Study Number : 95JH188

Report Title : R173642 and R223068 : Analytical Method and Validation of a Method for the Determination of Residues in Soil.

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SUMMARY

A series of procedural recovery experiments were carried out in which soil samples were fortified at various levels with R173642 and R223068 and analysed. The method uses high performance liquid chromatography with triple quadrupole mass spectrometry (HPLC-MS-MS).

The limit of determination achieved was 0.01 mg kg^{-1} soil for both R173642 and R223068. Fortification levels used ranged from 0.01 - 0.50 mg kg^{-1} and satisfactory recovery levels and relative standard deviation values were obtained for all of the analyses, including those at the required limits of determination. The detector response was linear in the range of 0 to 0.50 µg cm^{-3} for both R173642 and R223068 when analysed by HPLC-MS-MS.

Therefore, it was concluded that the method described in this report (to be issued as SOP RAM 278/01, see Appendix 1) was successfully validated for the analysis of R173642 and R223068 residues in soil using external standardisation with HPLC-MS-MS determination.

1 INTRODUCTION

Tralkoxydim is a ZENECA proprietary herbicide for the selective control of graminaceous weeds in cereal crops. Its major soil metabolites are R173642 (Figure 1) and R223068 (Figure 2).

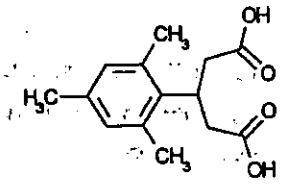

Figure 1 : 3-(2,4,6-trimethylphenyl) pentanedioic acid

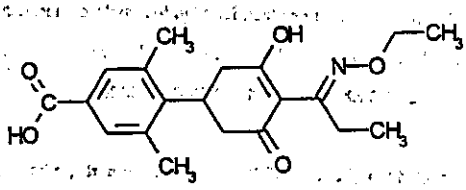

Figure 2 : 4-(-2-(1-(ethoxyimino)propyl)-3-hydroxycyclohex-2-enone-5-yl)-3, 5-dimethylbenzoic acid.

Methodology was developed to determine residues of R173642 and R223068 in soil. This method utilises extraction with sodium hydroxide followed by a liquid-liquid partition clean-up and quantitative determination by high performance liquid chromatography with triple quadrupole mass spectrometry (HPLC-MS-MS).

This report describes the analytical procedures and presents R173642 and R223068 validation data obtained when using the method described for the analysis of soil samples.

2 MATERIALS AND METHODS

2.1 Test Material

R173642 analytical standard ref: ASJ10043-01S (purity 100%) and R223068 analytical standard ref: ASJ10085-01S (purity 99%) were used for the method validation. They were obtained from ZENECA Agrochemicals Product Characterisation Section, Jealott's Hill Research Station, Bracknell, Berkshire, RG42 6ET, UK. R173642 analytical standard is stable at 25°C for 2 years (Ref: ASJ0043/25STAB/1). R223068 analytical standard has been shown to be stable at 25°C for at least 14 months (Ref: ASJ0085/25STAB/1).

Standard solutions in acetone were prepared for R173642 and R223068 and serial dilutions were made and used for sample fortification. Standard solutions in acetone have been shown to be stable for up to 4 months when stored at <7°C

2.2 Test System

The soil samples used for this study were obtained from study 94JH105 originating from Jealott's Hill Research Station, Bracknell, Berkshire, RG42 6ET.

2.3 Analytical Procedures

The analytical procedures were validated by fortifying untreated soil samples with R173642 and R223068. The samples were then subjected to the full analytical procedures (Appendix 1) and were analysed by HPLC-MS-MS.

The results for the analyses were then evaluated to check that the R173642 and R223068 recoveries were within acceptable ranges and that the required limit of determination could be achieved.

The detector linearities of R173642 and R223068 on HPLC-MS-MS were confirmed by measurement of the detector responses for a series of standards using the appropriate HPLC conditions described in Appendix 1. For LC-MS-MS linearity determination it was necessary to prepare standards in soil matrix as different responses are obtained in the presence of matrix.

Summary of samples analysed:

Control	- 4 or 5 replicates
Recovery 0.01 mg kg^{-1}	- 3 replicates; fortified with both R173642 and R223068
Recovery 0.05 mg kg^{-1}	- 3 replicates; fortified with both R173642 and R223068
Recovery 0.10 mg kg^{-1}	- 3 replicates; fortified with both R173642 and R223068
Recovery 0.50 mg kg^{-1}	- 3 replicates; fortified with both R173642 and R223068

2.3.1 Extraction

Soil samples (20 g) were weighed out in extraction vessels and fortified (excluding controls) with known amounts of R173642 and R223088. All samples were extracted in 1M sodium hydroxide. An aliquot of the extract was cleaned up by liquid-liquid partition with diethyl ether, after acidification. The organic phase was evaporated to dryness and redissolved in a known volume of suitable solvent prior to analysis by HPLC-MS-MS. Full details of the procedures and equipment used are given in Appendix 1.

1 SCOPE

The analytical procedures described are suitable for the determination of residues of R173642 (Figure 1) and R223068 (Figure 2) in soil.

To date, in these laboratories, the method has been applied to a variety of soil samples and the limit of determination of the method is 0.01 mg kg^{-1} for R173642 and R223068.

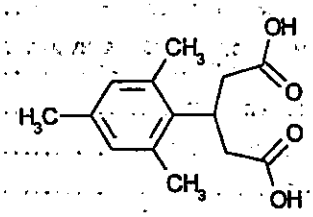

Figure 1 : 3-(2,4,6-trimethylphenyl) pentanedioic acid

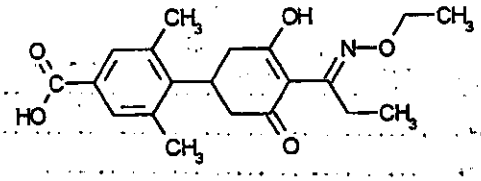

Figure 2 : 4-(2-(1-(ethoxyimino)propyl)-3-hydroxycyclohex-2-enone-5-yl)-3,5 dimethylbenzoic acid.

2 SUMMARY

R173642 and R223068 residues in soil samples are extracted in 1M sodium hydroxide. An aliquot of the extract is subjected to liquid-liquid partition with diethyl ether after acidification. An aliquot of the diethyl ether layer is taken, evaporated to dryness and resuspended in acetonitrile:water (40:60) for quantitative determination of R173642 and R223068 residues using high performance liquid chromatography with triple quadrupole mass spectrometry.

3 PROCEDURE

3.1 Sample preparation

All samples should be stored frozen at <-15°C. Samples should be thawed and then homogenised using a Tecator Homogeniser to produce a representative subsample. Prepared samples should also be stored frozen <-15°C prior to analysis and should only be allowed to thaw for the minimum time before analysis to prevent partition of the endogenous water content.

3.2 Extraction

a) Thoroughly mix the sample and weigh a representative aliquot (20 g), into a screw top Nalgene bottle (250 cm^3).

b) Fortify a minimum of two control samples with an accurately known amount of R173642 and R223068 as external standard recovery samples.

c) Add 1M sodium hydroxide (50 cm^3) and shake for 1 hour (at 130 +/- 20 rpm).

d) Centrifuge for 15 minutes (at 3500 +/- 500 rpm).

e) Transfer an aliquot (25 cm^3) of the supernatant extraction solution to a round-bottomed flask (250 cm^3).

3.3 Liquid-liquid partition

a) While cooling each sample in ice, acidify by a single addition (i.e. all at one time) of 12M hydrochloric acid (3 cm^3), and immediately partition with diethyl ether (25 cm^3). Care should be taken while shaking as the partition generates some pressure. (Release pressure by removing stopper after initial shake).

b) Transfer each sample to a graduated centrifuge tube (50 cm^3), and centrifuge the samples as soon as possible for five minutes (at 3500 +/- 500 rpm).

3.4 **Preparation of R173642 and R223068 Samples for Analysis**

(a) Transfer an aliquot of the diethyl ether phase (equivalent to 4/5 of the total volume of the organic diethyl ether phase) into a round-bottomed flask (50 cm^3) and evaporate to dryness under reduced pressure at a temperature of ≤40°C.

(b) Resuspend the residuum in acetonitrile:water 40:60 (v/v) (2 cm^3) using ultrasonication. The actual sample concentration is dependent on the soil moisture content of the soil and is determined as detailed in Section 5(d).

(c) Transfer the samples to HPLC vials for quantitative analysis by high performance liquid chromatography with triple quadrupole mass spectrometry as described in Section 4.

Standards for HPLC-MS-MS analysis must be prepared in the presence of soil matrix prior to analysis as different responses are achieved in the presence of different matrices and when matrix is absent. Standards are prepared as follows:

a) Transfer an appropriate amount of standard to a disposable tube and evaporate to dryness using a stream of clean, dry air.

b) Resuspend, using ultrasonication, the standard in a suitable volume of a control matrix in acetonitrile:water (40:60 (v/v)) produced in 3.4 (b) to give the required final standard concentration.

c) Analyze standards alongside samples on HPLC-MS-MS.

d) Extra control sample(s) may need to be taken through the method for use in generation of standards in the presence of matrix.

NOTES:

(1) An analytical run should be completed in a day for R223068 if possible, as it is relatively unstable in the presence of acids and acetonitrile. If samples have to be stored, the diethyl ether extract from 3.4 (a) should be taken to dryness and then the flask stored in a freezer until such time as analysis by HPLC-MS-MS can take place.

(2) Before each analytical run an extra standard should be injected onto the HPLC-MS-MS system to ensure that the instrument is functioning correctly and, in particular the sensitivity is satisfactory. It may also be necessary to prime the system with a few standards before initiating the run to ensure that the standard response is reproducible.

(3) Samples and standards should be injected from each vial only once and therefore standards should be placed in individual vials for each injection required throughout the analytical run.

4 **HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH TRIPLE QUADRUPOLE MASS SPECTROMETRY.**

The conditions for analysis by HPLC-MS-MS will depend upon the equipment available. The operating manuals for the instrumentation should always be consulted to ensure safe optimum use.

The following conditions for the analysis of R173642 and R223068 using a Perkin Elmer Binary L 250 pump fitted with a Perkin Elmer Advanced L sample processor ISS200 and a PE-SCIEX API 111 triple quadrupole mass spectrometer are:

(i)	Column	:	Kromasil KR100-5C18 (25 cm X 4.6 mm i.d.)
(ii)	Mobile Phase	:	60% water:40% acetonitrile + 0.4% (v/v) glacial acetic acid.
(iii)	Flow Rate	:	1 cm^3 min^{-1}
(iv)	Injection Volume	:	100 µl

Protonated molecular ions generated in the ion source (R173642, m/z 249 and R223068, m/z 358) are selected and subjected to further fragmentation by collisional activation. The largest ion (R173642, m/z 205 and R223068, m/z 284) in the resulting daughter spectra is then monitored and used for quantitative analysis.

Under these conditions the retention times of R173642 and R223068 were approximately 4 and 20 minutes, respectively.

5 **CALCULATION OF RESIDUE RESULTS**

a) Make repeated injections of 100 µl of a standard solution containing a mixture of R173642 and R223068 into the HPLC-MS-MS operated under conditions described in Section 4. When a consistent response is obtained measure the peak heights/areas obtained for each analyte.

b) Make an injection of each sample solution (100 µl) and measure the peak heights/areas of the peaks corresponding to the analyte.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) To determine the amount of soil equivalent taken in the 25 cm^3 aliquot removed in Section 3.2 e) and hence the final sample concentration, the percentage moisture content (MC) of the soil must first be determined.

Taking into account the water content of the soil, the total effective extraction volume (EV) in Section 3.2 c) is given by:

$$EV = 50 + \frac{(20 \times MC)}{100 + MC} \ cm^3$$

The wet soil weight equivalent (SWE) taken in Section 3.2 e) is then given by:

$$SWE = 20 \times (25/EV) \ g$$

Final sample concentration is given by:

Sample concentration = SWE * 0.4 g cm^3

e) Calculate the wet weight residue in the sample, expressed as mg kg^{-1} by proportionation of the analyte peak heights or area measured for the sample against that for the analytical standard solutions.

$$\text{Residue} = \frac{\text{peak area/height in sample}}{\text{peak area/height in standard}} \times \frac{\text{conc}^n \text{ of analyte in standard}}{\text{conc}^n \text{ of sample solution}}$$

where analyte = R173642 or R223068

Correct the measured residue value for the mean percentage recovery of the fortified control samples.

I.e. for a mean 80% recovery,

$$\text{corrected residue} = \text{measured residue} \times \frac{100}{80}$$

However, if the mean percentage recovery of the fortified control samples is ≥100%, the measured residue value should not be corrected.

NOTE:
In the case where laboratory data systems or computing integrators are used the computer algorithm may adopt a slightly different method for calculation of results. The final calculated results are of course, the same as the above manual calculation.

6 CONTROL AND RECOVERY EXPERIMENTS

At least one untreated sample must be analysed alongside any set of samples, using exactly the same method. This ensures that no unobserved contamination of the samples occurred prior to, or during, the analysis from the matrix, solvent or materials.

At least two control samples, accurately fortified with a suitable known amount of R173642 and R223068 should be analysed alongside every batch of treated samples. Fortification amounts should be based on anticipated residue levels. When no residues are expected, the recoveries should be fortified at low levels, typically 0.01-0.05 mg kg^{-1}. Reagent blanks may also be analysed to ensure that no contamination occurs during analysis due to the solvents or materials used.

7 LIMIT OF DETERMINATION

The limit of determination of the method can be assessed by carrying out recovery experiments at low levels of fortification (0.01 - 0.05 mg kg^{-1}). In these laboratories the limit of determination for R173642 and R223068 in soil has been set at 0.01 mg kg^{-1}.

1 **Apparatus**

a) Equipment for initial sample preparartion: Tecator homogeniser, available from Thompson and Capper Ltd., 9-11 Hardwick Road Astmoor Industrial Estate, Runcorn, Cheshire, UK. or equivalent to the Waring Laboratory Micromizer (Cat. No. FPC 60) Waring Products Division, Dynamic Corporation of America, New Hartford, CT 06057, USA.

b) Nalgene centrifuge bottles (250 cm^3 capacity) for sample extraction; available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, UK or VWR Scientific, P.O. Box 7900, San Fransisco, CA 94120, USA.

c) Flask shaker; available from Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH, UK. or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

d) Centrifuge; available from Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH, UK. or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

e) Graduated Conical Centrifuge Tubes (50 cm^3) available from BDH, Merck Ltd., Merck House, Poole, Dorset, BH15 1TD, UK. or Gallard Schlesinger Industries Inc., 584 Mineola Av. Carle Place, New York 11514-1731, USA.

f) Ultrasonic bath; available from Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH, UK. or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

g) Rotary evaporator with thermostatically controlled waterbath; available from Buchi via Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH, UK. or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

h) Hewlett Packard vials for HPLC analysis; available from Hewlett Packard Ltd., Heathside Park Road, Cheadle Heath, Stockport, Cheshire SK3 0RB, UK. or Hewlett Packard Co., PO Box 1000, Avondale, PA 19311-1000, USA.

i) HPLC column, Kromasil KR100-5C18 (25 cm x 4.6 mm internal diameter) available from Hichrom Ltd., 6 Chiltern Enterprise Centre, Station Road, Theale, Reading, Berkshire RG7 4AA, UK. or Phase Separations Inc., 140 Water Street, Norwalk, CT06854, USA.

j) Perkin Elmer Binary L250 pump fitted with a Perkin Elmer Advanced L sample processor ISS200 available from Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, HP9 1QA, UK.or Perkin Elmer Ltd., 761 Main Avenue, Norwalk CT06859-0001, USA.

k) PE-Sciex API 111 triple quadrupole mass spectrometer; available from Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, HP9 1QA , UK. or SCIEX, Division of MDS Health Group Ltd., Toronto, Canada.

2 **Reagents**

a) Solvents: acetonitrile, and diethyl ether (distilled in glass); available from Rathburn Chemicals Ltd., Walkerburn, Scotland, United Kingdom or B & J Brand Solvents, from Scientific Products Division of Baxter Healthcare Corporation, USA (Tel: 312-689-8410).

b) Analytical grade glacial acetic acid (99%); available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom or Aldrich Chemical Co. Ltd., 940 W St Paul Ave, Milwaukee, Wisconsin 53233, USA.

c) Analytical grade sodium hydroxide (98%); available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom or Aldrich Chemical Co. Ltd., 940 W St Paul Ave, Milwaukee, Wisconsin 53233, USA.

d) Analytical grade hydrochloric acid (35.5-37.5%); available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom or Aldrich Chemical Co. Ltd., 940 W St Paul Ave, Milwaukee, Wisconsin 53233, USA.

f) A sample of R173642 and R223068 of known purity (>95%).

g) Ultra-pure water e.g. as produced by the Millipore Water still

3 **Hazards**

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate safety manual (e.g. ZENECA Laboratory Safety Manual) which contains recommendations and procedures for handling chemicals or a monograph such as 'Hazards in the Chemical Laboratory', Edited by G D Muir, The Chemical Society, London.

a) Solvent Hazards

	Acetonitrile	Diethyl ether
Harmful vapour	Yes	Yes
Highly flammable	Yes	Yes
Harmful by skin absorption	Yes	No
TLV mg/m³	70	400

In all cases avoid breathing vapour. Avoid contact with skin and eyes.

R173642 has a ZENECA Divisional Toxicity class of 3.
R223068 has a ZENECA Divisional Toxicity class of 3.

4 **Preparation of Analytical Standards**

The following handling precautions must be taken when weighing out the analytical standard material:

a) Ensure good ventilation.
b) Wear appropriate protective clothing. Gloves, lab. coat and safety glasses.
c) Avoid inhalation and contact with mouth.
d) Wash contaminated area immediately.

Weigh out accurately using a five figure balance, sufficient R173642 and R223068 solids to allow dilution in acetone to give 1000 µg cm^{-3} stock solutions in volumetric flasks. Make serial dilutions from the stock to give 100, 10 and 1 µg cm^{-3} standard solutions. These solutions of R173642 and R223068 are to be used for fortification of recovery samples.

Also a standard solution in matrix should be prepared in acetonitrile:water (40:60) for HPLC-MS-MS just prior to analysis.

When not in use, always store the standard solutions, securely stoppered, in a refrigerator at ≤8°C to prevent decomposition, concentration of the solvent strength and other forms of standard degradation. Analytical standards should be freshly prepared from the solid material after four months of use.