Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pestcide Name: Spiroxamine

MRID #: 450904-07

Matrix: Soil

Analysis: LC/MS/MS

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Bayer AG
Plant Protection Development
Institute for Metabolism Research
and Residue Analysis
D-51368 Leverkusen

Title

Method 00433 (MR-248/96) for Liquid Chromatographic Determination of KWG 4168 and the Metabolites Desethyl-KWG 4168 (KWG 4557), Despropyl-KWG 4168 (KWG 4669) and KWG 4168 N-Oxide (WAK 6301) in Soil

Purpose

Determination of Recovery Rates and Blind Values of Control Samples, Reproducibility and Detector Linearity for Validation of the Residue Analytical Method 00433 (MR-248/96)

Author

Dr. H. Sommer

Study Completion Date

January 8, 1997

Testing Facility

Bayer AG
PF-E/MR
D-51368 Leverkusen

P 60150057

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Statement of Compliance

Study Number: P 60150057

Test Substances: KWG 4168
Desethyl-KWG 4168 (KWG 4557)
Despropyl-KWG 4168 (KWG 4669)
KWG 4168 N-Oxide (WAK 6301)

Study Director: Dr. H. Sommer

Testing Facility: Bayer AG
PF-E/MR
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Title of the Study: Method 00433 (MR-248/96) for Liquid Chromatographic Determination of KWG 4168 and the Metabolites Desethyl-KWG 4168 (KWG 4557), Despropyl KWG 4168 (KWG 4669) and KWG 4168 N-Oxide (WAK 6301) in Soil

I hereby certify that this study has been performed in agreement with the rules of "Good Laboratory Practice (GLP)" (German Chemikaliengesetz, attachment 1 of § 19a, dated July 25, 1994 (1) and OECD Principles of Good Laboratory Practice (GLP), dated May 12, 1981 [C (81) 30 (Final)] (2).

H. S

(Dr. H. Sommer)

01/08/97

(Date)

Certification of Authenticity

The result of following trial is presented in this report:

P 60150057

Signatures

(Dr. H. Sommer)	Study Director and Analytical Laboratory	01/08/97 (Date)

Signature of Management

(Dr. J. Köhler)	Manager Testing Facility (PF-E/MR)	1/13/97 (Date)

For requests contact:

Dr. H. Sommer
Bayer AG
PF-E/MR
D-51368 Leverkusen

Referat GLP	
Quality Assurance Statement	
Report No.: MR-248/96	**Study No.:** P 60150057
Title of report: Method 0043 (MR-248/96) for Liquid Chromatographic Determination of KWG 4168 and the Metabolites Desethyl-KWG 4168 (KWG 4557), Despropyl-KWG 4168 (KWG 4669) and KWG 4168 N-oxide (WAK 6301) in Soil	

The conduct of this study has been periodically inspected and this report has been audited by the Quality Assurance Unit. The dates of inspection are given below.

Date of Protocol Inspection:	Date of Report to Management:
March 28, 1996	March 28, 1996
Date of Study Inspection:	Date of Report to Management:
April 2, 1996	April 2, 1996
Date of Final Report Audit:	Date of Report to Management:
November 20, 1996	November 20, 1996

The results reported in this study accurately reflect the raw data.

K. Ertz 11/13/97
Quality Assurance Unit, PF-S

Date: L. Ertz

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.

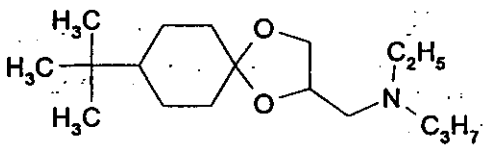
The mean recoveries of the method, which were determined in the range of 5 to 400 µg/kg were 79.8 % for KWG 4168, 90.4 % for Desethyl-KWG 4168, 101 % for Despropyl-KWG 4168 and 94.9 % for KWG 4168 N-Oxide with relative standard deviations of 8.8 % for KWG 4168, 4.7 % for Desethyl-KWG 4168, 11.7 % for Despropyl-KWG 4168 and 5.3 % for KWG 4168 N-Oxide.

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:	$C_{18}H_{35}NO_2$
Molar mass:	297.5 g
Appearance:	colorless liquid
vapor pressure:	$4.0 * 10^{-3}$ Pa at 20°C (Isomer A) $5.7 * 10^{-3}$ Pa at 20°C (Isomer B)

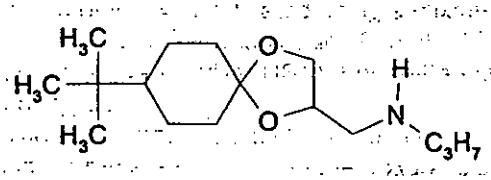
solubility [g/l at 20°C]:	water:		
	pH3	> 200	(Isomer A and B)
	pH7	0.47	(Isomer A)
		0.34	(Isomer B)
	pH9	0.014	(Isomer A)
		0.010	(Isomer B)

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 P_{OW}	
		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

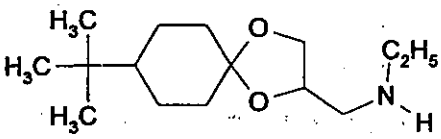
Technical KWG 4168 is a mixture of Diastereomers A and B at a ratio of 55 : 45.



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

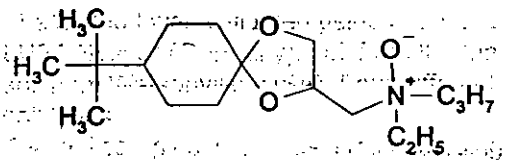
KWG 4557 (Desethyl-KWG 4168) is a mixture of Diastereomers A and B at a ratio of 42 : 56.



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

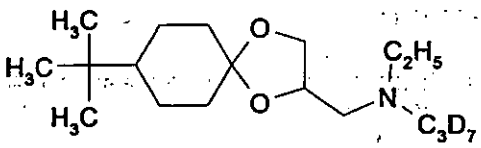
KWG 4669 (Despropyl-KWG 4168) is a mixture of Diastereomers A and B at a ratio of 55 : 43.



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:	$C_{18}H_{35}NO_3$
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:

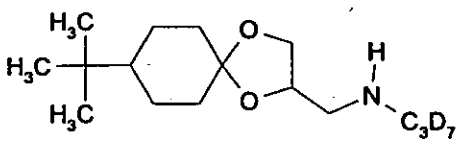


KWG 4168-D7 (KWG 7498)

Chemical Name:	8-(1,1-Dimethylethyl)-N-ethyl-N-(D_7-propyl)-1,4-dioxaspiro[4.5]decane-2-methanamine
Total formula:	$C_{18}H_{28}D_7NO_2$
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.

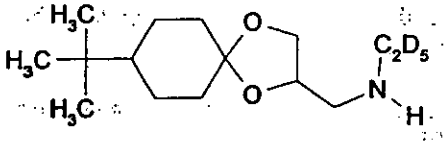
The compound KWG 4557-D7 (Desethyl-KWG 4168-D7, KWG 7566) is used as internal standard and has the following chemical structure:



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

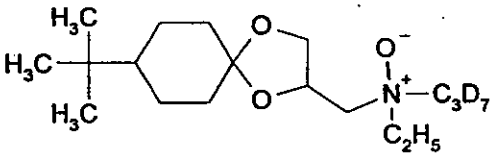
Chemical Name:	8-(1,1-Dimethylethyl)-N-(D_7-propyl)-1,4-dioxaspiro[4.5]decane-2-methanamine
Total formula:	$C_{16}H_{24}D_7NO_2$
Molar mass:	276.5 g

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



Despropyl-KWG 4168-D5 (KWG 4669-D5, KWG 7718)

Chemical Name:	8-(1,1-Dimethylethyl)-N-(D_5-ethyl)-1,4-dioxaspiro[4.5]decane-2-methanamine
Total formula:	$C_{15}H_{24}D_5NO_2$
Molar mass:	260.4 g
Appearance:	yellowish liquid



KWG 4168 N-oxide D7 (WAK 7211/1)

Chemical Name:	8-(1,1-Dimethylethyl)-N-ethyl-N-(D_7-propyl)-1,4-dioxaspiro[4.5]decane-2-methanamine-N-oxide
Total formula:	$C_{18}H_{28}D_7NO_3$
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 µm, 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_3 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 (KWG 4557)	921103ELB02 purity: 98 % 42 % Isomer A, 56 % Isomer B expiry date: November 1997
Despropyl-KWG 4168 (KWG 4669)	921103ELB03 purity: 98 % 55 % Isomer A, 43 % Isomer B expiry date: November 1997
KWG 4168 N-Oxide (WAK 6301)	950209ELB01 purity: 93 % expiry date: April 1997

Reference Substances (Internal Standards)

KWG 4168-D7 (KWG 7498)	940627ELB01 purity: 97.5 % 50.6 % Isomer A, 47 % Isomer B expiry date: June 1996
Desethyl-KWG 4168-D7 (KWG 4557-D7) (KWG 7566)	940729ELB01 purity: 88 % 40 % Isomer A, 48 % Isomer B expiry date: August 1996
Despropyl-KWG 4168-D5 (KWG 4669-D5) (KWG 7718)	941124ELB01 purity: 93 % 45 % Isomer A, 48 % Isomer B expiry date: January 1997
KWG 4168 N-Oxide-D7 (WAK 7211/1)	960423ELB01 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%) / l
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_2
Nebulizer gas:	18 l/h N_2
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
MS1-Parameter:	
LM Resolution	8
HM Resolution	8
Ion Energy	2
Ion Energy Ramp	0
Lens 6	5
Multiplier	750
MS2-Parameter:	
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.

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

$$c_i = \frac{A_i}{A_{iSi}} * \frac{c_{iS}}{RF_i}$$

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

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

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

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 (µg/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]

10. Detector Linearity

Standard solutions of KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168 and KWG 4168 N-Oxide are measured in a concentration range of about 0.01 to 3.4 µg/ml.
The concentrations of the internal standard substances KWG 4168 D7, Despropyl-KWG 4168-D7, Despropyl-KWG 4168-D5 and KWG 4168 N-Oxide-D7 were fixed.
For the four analytes the detector shows linear correlation between concentration and peak height (Tables 5 - 6). Graphical representations of the results are included in the Appendix (Figures 11 - 18)

Table 5: Correlation between Concentration and Peak Height from Standards in Solvent

Substance	Concentration Range [ng/ml]	Correlation Coefficient
KWG 4168	14.20 to 3408	0.9989
Desethyl-KWG 4168 (KWG 4557)	12.36 to 2966	0.9999
Despropyl-KWG 4168 (KWG 4669)	13.18 to 3162	0.9999
KWG 4168 N-Oxide (WAK 6301)	13.62 to 3270	0.9997

Table 6: Correlation between Concentration and Peak Height from Standards in Matrix

Substance	Concentration Range [ng/ml]	Correlation Coefficient
KWG 4168	14.20 to 3408	0.9999
Desethyl-KWG 4168 (KWG 4557)	12.36 to 2966	0.9999
Despropyl-KWG 4168 (KWG 4669)	13.18 to 3162	0.9995
KWG 4168 N-Oxide (WAK 6301)	13.62 to 3270	0.9998

11 Reproducibility

Standard solutions of about 10 ng/ml KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168, KWG 4168 N-Oxide and internal standards of about 100 ng/ml KWG 4168-D7, Desethyl-KWG 4168-D7, Despropyl-KWG 4168-D5 and KWG 4168 N-Oxide-D7 are injected 10 times into the liquid chromatograph. The peak heights for KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168, KWG 4168 N-Oxide, KWG 4168-D7, Desethyl-KWG 4168-D7, Despropyl-KWG 4168-D5 and KWG 4168 N-Oxide-D7 are determined. The response factors and retention-times for KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168 and KWG 4168 N-Oxide are calculated and listed in Table 7.

Table 7: Reproducibility of the Response Factors and Retention Times

Substance	Concentration [ng/ml]	Response Factors	RSD [%]	Retention Time (min.)	RSD [%]
KWG 4168	14.20	0.111	4.6	8.075	0.00
Desethyl-KWG 4168 (KWG 4557)	12.36	0.100	3.6	7.720	0.20
Despropyl-KWG 4168 (KWG 4669)	13.18	0.137	3.7	7.304	0.24
KWG 4168 N-Oxide (WAK 6301)	13.62	0.174	3.8	8.188	0.14

12 Control Samples

The analytical results of all control samples were below 2 µg/kg for KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168 and KWG 4168 N-Oxide.

13 Limit of Quantification and Limit of Detection

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

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

14 Determination of the Recovery Rates

A mixture of the standard soils 2.1, 2.2 and 2.3 (1 + 1 + 1 by weight) of LUFA Speyer, soil Höfchen, soil Fresno and soil Watsonville were fortified with the active ingredient and the metabolites.

For this method validation 36 recovery experiments were conducted, each sample was injected one time into the HPLC instrument.

Fortification levels of the different soils with KWG 4168, Desethyl-KWG 4168, Despropyl-KWG 4168 and KWG 4168 N-Oxide, recoveries and standard deviations are presented in Tables 8 to 11.

Table 8: Recovery Rates of KWG 4168

Fortification Level [µg/kg]	Soil	Single Values [%]	Mean Value [%]	RSD [%]
4.733	soil mixture 2.1, 2.2, 2.3	79.3 / 73.5 / 84.2	85.3	7.8
	Höfchen	92.0 / 86.3 / 85.4		
	Fresno	80.8 / 79.5 / 82.6		
	Watsonville	94.6 / 91.7 / 94.2		
47.33	Fresno	74.6 / 77.5 / 77.1	78.2	5.2
	Watsonville	85.4 / 80.2 / 74.5		
94.67	soil mixture 2.1, 2.2, 2.3	75.7 / 75.1 / 68.4	76.3	6.4
	Höfchen	80.1 / 75.7 / 82.7		
378.7	soil mixture 2.1, 2.2, 2.3	73.4 / 80.2 / 76.8	76.9	8.6
	Höfchen	83.6 / 79.8 / 81.8		
	Fresno	66.6 / 66.9 / 76.0		
	Watsonville	77.1 / 71.6 / 89.0		
	over all single values		79.8	8.8

RSD = relative standard deviation

Table 9: Recovery Rates of Desethyl-KWG 4168 (KWG 4557)

Fortification Level [µg/kg]	Soil	Single Values [%]	Mean Value [%]	RSD [%]
4.120	soil mixture 2.1, 2.2, 2.3	93.1 / 90.0 / 96.7	91.2	4.9
	Höfchen	94.2 / 88.8 / 93.6		
	Fresno	94.0 / 91.8 / 89.3		
	Watsonville	96.5 / 84.5 / 82.4		
41.20	Fresno	90.6 / 91.7 / 87.8	90.2	4.4
	Watsonville	94.2 / 83.6 / 93.5		
82.40	soil mixture 2.1,2.2,2.3	92.1 / 92.1 / 83.4	89.7	4.0
	Höfchen	88.5 / 88.9 / 93.2		
329.6	soil mixture 2.1, 2.2, 2.3	93.9 / 98.0 / 94.8	90.1	5.4
	Höfchen	92.9 / 90.3 / 90.0		
	Fresno	89.6 / 85.3 / 89.7		
	Watsonville	79.4 / 86.3 / 91.1		
	over all single values		90.4	4.7

RSD = relative standard deviation

Table 10: Recovery Rates of Despropyl-KWG 4168 (KWG 4669)

Fortification Level [µg/kg]	Soil	Single Values [%]	Mean Value [%]	RSD [%]
4.393	soil mixture 2.1, 2.2, 2.3	109 / 110 / 113	116	5.3
	Höfchen	113 / 111 / 114		
	Fresno	128 / 111 / 119		
	Watsonville	120 / 123 / 123		
43.93	Fresno	99.5 / 91.7 / 95.1	94.0	4.3
	Watsonville	93.0 / 87.9 / 96.7		
87.87	soil mixture 2.1, 2.2, 2.3	95.6 / 97.3 / 89.8	93.3	3.0
	Höfchen	91.4 / 91.8 / 94.1		
351.3	soil mixture 2.1, 2.2, 2.3	99.0 / 100 / 98.1	93.7	5.5
	Höfchen	93.3 / 92.0 / 91.9		
	Fresno	92.6 / 91.3 / 101		
	Watsonville	82.5 / 90.3 / 92.8		
	over all single values		101	11.7

RSD = relative standard deviation

Table 11: Recovery Rates of KWG 4168 N-Oxide (WAK 6301)

Fortification Level [µg/kg]	Soil	Single Values [%]	Mean Value [%]	RSD [%]
4.540	soil mixture 2.1, 2.2, 2.3	103 / 99.9 / 102	94.0	5.9
	Höfchen	95.0 / 93.9 / 88.5		
	Fresno	90.0 / 94.7 / 86.0		
	Watsonville	92.6 / 88.3 / 97.6		
45.40	Fresno	97.7 / 101 / 103	97.9	3.9
	Watsonville	92.9 / 94.4 / 98.3		
90.80	soil mixture 2.1, 2.2, 2.3	100 / 103 / 99.1	96.0	5.8
	Höfchen	88.4 / 92.5 / 93.1		
363.3	soil mixture 2.1, 2.2, 2.3	99.1 / 99.7 / 102	93.5	4.8
	Höfchen	92.6 / 93.1 / 92.8		
	Fresno	89.4 / 88.0 / 90.8		
	Watsonville	92.9 / 88.9 / 92.8		
	over all single values		94.9	5.3

RSD = relative standard deviation

The mean recovery during method validation was
79.8 % for KWG 4168 with a relative standard deviation of 8.8 %,
90.4 % for Desethyl-KWG 4168 (KWG 4557) with a relative standard deviation of 4.7 %,
101 % for Despropyl-KWG 4168 (KWG 4669) with a relative standard deviation of 11.7 % and
94.9 % for KWG 4168 N-Oxide (WAK 6301) with a relative standard deviation of 5.3 %.

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.

15 Organisation

Sponsor:	Dr. J. Köhler Bayer AG, PF-E/MR 51368 Leverkusen
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Analytics:	M. Schuld, U. Weiß Bayer AG, PF-E/MR 51368 Leverkusen
Reference Compounds:	H.-J. Ott, Dr. W. Krämer, D. K. Wagner Bayer AG, PF-F 51368 Leverkusen
Testing Facility:	Institute for Metabolism Research and Residue Analysis Bayer AG, PF-E/MR 51368 Leverkusen
Manager of Testing Facility:	Dr. J. Köhler Institute for Metabolism Research and Residue Analysis Bayer AG, PF-E/MR

15.1 Archiving

All raw data pertaining to this study and the original report are stored in the central GLP archive PF-F, Bayer AG, D-51368 Leverkusen, for at least as long as required by GLP-principles.
Test and reference substances are stored in the archives of PF-E/FT-EA, Bayer AG, D-51368 Leverkusen, as long as their quality still guarantees an evaluation.

15.2 Time Schedule

Approval of the Study by the Study Director:	March 28, 1996
Start of Experimental Phase:	March 28, 1996
End of Experimental Phase:	April 9, 1996

Appendix

Figure 1: **Flow Diagram of Analysis Procedure**

30 g Soil

⇩

Extract with 100 ml of methanol / water / ammonia (25%) (800 + 200 + 10 parts by volume) on a mechanical shaker for 1h

⇩

Filter and transfer an aliquot of 40 ml into a 50 ml centrifuge tube

⇩

Evaporate to the aqueous remainder of about 5 ml
(do not evaporate extracts to dryness !)

⇩

Add internal standard
Adjust the volume to 10 ml using water / methanol / ammonia solution (25%)
(750 + 250 + 10 parts by volume)

⇩

Centrifuge an aliquot of 1.5 ml at 13000 rpm and fill into a HPLC vial

⇩

HPLC-MS-MS

Figure 2 Mass spectrum of KWG 4168, KWG 4168-D7, Desethyl-KWG 4168 (KWG 4557), Desethyl-KWG 4168-D7 (KWG 4557-D7),Despropyl-KWG 4669 (KWG 4669), Despropyl-KWG 4168-D5 (KWG 4669-D5) KWG 4168 N-Oxide (WAK 6301) and KWG 4168 N-Oxide D7 (WAK 7211/1)

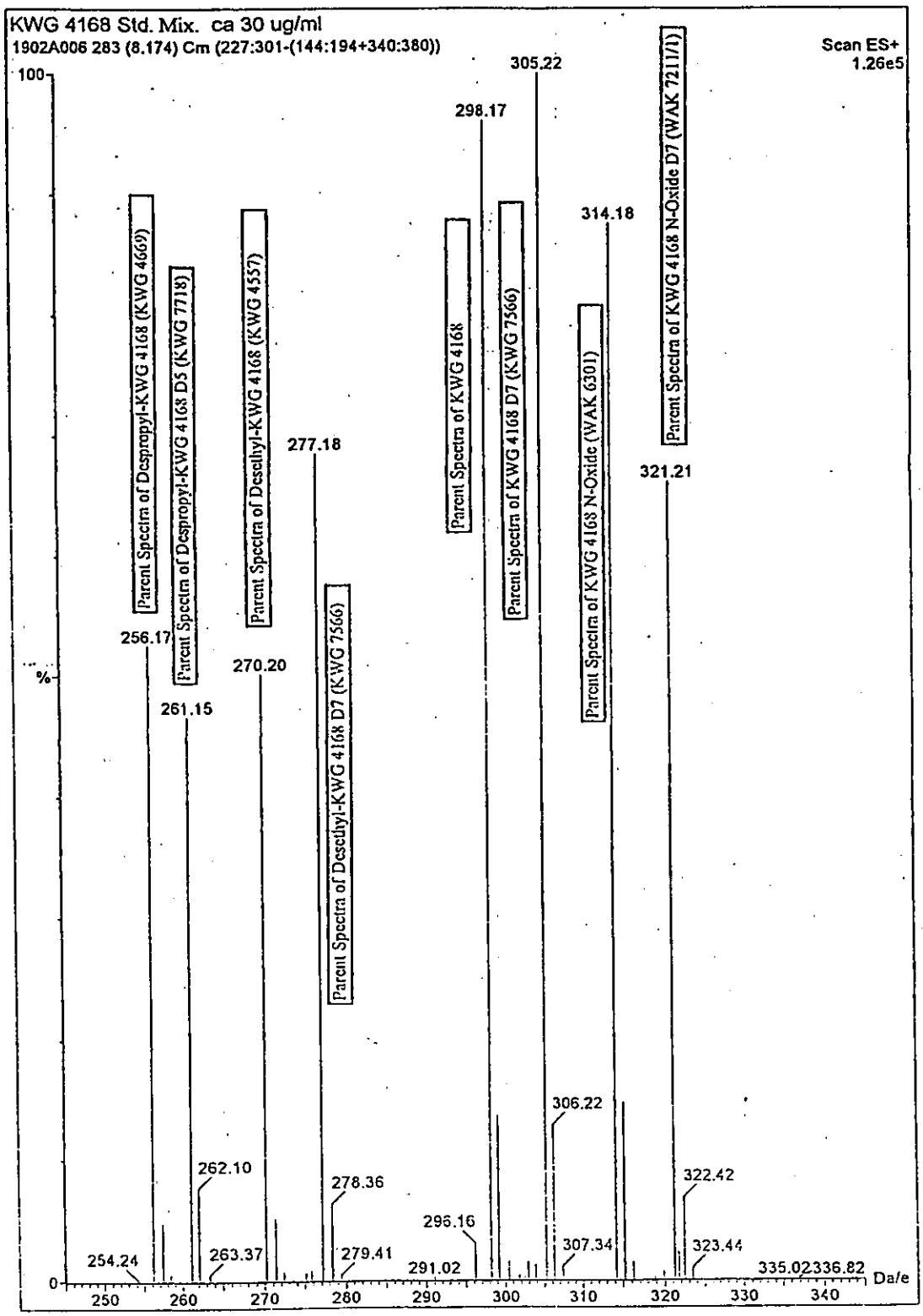


Figure 3 Mass spectrum of the daughter ions of KWG 4168

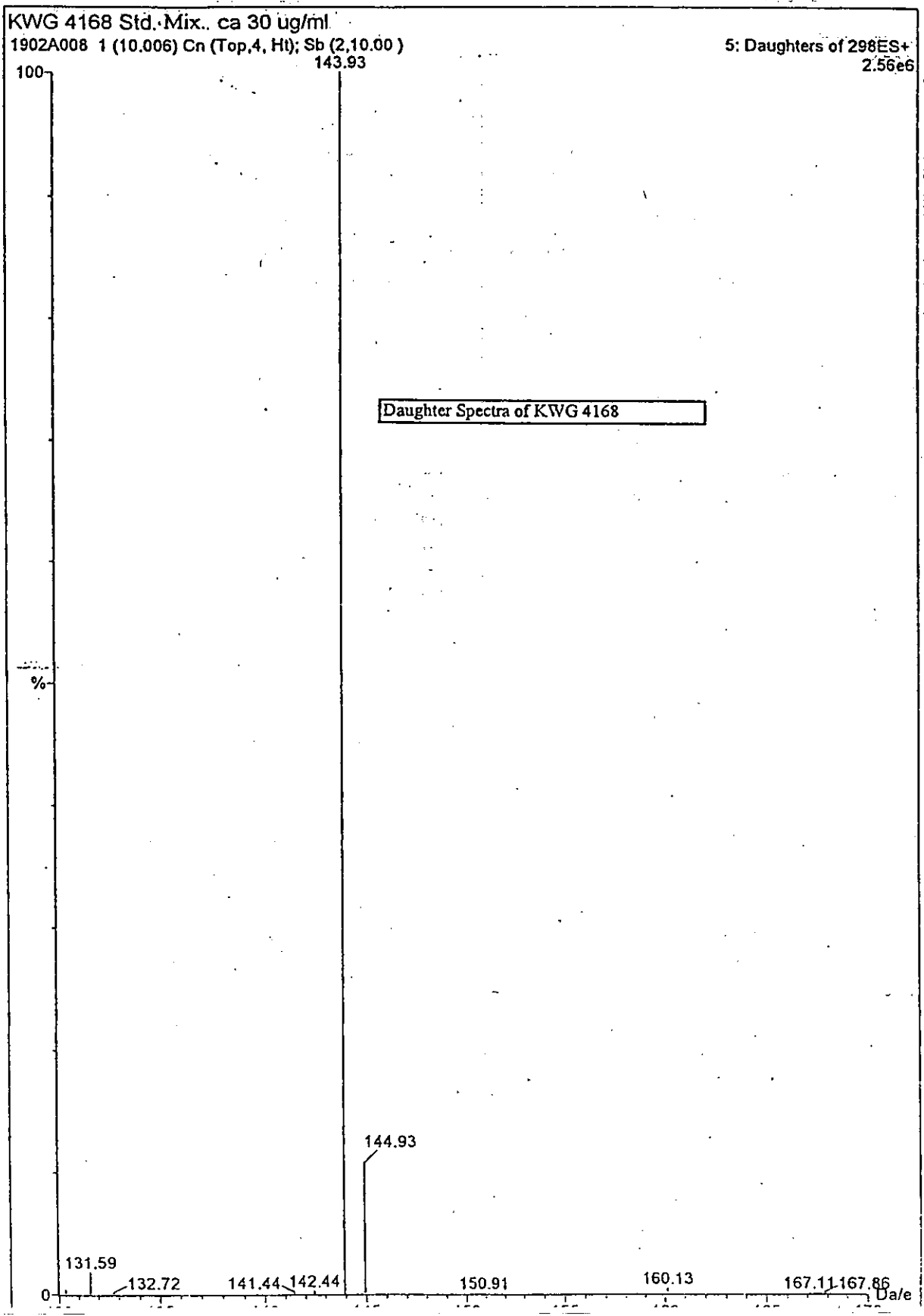


Figure 4 Mass spectrum of the daughter ions of Desethyl-KWG 4168 (KWG 4557)

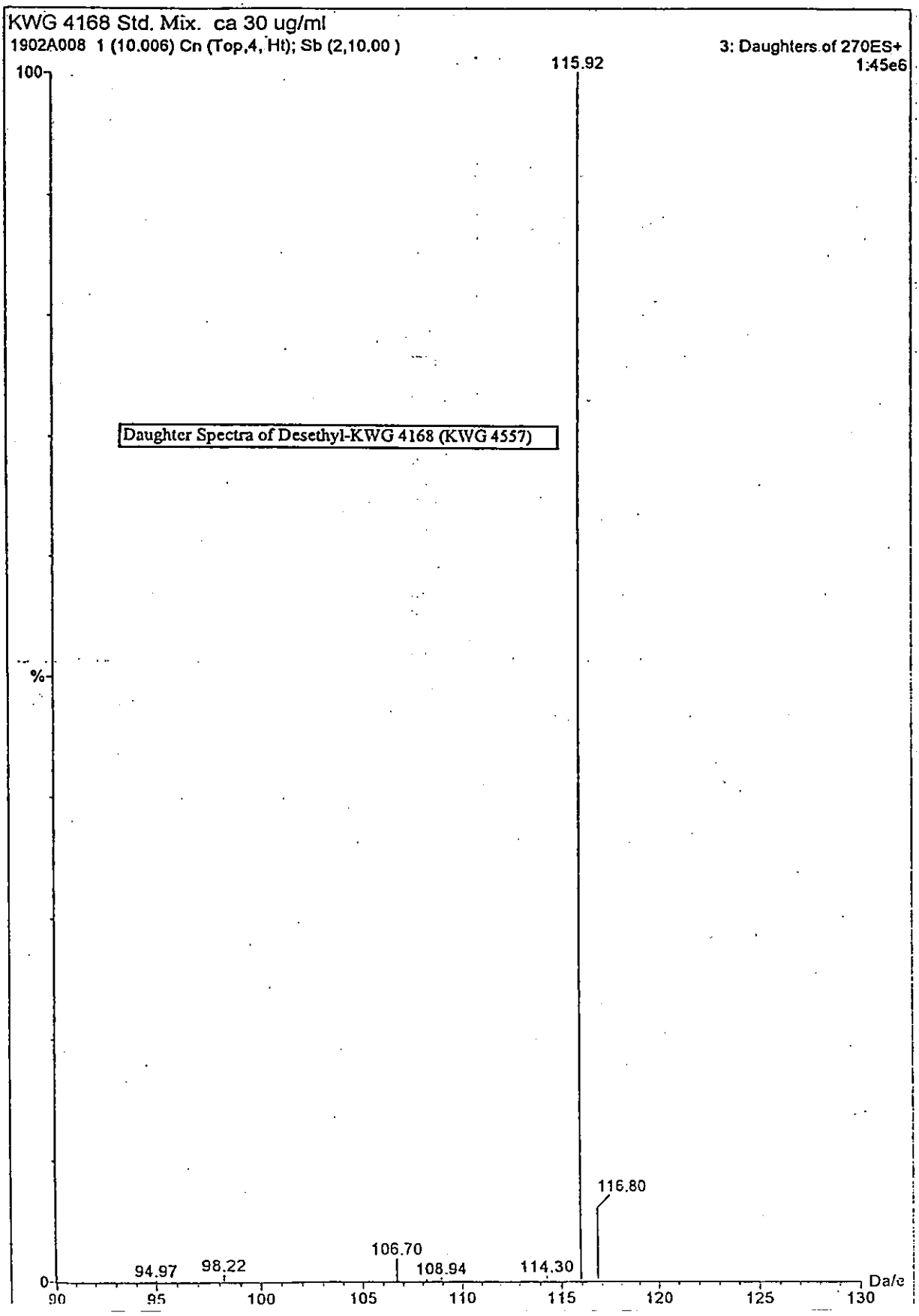


Figure 5 Mass spectrum of the daughter ions of Despropyl-KWG 4669 (KWG 4669)

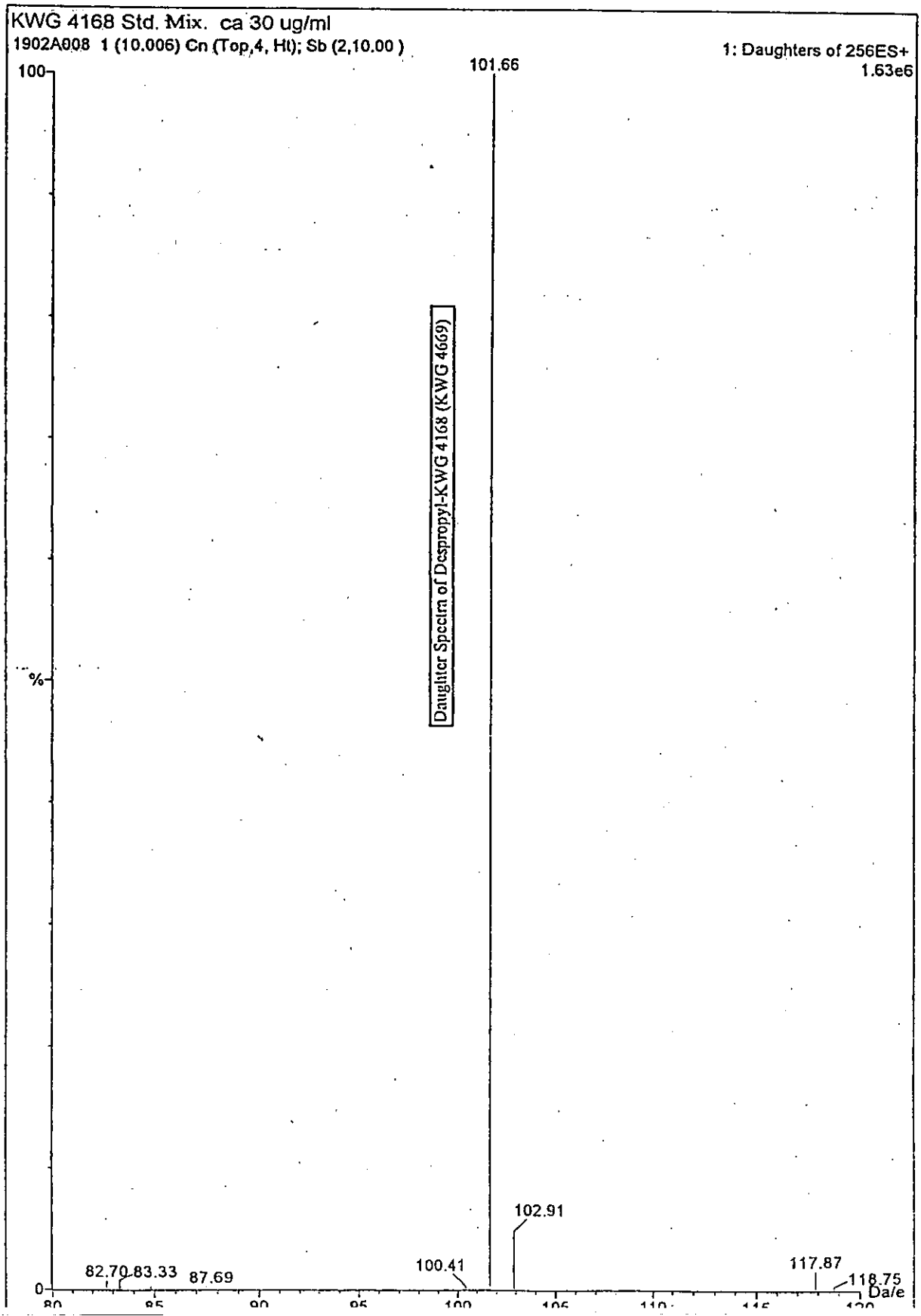


Figure 6 Mass spectrum of the daughter ions of KWG 4168 N-Oxide (WAK 6301)

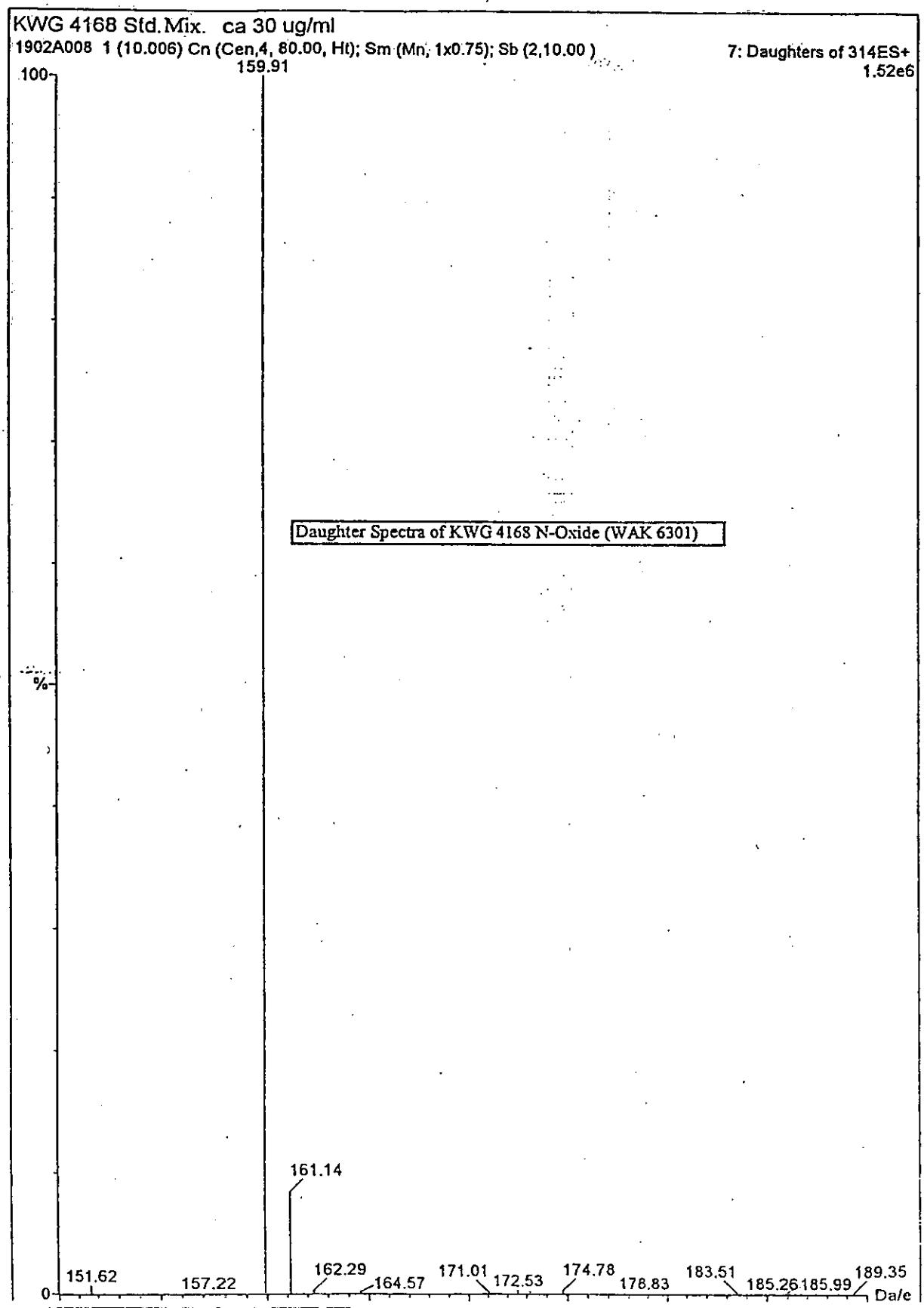


Figure 7 Mass spectrum of the daughter ions of KWG 4168-D7

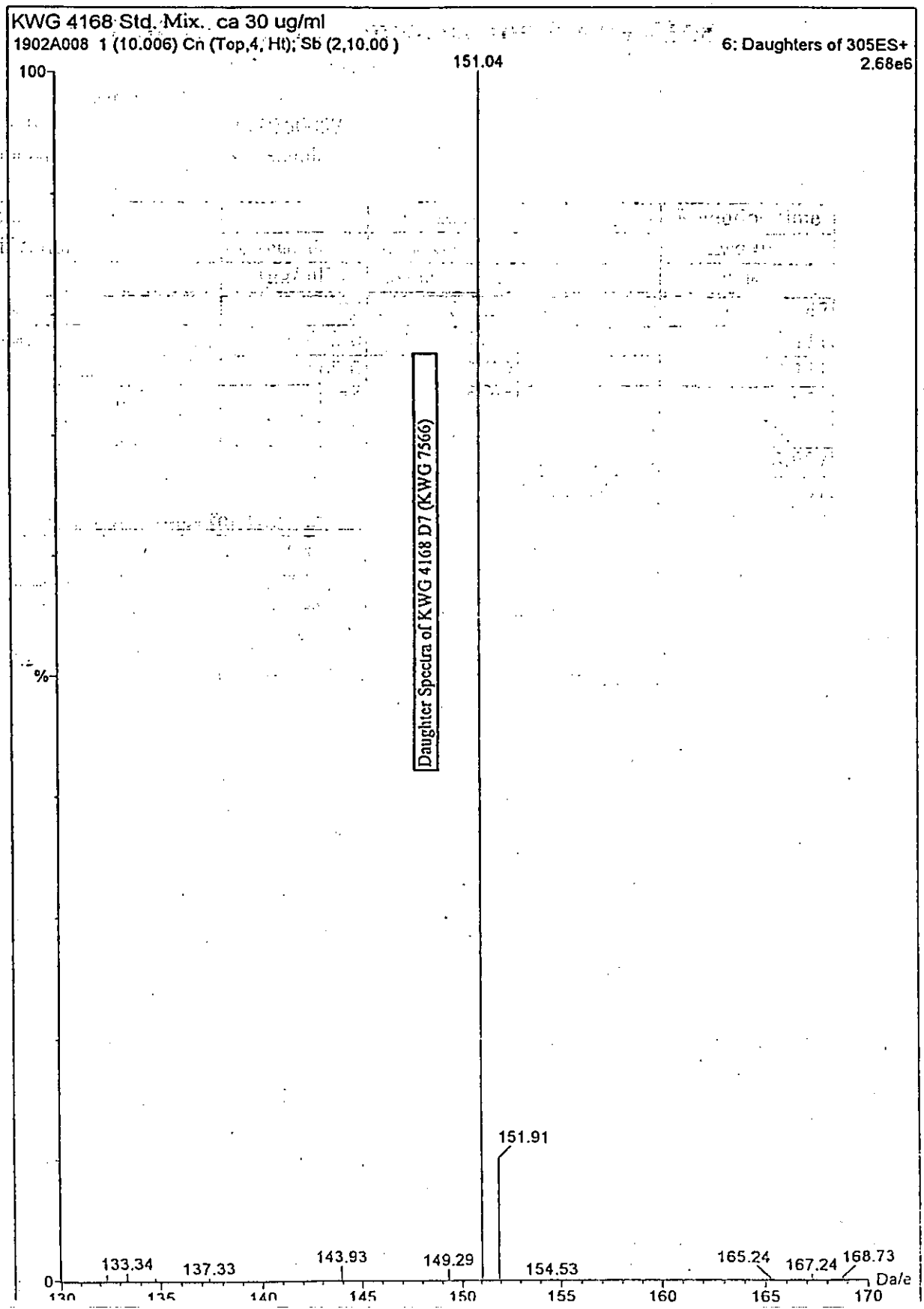


Figure 8 Mass spectrum of the daughter ions of Desethyl-KWG 4168-D7 (KWG 4557-D7)

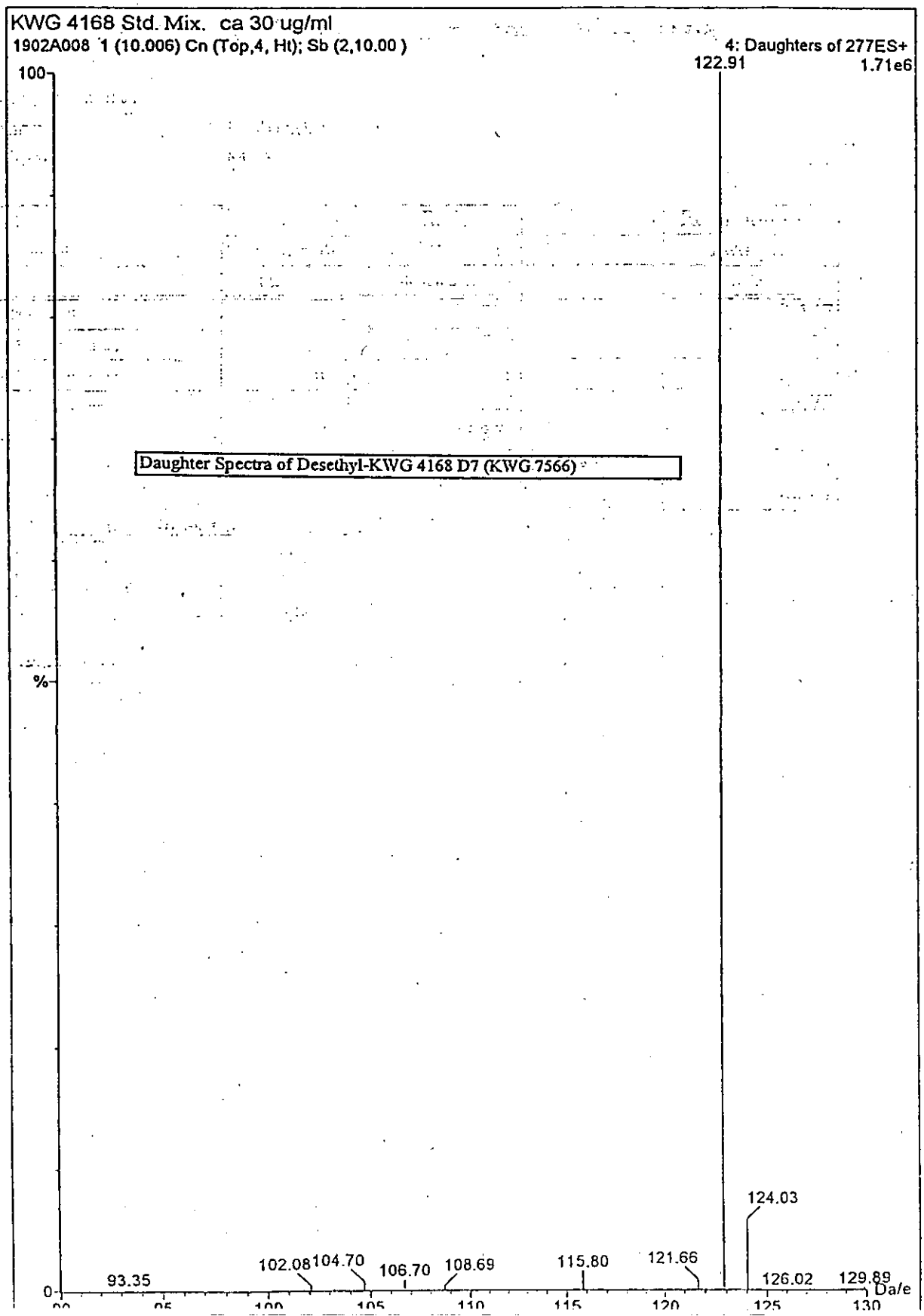


Figure 9 Mass spectrum of the daughter ions of Despropyl-KWG 4168-D5 (KWG 4669-D5)

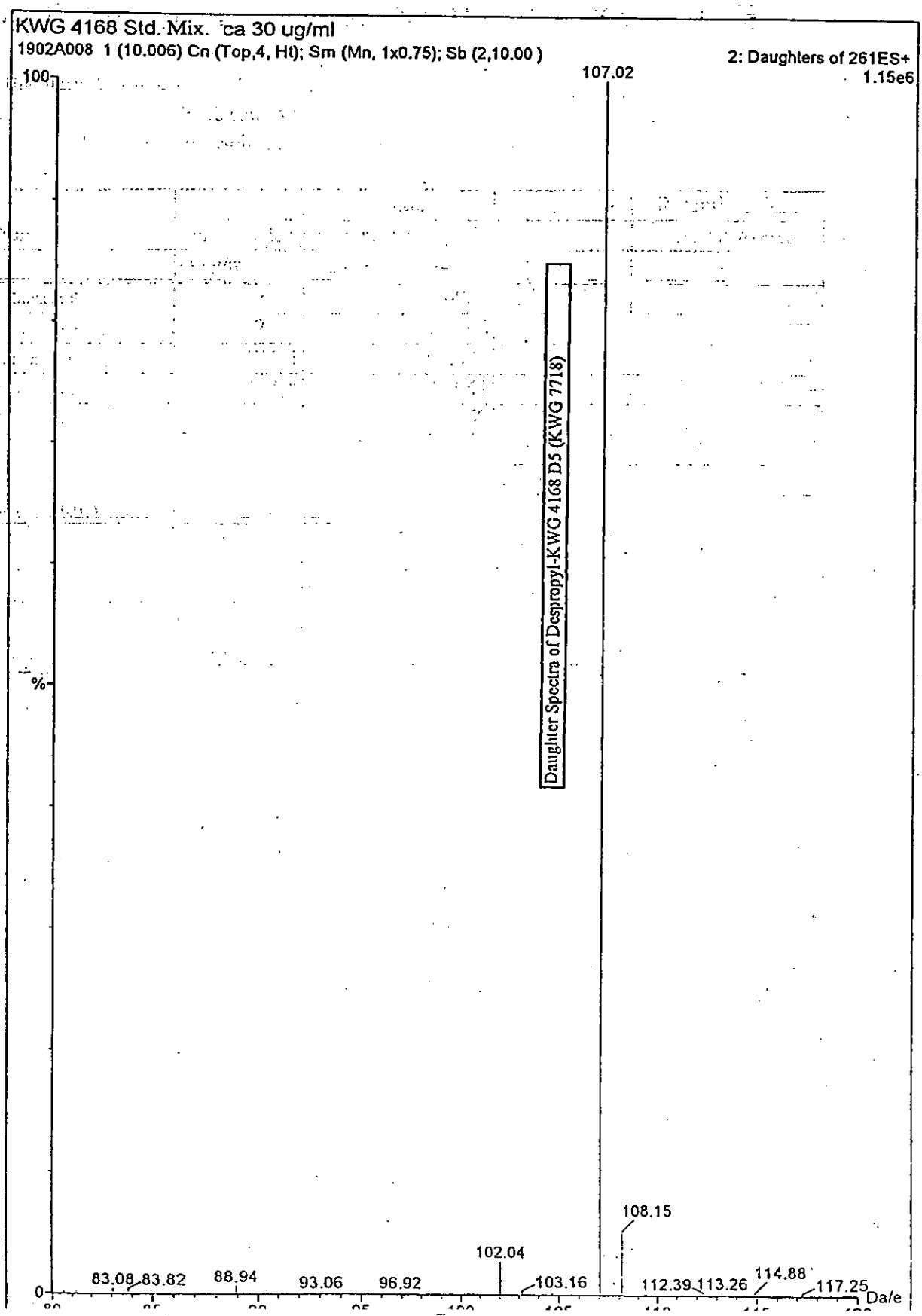
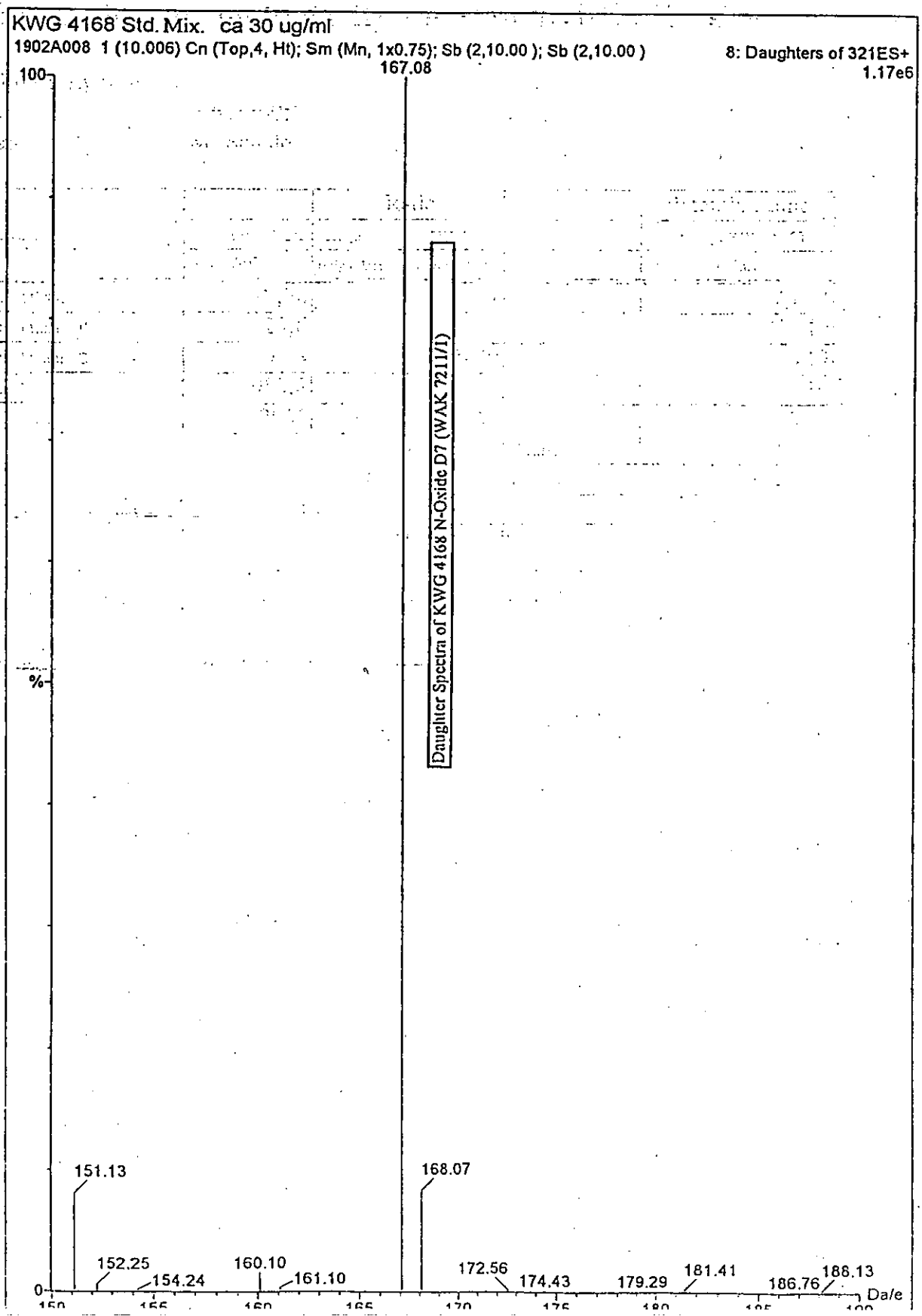


Figure 10 Mass spectrum of the daughter ions of KWG 4168 N-Oxide D7 (WAK 7211/1)



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