INTRODUCTION

The objective of this study was to validate a method of analysis for the determination of TKC-94 and its major metabolite MET-42 in soil, supplied from four sites and water from one site, which were used in dissipation studies during 1998/99 (Study numbers TON/021 and TON/022, Report numbers TON 021/002214 and TON 022/002274).

To determine the validity of the analytical method, it was necessary to determine:

- linearity
- recovery
- accuracy
- precision (repeatability)
- limit of detection
- limit of quantitation

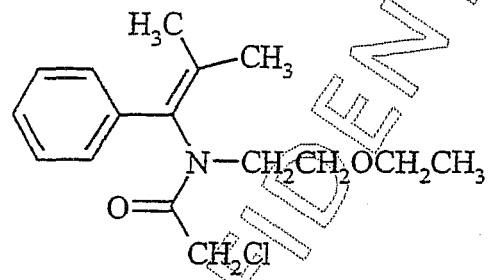
The protocol was signed by the Study Director and the Head of Department on 26 June 1998 and by the Sponsor on 1 July 1998.

The analytical phase was undertaken at Huntingdon Life Sciences, Eye between 16 July 1998 and 3 April 2000.

The signed protocol, a copy of the final report and the primary data pertaining to the study have been retained in the archives of Huntingdon Life Sciences. These records will be retained for a minimum period of ten years from the date of issue of the final report.

MATERIALS

Test substance

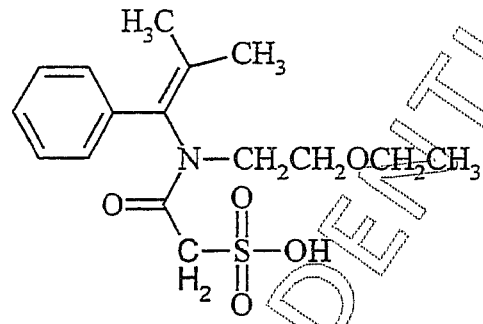
Code name:	TKC-94
Chemical name	2-Chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl) acetamide
Structure:	
Appearance:	White crystalline solid
Storage conditions:	4°C in the dark
Batch number:	TP-960418A
Receipt date:	13 May 1998
Purity:	min. 99.9%
Expiry date:	18 June 2000

Test substance

Code name: MET-42 (TKC-94 sulphonic acid)

Chemical name: N-(2-Ethoxyethyl)-N-(2-methyl-1-phenyl-propenyl) -2-sulfoacetamide

Structure:



Appearance: White powder

Storage conditions: 4°C in the dark

Batch number: 10.12.98/8

Receipt date: 10 December 1998

Soil samples

Samples of soil taken prior to treatment from each of the four sites used in the soil dissipation studies and ground water from the UK site used in the leaching study (Report numbers: TON 021/002214 and TON 022/002274) were supplied for validation purposes. Soil samples from trials TON/021-01 (Spain), TON/021-03 (France) and TON/021-04 (Spain) were sent to Levington Agriculture for characterisation, and the soil from trial TON/022-01 was sent to the Survey and Land Research Centre for characterisation. The soil characterisation reports are presented in Appendix 3, and a summary of the soils from the treated plots is presented below.

	TON/021-01 (SH98-1114)	TON/021-03 (SH98-1232)	TON/021-04 (SH99-3240)	TON/022 (176/98/1)
pH	7.5	6.9	7.3	7.2
Organic carbon (%)	0.8	0.8	0.8	3.0
CEC (meq/100 g)	19.1	14.8	16.3	20.2
Clay (%)	37	24	31	61
Silt (%)	23	46	16	24
Sand (%)	40	30	53	15

METHOD

Pre-treatment soils were supplied from each of the four trial sites and water from the UK site used in the field dissipation studies (TON/021 and TON/022). The methodology for the determination of TKC-94 and MET-42 was validated in all four soils and in water as described below.

Validation

Sub-samples of each of the four soils were fortified at known concentrations of TKC-94 and MET-42 according to the following regime:

5 sub-samples of untreated soil
5 sub-samples of untreated soil fortified at 10 ng/g
5 sub-samples of untreated soil fortified at 100 ng/g
5 sub-samples of untreated soil fortified at 2500 ng/g

These samples were then subjected to the analytical methodology described.

Sub-samples of water were fortified at known concentrations of TKC-94 and MET-42 according to the following regime:

5 sub-samples of untreated water
5 sub-samples of untreated water fortified at 0.1 µg/l
5 sub-samples of untreated water fortified at 0.5 µg/l
5 sub-samples of untreated water fortified at 5.0 µg/l

These samples were then subjected to the analytical methodology described.

Homogenisation of soil cores

In order to confirm the efficiency of the soil homogenisation procedure the following experiment was performed:

Twenty horizons (20 to 30 cm) were cut from the remainder of the twenty cores comprising an untreated soil sample. Each core was spiked with 250 µg of TKC-94 (500 µl of a 500 µg/ml standard in acetone). The solvent was allowed to evaporate before the soils were removed from the acetate tubes onto a tray lined with polyethylene. The tray was placed into the 4°C room overnight. The following morning the tray was removed from the 4°C room and any stones and organic debris removed from the soil. The soil was mixed by hand until homogeneous and a number of sub-samples were taken and subjected to the analytical methodology, together with an untreated sample and an untreated sample fortified TKC-94 to act as a procedural recovery.

Thawing of soil-stability check

To establish the stability of both compounds during the thawing period during preparation (approximately sixteen hours at 4°C), the following experiment was performed:

Twelve untreated sub-samples (20 g) of each of the four soils were weighed into polyethylene bottles. Two of the twelve sub-samples were fortified with TKC-94 at 100 ng/g and two with MET-42 at 100 ng/g. All the samples were then placed in the 4°C room overnight. The samples were removed from the 4°C room and four sub-samples of each soil were spiked as previously described to act as procedural recovery samples. All twelve sub-samples for each of the four soils were then subjected to the analytical methodology.

Storage stability

To establish the stability of both compounds in each of the four soils during storage at approximately -18°C for a period of time representing the storage of samples from the field dissipation studies TON/021 and TON/022, the following experiment was performed:

Four sub-samples of each soil type were spiked at 2500 ng/g with TKC-94 and four samples were spiked at 2500 ng/g with MET-42. Two sub-samples of each soil spiked with each of the compounds were subjected to the analytical methodology immediately (time = 0 data point). The remaining four sub-samples of each soil (two spiked with TKC-94, two spiked with MET-42) and four unfortified sub-samples of each soil type were placed in freezer storage at approximately -18°C pending analysis. The period of storage incurred in the field dissipation study was approximately six* months. After a six* month period the spiked sub-samples were removed from freezer storage and subjected to the analytical method along with untreated sub-samples and untreated sub-samples fortified with TKC-94 and MET-42 respectively (procedural recovery samples). These samples were then subjected to the analytical method in order to determine the stability over the period of freezer storage.

To establish the stability of both compounds in ground water during storage at approximately -18°C for a period of time representing the storage of samples from the field leaching study TON/022, the following experiment was performed:

Four sub-samples of untreated water were spiked at 5 µg/l with TKC-94 and four samples were spiked at 5 µg/l with MET-42. Two sub-samples spiked with each of the compounds were subjected to the analytical methodology immediately (time = 0 data point). The remaining four sub-samples (two spiked with TKC-94, two spiked with MET-42) and four un-fortified sub-samples of water were placed in freezer storage at approximately -18°C pending analysis. The maximum period of storage incurred in the leaching study was approximately six months for TKC-94 analysis and 13 months for MET-42 analysis. After the appropriate period of time the spiked sub-samples were removed from freezer storage and subjected to the analytical method along with untreated sub-samples and untreated sub-samples fortified with TKC-94 and MET-42 respectively (procedural recovery samples). These samples were then subjected to the analytical method in order to determine the stability over the period of freezer storage.

* For trial TON/021-04 the maximum storage period was approximately four months.
For Trial TON/021-01, the maximum period of storage was nineteen months (this represented the time period between analysis of the day zero samples and analysis of the 20-30 cm horizons reported in Report number TON 021/ 002214). Storage stability data covering this period is presented in Table 47 (Appendix 1).
For trial TON/021-03 the maximum storage period was approximately 9 months.

General principles

Two methods were developed and validated for the determination of TKC-94 in soil. The first method comprised of extraction with an acetone:water mixture followed by a liquid:liquid partition with hexane. The soil extracts were cleaned-up by solid phase extraction (SPE) using a silica cartridge, prior to quantitation by gas chromatography using electron capture detection (GC - ECD). The second method comprised of extraction with an acetone:water mixture followed by quantitation by liquid chromatography using mass spectroscopy (LC-MS).

Two methods were developed and validated for the determination of TKC-94 in water. The first method comprised of a liquid:liquid partition with dichloromethane followed by a clean-up by solid phase extraction (SPE) using a silica cartridge, prior to quantitation by gas chromatography using electron capture detection (GC - ECD). The second method comprised of a clean-up by solid phase extraction (SPE) using a C_{18} cartridge, prior to quantitation by liquid chromatography using mass spectroscopy (LC-MS).

The method for the determination of MET-42 in soil comprised of extraction with an acetone:water mixture followed by quantitation by liquid chromatography using mass spectroscopy (LC-MS).

The method for the determination of MET-42 in water comprised of a clean-up by solid phase extraction (SPE) using a C_{18} cartridge, prior to quantitation by liquid chromatography using mass spectroscopy (LC-MS).

Apparatus

Cut-off saw, Makita 2414B
Centrifuge, Mistral 3000E
Mechanical shaker, IKA KS501
Polyethylene bottles, 500 ml, wide neck
Polypropylene tubes, 50 ml
Round bottom flasks, 100 ml, glass
Rotary Evaporator, Buchi
Sample concentrator, Techni
Ultrasonic bath, Decon Ltd.
Vacuum Manifold, Supelco
Volumetric flasks, Various sizes
Volumetric pipettes, Various sizes
Drying cartridge - A Bond Elut reservoir (6 ml capacity), with a glass wool plug, containing approximately 5 g of anhydrous sodium sulphate

Materials	Grade	Supplier
Acetic acid	Glacial	Fisher Scientific, Loughborough, England
Acetone	Distol	Fisher Scientific, Loughborough, England
Acetonitrile	Distol	Fisher Scientific, Loughborough, England
Ammonia acetate	AR	Fisher Scientific, Loughborough, England
Hexane	Distol	Fisher Scientific, Loughborough, England
Dichloromethane	Distol	Fisher Scientific, Loughborough, England
Diethyl ether	Analytical Reagent	Fisher Scientific, Loughborough, England
Sodium chloride	Laboratory Reagent	Fisher Scientific, Loughborough, England
Sodium sulphate	Anhydrous	Sigma-Aldrich, Poole, Dorset, England
Toluene	Distol	Fisher Scientific, Loughborough, England
Water	Ultra Pure	Elga Maxima, Huntingdon Life Sciences
Si SPE cartridges (1 g, 6cc) part no. 1225-6008		Varian Ltd, Walton-on-Thames, England
C_{18} SPE cartridges (500 mg, 3cc) part no. 1210-2028		Varian Ltd, Walton-on-Thames, England

Test substance stock and fortifying solutions

An appropriate amount of TKC-94 (corrected for purity) was accurately weighed and dissolved in acetone to give a stock standard solution. An appropriate amount of MET-42 was also accurately weighed and dissolved in acetone to give a stock standard solution. The stock standard solutions were diluted with acetone to give fortification standard solutions.

An appropriate volume of the TKC-94 fortification standard solution in acetone was diluted progressively with toluene to produce calibration standard solutions in the range 5 to 100 ng/ml for quantitation by GC-ECD.

An appropriate volume of the TKC-94 fortification standard solution in acetone was diluted progressively with acetonitrile:water (50:50 v:v) to produce calibration standard solutions in the range 1 to 100 ng/ml for quantitation by LC-MS.

An appropriate volume of the MET-42 fortification standard solution in acetone was diluted progressively with acetonitrile:water:acetic acid (20:80:1 v:v:v) to produce calibration standard solutions in the range 1 to 100 ng/ml.

ANALYTICAL PROCEDURE

Soil analysis

Extraction of soil

A sub sample (20 g) of soil was weighed into a polyethylene bottle. If necessary the sample was spiked at this point with a fortification standard. An aliquot (200 ml) of acetone:water (3:1 v:v) was added, the sample shaken for 2 hours and then centrifuged to separate the phases.

TKC-94 analysis by GC-ECD

An aliquot (10 ml) of the supernatant was transferred to a polypropylene tube (50 ml) and aliquots (10 ml) of sodium chloride solution (10% w:v) and hexane were added. The tube was shaken vigorously and the phases allowed to separate. The upper (hexane) phase was transferred to a round bottom flask via a drying cartridge. The partition was repeated with a further aliquot (10 ml) of hexane, and the hexane extracts combined. The solvent was removed by rotary evaporation at approximately 40°C. The residue was reconstituted in hexane (5 ml) prior to clean-up by SPE on a silica cartridge.

The silica cartridge was washed with aliquots (6 ml) of diethyl ether followed by hexane. The hexane extract was loaded onto the cartridge and allowed to elute through at a steady rate (dropwise). The cartridge was washed with aliquots (6 ml) of hexane followed by hexane:diethyl ether (80:20 v:v), discarding the eluates. The cartridge was eluted with an aliquot (6 ml) of diethyl ether. The eluate was then evaporated to dryness under a stream of nitrogen at 40°C and the residue reconstituted in an appropriate volume of toluene, prior to quantitation by GC-ECD.

TKC-94 analysis by LC-MS

An aliquot (10 ml) of the supernatant was transferred to a round bottom flask and the solvent removed by rotary evaporation at approximately 40°C (acetonitrile was added to the flask to assist in the evaporation of any water present). The residue was reconstituted in an appropriate volume of acetonitrile:water (50:50 v:v), prior to quantitation by LC-MS.

MET-42 analysis by LC-MS

An aliquot (10 ml) of the supernatant was transferred to a round bottom flask and the solvent removed by rotary evaporation at approximately 40°C (acetonitrile was added to the flask to assist in the evaporation of any water present). The residue was reconstituted in an appropriate volume of acetonitrile:water:acetic acid (20:80:1 v:v:v), prior to quantitation by LC-MS.

Water analysis

TKC-94 analysis by GC-ECD

An aliquot (100 ml) of water was transferred to a separating funnel and fortified if required. Aliquots (50 ml and 10 ml) of sodium chloride solution (30% w:v) and dichloromethane respectively were added and the funnel was shaken vigorously. The phases were allowed to separate and the lower (dichloromethane) phase was collected through anhydrous sodium sulphate into a round bottom flask. A further aliquot (10 ml) of dichloromethane was added and the partition repeated. The dichloromethane phases were combined and the solvent was removed by rotary evaporation at approximately 40°C. The residue was reconstituted in hexane (5 ml) with the aid of ultrasonication, prior to clean-up by SPE on a silica cartridge.

The silica cartridge was washed with aliquots (6 ml) of diethyl ether followed by hexane. The hexane extract was loaded onto the cartridge and allowed to elute through at a steady rate (dropwise). The cartridge was washed with aliquots (6 ml) of hexane followed by hexane:diethyl ether (80:20 v:v), discarding the eluates. The cartridge was eluted with an aliquot (6 ml) of diethyl ether. The eluate was then evaporated to dryness under a stream of nitrogen at 40°C and the residue reconstituted in an appropriate volume of toluene, prior to quantitation by GC-ECD.

TKC-94 analysis by LC-MS

The C_{18} cartridge was washed with aliquots (3 ml) of acetonitrile followed by water. An aliquot (100 ml) of the water sample (fortified if required) was loaded onto the cartridge and allowed to elute through at a steady rate (dropwise). The cartridge was washed with aliquots (20 ml and 3 ml respectively) of water followed by acetonitrile:water (50:50 v:v), discarding the eluates. The cartridge was eluted with an aliquot (2.5 ml) of acetonitrile. The eluate was then diluted to an appropriate volume with water, prior to quantitation by LC-MS.

MET-42 analysis by LC-MS

The C_{18} cartridge was washed with aliquots (3 ml) of methanol followed by water. An aliquot (25 ml) of the water sample (fortified if required) was loaded onto the cartridge and allowed to elute through at a steady rate (dropwise). The cartridge was washed with an aliquot (10 ml) of water, discarding the eluate. The cartridge was eluted with an aliquot (3 ml) of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 50°C. The residue was reconstituted in an appropriate volume of acetonitrile:water:acetic acid (20:80:1 v:v:v), prior to quantitation by LC-MS.

Instrumentation

GC-ECD (TKC-94)

Instrument:	Hewlett Packard 6890 Gas Chromatograph with ECD Detector	
Column:	J&W Scientific 122-1732, DB17 (30 m x 0.25 mm x 0.25µm film thickness)	
Temperature programme:	Initial 150°C held for 0.5 min; elevated at 10°C/min to 300°C and held for 5 min	
Injector temperature:	270°C	
Detector temperature:	300°C	
Flow rates:	Initial carrier flow Anode purge flow Make-up (N_2)	1.0 ml/min 6.0 ml/min 60 ml/min - carrier flow
Injection volume:	1 µl	
Retention time:	TKC-94 = approx. 12.2 minutes	

LC-MS (TKC-94)

Instrument:	Quattro LC/ZMD 2000
Ionisation mode:	Positive electrospray (ESP+)
Source temperature:	120°C
Atmospheric pressure ionisation	approx. 400 l/h
Ion monitoring details:	*m/z* 296.2
Column:	Phenomenex Columbus C_{18} (15 cm × 2 mm) + generic C_{18} guard
Mobile phase:	Isocratic - 20:80 A:B A = 0.01M ammonium acetate + 0.1% acetic acid in water : acetonitrile (80:20 v:v) B = 0.01M ammonium acetate + 0.1% acetic acid in water : acetonitrile (20:80 v:v)
Flow rate:	0.2 ml/min
Injection volume:	20 µl
Retention time:	TKC-94 = approx. 6.2 minutes

LC-MS (MET-42)

Instrument:	Quattro LC/ZMD 2000
Ionisation mode:	Negative electrospray (ESP-)
Source temperature:	120°C
Atmospheric pressure ionisation	approx. 400 l/h
Ion monitoring details:	*m/z* 340.1
Column:	Phenomenex Columbus C_{18} (15 cm × 2 mm) + generic C_{18} guard
Mobile phase:	Isocratic - 75:25 A:B A = 0.01M ammonium acetate + 0.1% acetic acid in water : acetonitrile (80:20 v:v) B = 0.01M ammonium acetate + 0.1% acetic acid in water : acetonitrile (20:80 v:v)
Flow rate:	0.2 ml/min
Injection volume:	20 µl
Retention time:	MET-42 = approx. 4 minutes

Calculation of results

Test samples were quantified using the following equation:

$$\text{Residue found (ng/g)} = x \times \frac{1}{M} \times D$$

Where x (residue concentration in final solution) was calculated using the linear regression

$y = m x + c$ where x (concentration in ng/ml) $= \frac{y - c}{m}$

c = intercept
m = slope
y = peak area of sample
M = matrix concentration (g/ml)
D = dilution factor

Example calculation of TKC-94 detected in soil from trial TON/021-03 soil fortified at 100 ng/g. The primary data for this sample is presented in Table 27, Appendix 1 (sample identification - F100 A)

Linear regression $y = m x + c$

$y = 3036.846x + 1693.561$

where $y = 31121.787$
m = 3036.846
c = 1693.561

Therefore, concentration of TKC-94 (x) $= \frac{31121.787 - 1693.561}{3036.846} = 9.69$ ng/ml

Matrix concentration = 0.5 g/ml
Dilution factor = 5

TKC-94 detected (ng/g) $= \frac{9.69 \text{ ng/ml} \times 5}{0.5 \text{ g/ml}} = 96.9$ ng/g

Recovery of TKC-94 $= \frac{96.9 \text{ ng/g}}{100 \text{ ng/g}} \times 100\% = 97\%$

For the batches presented in Tables 47 and 49, Appendix 1 the bracketing* standard method was used to quantify the samples, this was done as follows:

$$\text{Residue found (ng/g)} = \frac{\text{sample peak area}}{\text{mean bracketing standard peak area}} \times \text{standard concentration} \times \frac{1}{M} \times D$$

M = matrix concentration (g/ml)
D = dilution factor

Example calculation of TKC-94 residue detected in a soil sample 99/TON/874B F100 ng/g A from trial TON/021-04. The primary data for this sample is presented in Table 49, Appendix 1.

Peak area of sample = 20977

Mean bracketing standard peak area = $\frac{20646 + 20116}{2} = 20381$

Standard concentration = 5 ng/ml

M = 0.5 g/ml

D = 10

Residue found (ng/g) = $\frac{20977}{20381} \times 5 \text{ ng/ml} \times \frac{1}{0.5 \text{ g/ml}} \times 10 = 102.9 \text{ ng/g}$

Recovery of TKC-94 = $\frac{102.9 \text{ ng/g}}{100 \text{ ng/g}} \times 100\% = 103\%$

*The bracketing standard method of quantitation involves quantifying the peak area of the sample against the mean peak area of standards of known magnitude injected prior to and after the sample.