Hydrochloric acid:**

min. 37 % Riedel de Haen, No. 30721  
D-30926 Seelze, FRG

**Water:**

cleaned in a Milli-Q unit

**6 Test and Reference Substances**

**Test and Reference Substances**

**KWG 4168**

**920522ELB01**

purity: 99 %

53 % Isomer A, 46 % Isomer B

expiry date: February 1999

**Desethyl-KWG 4168**

**921103ELB02**

**(KWG 4557)**

purity: 98 %

42 % Isomer A, 56 % Isomer B

expiry date: November 1997

**Despropyl-KWG 4168**

**921103ELB03**

**(KWG 4669)**

purity: 98 %

55 % Isomer A, 43 % Isomer B

expiry date: November 1997

**KWG 4168 N-Oxide**

**950209ELB01**

**(WAK 6301)**

purity: 93 %

expiry date: April 1997

**Reference Substances (Internal Standards)**

**KWG 4168-D7**

**940627ELB01**

**(KWG 7498)**

purity: 97.5 %

50.6 % Isomer A, 47 % Isomer B

expiry date: June 1996

**Desethyl-KWG 4168-D7**

**940729ELB01**

**(KWG 4557-D7)**

**(KWG 7566)**

purity: 88 %

40 % Isomer A, 48 % Isomer B

expiry date: August 1996

**Despropyl-KWG 4168-D5**

**941124ELB01**

**(KWG 4669-D5)**

**(KWG 7718)**

purity: 93 %

45 % Isomer A, 48 % Isomer B

expiry date: January 1997

**KWG 4168 N-Oxide-D7**

**960423ELB01**

**(WAK 7211/1)**

purity: 73 %

expiry date: May 1998

The stock solutions of the test and reference substances are prepared in methanol. From this the measuring solutions are prepared by dilution of the stock solutions with water / methanol / ammonia solution (25%) (750 + 250 + 10 parts by volume).

For the correct determination of the used weight of the reference compounds the certified amounts have to be considered.

The concentration range of the measured solutions is between 0.01 and 3.5 µg/ml.

The internal standard solution is a mixture of the four deuterized analytes on fixed levels. The same level of internal standards is added to the measuring standard solutions. The solutions used for fortification contain only the four undeuterized analytes.

## 7 Safety Precautions

While processing this method the German guidelines for laboratories of the professional association (e.g. leaflet M006) or similar guidelines in other countries are to be considered.

The following, according to the German "Gefahrstoffverordnung" as very toxic, toxic or less toxic rated plant protection compounds and solvents are used. This classification is based on German guidelines and has to be adjusted to the national guidelines of other countries while using the method outside of Germany.

treated as very toxic: KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168, KWG 4168 N-Oxide, KWG 4168-D7, Desethyl-KWG 4168-D7, Despropyl-KWG 4168-D5, KWG 4168 N-Oxide-D7 (all not fully investigated)

toxic: Methanol, Acetonitrile, Ammonia solution, Hydrochloric acid

While working with these substances the relevant safety regulations are to be considered (see R- and S-rules).

## 8 Performance of Analysis

### 8.1 Fortification

The method is validated for the matrix by analyzing control samples and control samples fortified prior to the extraction procedure at and above the limit of quantification.

Sample fortification should be conducted such that no less than 100 µl and no more than 1.5 ml of the standard solution is used. The preparation of the fortification standards is described in section 6. After fortification the extraction bottle is allowed to sit for about 1 hour before the addition of the extraction solvent.

### 8.2 Extraction

30 g of soil are weighed into a 250-ml polyethylene bottle, 100 ml of a mixture of methanol / water / ammonia solution (25%) (800 + 200 + 10 parts by volume) are added and the soil is extracted on a mechanical shaker for 60 minutes (300 rpm). After about 30 min., to allow the soil particles to deposit on the ground of the bottle, the solvent is filtered through a folded filter into a 100 ml measuring cylinder up to a volume of 40 ml. This aliquot of 40 ml is transferred into a 50 ml centrifuge tube. The extract is evaporated on the Turbo Vap LV evaporator for about 1.5 hours at 40 °C until a volume of about 5 ml is achieved (do not evaporate extracts to dryness!).

1 ml of the internal standard solution mixture is filled into a 10 ml volumetric flask. The concentrated extract is transferred quantitatively into this flask. The centrifuge tube is rinsed two times each with about 1.5 ml of water / methanol / ammonia solution (25%) (750 + 250 + 10 parts by volume). Subsequently the volumetric flask is filled up to the calibration mark with water / methanol / ammonia solution (25%) (750 + 250 + 10 parts by volume).

After mixing an aliquot of 1.5 ml of the sample is transferred to an Eppendorf centrifuge tube and centrifuged at 13000 rpm for about 5 minutes. The sample is transferred into a HPLC vial and an aliquot of this solution (100µl) is injected into the HPLC-MS/MS instrument.

Identification and quantification of the residues of KWG 4168, Desethyl KWG 4168, Despropyl-KWG 4168 and KWG 4168 N-Oxide is done by high performance liquid chromatography with mass selective detector (MS-MS) in the Multiple-Reaction-Monitoring mode.

**8.3 Remarks**

The extracts of the samples are stable for the time of analysis. If an interruption of the analyses is necessary, extracts have to be stored in solution in a refrigerator.

**8.4 Liquid Chromatographic Conditions**

Liquid chromatograph: HP-1090

Detector: VG Quattro with electrospray interface,  
mass selective detector (MS-MS)

Column: PRP-1, length: 25cm, ID: 4 mm, particle size: 10 µm  
Hamilton No. 79427

Injection volume: 100 µl

Oven temperature: 40°C

Mobile phase: A: Water + 0.4 ml HCl (37%) / I  
B: Acetonitrile  
C: Methanol

**Table 2**

Time [min.]	0	1	3	4	8	13	14	18
% A	70	70	62	50	5	5	70	70
% B	23	23	30	50	95	95	23	23
% C	7	7	8	0	0	0	7	7

Runtime: 18 min.

Flow (column): 1.5 ml/min.

Flow (interface): 0.25 ml/min.

Switching times for the transfer of the mobile phase into the MS/MS detector:

0 - 4.0 min. solvent waste  
4.0 - 8.0 min. MS/MS detector  
8.0 - 18.0 min. solvent waste

Retention times: KWG 4168: approx. 6.8 min. (Isomer A and B)  
Desethyl-KWG 4168 (KWG 4557): approx. 6.5 min. (Isomer A and B)  
Despropyl-KWG 4168 (KWG 4669): approx. 5.9 min. (Isomer A and B)  
KWG 4168 N-Oxide (WAK 6301): approx. 6.9 min. (Isomer A and B)  
KWG 4168-D7 (KWG 7498): approx. 6.8 min. (Isomer A and B)  
Desethyl-KWG 4168-D7 (KWG 7566): approx. 6.5 min. (Isomer A and B)  
Despropyl-KWG 4168-D5 (KWG 7718): approx. 5.9 min. (Isomer A and B)  
KWG 4168 N-Oxide D7 (WAK 7211/1): approx. 6.9 min. (Isomer A and B)

### 8.5 Mass Spectroscopy

Detector: VG Quattro (Fisons Instruments) with electrospray interface, mass selective detector (MS-MS).

### 8.6 Principle of Measurement

The substances placed into the mass spectrometer are ionized using an electrospray interface. The desired ions are accelerated by the adequate voltage regulation and separated by mass in the first quadrupole (MS 1). The most intensive ions (the protonated ions, parent ions) of the analyte are impulsed with argon in the collision cell. Fragments of these ions (daughter ions) are separated by mass in the second quadrupole (MS 2) and detected. The analytes and the selected ions are shown in Table 3.

### 8.7 Mass Spectroscopic Parameters

The reported parameters are examples for an optimal adjustment of the mass spectrometer. Using these parameters the results in chapter 10 to 14 were obtained. These parameters have to be checked from time to time and adjusted if necessary.

Bath gas: 400 l/h N<sub>2</sub>  
Nebulizer gas: 18 l/h N<sub>2</sub>  
Reactant gas: Argon (3.5 bar)

Table 3

Tune Parameter for Electrospray plus	
Capillary	3.5 KV
HV Lens	0.20 KV
Cone	20 - 24*
Skimmer Offset	2
Skimmer	1.6
Source Temperature	190°C
<u>MS1-Parameter:</u>	
LM Resolution	8
HM Resolution	8
Ion Energy	2
Ion Energy Ramp	0
Lens 6	5
Multiplier	750
<u>MS2-Parameter:</u>	
LM Resolution	5
HM Resolution	5
Collision	12 - 20*
Ion Energy	2
Ion Energy Ramp	0
Lens 8	150
Lens 9	0
Multiplier	750

\* depends on the ions that are to be detected, see Table 4

Table 4 MS/MS Timetable

Detection time [min.]	Principle	Cone [V]	Collision-energy [eV]	m/z Parent	m/z Daughter	Substance
4 - 9	ESP+	24	20	298.2	143.9	KWG 4168
4 - 9	ESP+	20	15	270.2	115.9	Desethyl-KWG 4168 (KWG 4557)
4 - 9	ESP+	20	15	256.2	101.7	Despropyl-KWG 4168 (KWG 4669)
4 - 9	ESP+	22	12	314.2	159.9	KWG 4168 N-Oxide (WAK 6301)
4 - 9	ESP+	24	20	305.2	151.0	KWG 4168-D7 (KWG 7498)
4 - 9	ESP+	20	15	277.2	123.0	Desethyl-KWG 4168-D7 (KWG 7566)
4 - 9	ESP+	20	15	261.2	107.0	Despropyl-KWG 4168-D5 (KWG 7718)
4 - 9	ESP+	22	12	321.2	167.1	KWG 4168 N-Oxide-D7 (WAK 7211/1)

ESP+ = electrospray, positive ions

## 9 Calculation of Residues

The evaluation is made in comparison to internal standard solutions of KWG 4168-D7, Desethyl-KWG 4168-D7, Despropyl-KWG 4168-D5 and KWG 4168 N-Oxide-D7 in water / methanol / ammonia solution (25%) (750 + 250 + 10 parts by volume), added to the sample after the extraction.

The response factors of the analytes are determined in independent experiments by injection of standards containing the test compounds as well as the deuterized reference compounds. The concentration of the deuterized analytes is on the same level in all standard solutions used. For each concentration level of the external standard from one sequence the mean response factor is calculated.

The concentration of the respective analyte is calculated by comparison of the response factor of the analyte in the sample to the response factor of the analyte in the standard solution with the similar concentration.

$$RF_i = \frac{A_{iS}}{A_{iSi}}$$

$$C_i = \frac{A_i}{A_{iSi}} \cdot \frac{C_{iS}}{RF_i}$$

When calculating the content of residue relative to the dry substance, the water content of the soil must be taken into consideration.

$$V_{ex'} = V_{ex} + \frac{F \cdot EW}{100}$$

$$R = C_i \cdot \frac{V_{end}}{EW} \cdot \frac{100}{100 - F} \cdot \frac{V_{ex'}}{V_A}$$

- $A_i$  = peak area or peak height of the sample solution [area counts or peakheight]
- $A_{iS}$  = peak area or peak height of the standard [area counts or peakheight]
- $A_{iSi}$  = peak area or peak height of the internal standard [area counts or peakheight]
- $C_i$  = concentration of the sample solution [ng/ml]
- $C_{iS}$  = concentration of the standard solution [ng/ml]
- $EW$  = weight of the sample [kg]
- $F$  = water content of the sample [%]
- $R$  = concentration of active ingredient in the soil sample ( $\mu\text{g}/\text{kg}$  of dry weight of soil)
- $RF_i$  = response factor of the analyte
- $V_{end}$  = final volume of the sample solution [ml]
- $V_{ex}$  = extraction volume [ml]
- $V_{ex'}$  = corrected extraction volume [ml]
- $V_A$  = Aliquot of the extract [ml]

**Appendix**

**Figure 1:**

**Flow Diagram of Analysis Procedure**

