REPORT TITLE

Validation of an Analytical Method for the Determination of Residues of Metamitron and Desaminometamitron in Soil

DATA REQUIREMENTS

Council Directive 91/414/EEC Annex II (Part A, Section 4.2.2), amended by Commission Directive 96/46/EC, detailed in the EC Guidance document on residue analytical methods SANCO/825/00 rev. 7 (17/03/2004).

US EPA OCSPP 860.1340

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1. INTRODUCTION

Background and Objective:

The objective of the study was to develop and to validate an analytical method for the determination of metamitron and desaminometamitron in soil exemplified by two standard soils, with a limit of quantification (LOQ) of 0.05 mg/kg (50 ppb).

Principle of the Method:

After acidification with acetic acid, a 25-g soil specimen is extracted with 50 mL of methanol using a mechanical shaker. After centrifugation and filtration of the supernatant the filtrate is re-extracted twice with methanol. The combined raw extract is adjusted to a final extract volume of 200 mL using methanol. Finally the raw extract is diluted with water by a factor of 10 and subsequently analyzed by LC/MS/MS using two MRM transitions.

Method Validation:

The analytical method was validated by analyzing for each of the two soil types (LUFA Speyer loamy sand 2.2 and sandy loam 2.3) 2 blank control specimens, 5 replicate specimens fortified at LOQ (0.05 mg/kg per analyte), and 5 replicate specimens fortified at 10xLOQ (0.50 mg/kg per analyte).

2. EXPERIMENTAL

2.1 Test Systems LUFA Speyer Standard Soils

The following European standard soils originating from LUFA Speyer were used:

Standard soil 2.2: Loamy sand with a relatively high organic carbon (≈ 2.3 %) portion. Standard soil 2.3: Sandy loam with a relatively low organic carbon (≈ 1.2 %) portion.

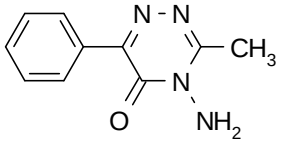
Details on the soil characteristics as provided with the soils are given in Appendix 3. The soils were collected in Jan-2003 at LUFA Speyer.

The soils were stored dry at PTRL Europe, but prior to use in the present study the water content was adjusted to approximately 40 % of their maximum water holding capacity, so that the loamy sand 2.2 contained about 0.19 kg water per kg wet soil weight, and sandy loam 2.3 contained about 0.13 kg water per kg wet soil weight.

2.2 Test / Reference Items

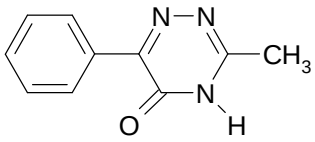
The analytical test and reference items were provided by the Sponsor with the following information (see Appendix 1):

Metamitron



IUPAC Name:	4-amino-4,5-dihydro-3-methyl-6-phenyl-1,2,4-triazin-5-one		
Molecular formula:	$C_{10}H_{10}N_4O$	Molar mass:	202.2 g/mol
Sample number:	FC 1434.2	Purity:	99.4 %

Desaminometamitron



IUPAC Name:	4,5-dihydro-3-methyl-6-phenyl-1,2,4-triazin-5-one		
Empirical formula:	$C_{10}H_9N_3O$	Molar mass:	187.2 g/mol
Sample number:	FC 1952	Purity:	99.7 %

2.3 Equipment and Instrumentation

2.3.1 Equipment for Extraction and Specimen Clean-up

Sartorius analytical balance RC 210 D used for analytical standards.

Scaltec SBC 41 laboratory balance used for specimens.

IKA horizontal shaker HS 250 B.

Hettich Rotixa 50 S centrifuge.

Ultrasonic bath Elma Transsonic 460.

Vortex mixer Assistant Reamix.

PE bottles, 250 mL.

Common laboratory glassware:

All glassware was cleaned in a laboratory dishwasher and air-dried before use.

2.3.2 LC/MS/MS Instrumentation

Agilent 1200 Series HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.

Applied Biosystems MDS Sciex API 4000 triple quadrupole LC/MS/MS system with TurboIonspray ESI source. Analyst 1.4.2 Instrument control and data acquisition software.

Thermo Aquasil ODS C_{18} column (length: 150 mm, i.d.: 3.0 mm, particle size: 3 µm). Pre-column: Phenomenex C_{18} (length: 4 mm, i.d.: 3.0 mm).

2.4 Reagents, Chemicals and Miscellaneous

Solvent/Chemical/Supply	Grade	Supplier
Acetonitrile	HPLC Grade	Promochem
Methanol	HPLC Grade	Promochem
Ampuwa	Bi-distilled water	Fresenius
Glacial acetic acid	100 %	Merck
Formic acid	98 % – 100 %	Riedel de Haën
Fluted cellulose filter	Not applicable	Schleicher & Schuell

Mixtures of 0.1 % formic acid in water or acetonitrile were prepared as components of the RP-HPLC mobile phase.

2.5 Standard Solutions

See Appendix 1 for information provided with the analytical test/reference items used.

2.5.1 Stock Solutions

Stock solutions of the test substances were prepared in methanol at a concentration level of approx. 1.00 mg/mL of each test substance. For calculation of concentrations purity was taken into account.

2.5.2 Fortification Solutions of Analytes

Fortification solutions containing both analytes were prepared in methanol at concentrations (per analyte) of 2.5 µg/mL (LOQ fortification) and 25 µg/mL (10xLOQ fortification).

2.5.3 Calibration Solutions

The stock standard solutions were used to prepare an intermediate solution by volumetric dilution into acetonitrilel/water (1/1 v/v) containing each analyte at a concentration of 5000 ng/mL. The intermediate solution was used for further volumetrical dilutions into acetonitrile/water (1/1 v/v) to obtain calibration solutions with concentrations per analyte ranging from 0.10 ng/mL to 10 ng/mL. These solutions were used for LC/MS/MS calibration.

2.5.4 Storage and Stability of Standard Solutions

All standard solutions were stored refrigerated when not in use. Stability was not tested specifically, however, the solutions are considered stable for several days (at least 10) when kept at < 8 °C, as demonstrated by consistent LC/MS/MS results.

3. ANALYTICAL PROCEDURE

3.1 Fortification of Soil Specimens for Recovery Experiments

25-g soil specimens for recovery control were fortified with 0.50 mL of either the 2.5 µg/mL or the 25 µg/mL fortification solutions, to obtain fortification levels of 0.05 mg/kg (LOQ) or 0.50 mg/kg (10xLOQ) of metamitron and desaminometamitron.

3.2 Extraction and Preparation of Final Extract

1. Weigh 25-g of soil into 250-mL screw-capped PE bottle.
2. Fortify specimen with analytes, if applicable.
3. Acidify specimen using 1.0 mL of concentrated acidic acid.
4. Add 50 mL of methanol.
5. Shake on a horizontal shaker for 30 min (270 rpm).
6. Centrifuge the extract for 2 min (4000 rpm).
7. Filter supernatant through folded filter into a measuring cylinder.
8. Re-extract filtrate twice using 50 mL portions of methanol (for details see point 4. to 7.)
9. Combine raw extract in a measuring cylinder.
10. Adjust raw extract volume to V_{Ex} = 200 mL using methanol.
11. Transfer an aliquot of the raw extract (V_1 = 0.10 mL) into an autosampler vial.
12. Add 0.90 mL of purified water to establish a final volume V_{End} of 1.0 mL.

3.3 LC/MS/MS Determination

3.3.1 RP-HPLC/MS/MS Method

The following RP-HPLC method was used:

HPLC System	Agilent 1200 SL HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.
HPLC Column	Thermo Aquasil C_{18} column (length: 150 mm, i.d.: 3.0 mm, particle size: 3 µm). Pre-column: Phenomenex C_{18} (4 mm, i.d.: 3.0 mm).
HPLC Injection Volume	20 µL.
HPLC Method	Solvent A: 0.1 % formic acid in water. Solvent B: 0.1 % formic acid in acetonitrile. Mobile Phase Composition: Time (min) / Flow rate (mL/min) / % A / % B 0.0 / 0.50 / 85 / 15 1.0 / 0.50 / 85 / 15 1.01 / 0.50 / 35 / 65 6.0 / 0.50 / 35 / 65 6.01 / 0.50 / 0 / 100 8.0 / 0.50 / 0 / 100 8.01 / 0.50 / 85 / 15 11.0 / 0.50 / 85 / 15
Retention Time	Metamitron: approx. 4.7 min Desaminometamitron: approx. 4.4 min.

LC/MS/MS employed electron spray ionization with positive source polarity and used the pseudomolecular ions of the analytes $[M+H]^+$ as parent ions for MS/MS detection (MRM mode). Two daughter ions were monitored per analyte for highly specific MS/MS detection.

The following LC/MS/MS method was used for determination of the analytes:

MS System	Applied Biosystems MDS Sciex API 4000 triple quadrupole LC/MS/MS system with TurboIonspray (ESI) source
Ion Source Conditions ESI Positive Polarity	Source temperature: 550 °C Gas supply GS 1: 40 (arbitrary units) Gas supply GS 2: 70 (arbitrary units) Curtain gas: 20 (arbitrary units) Entrance potential: 10 V IonSpray voltage: 5000 V Declustering potential: 50 V (both analytes)
MS/MS Conditions	*Metamitron:* Transition 203 m/z > 175 m/z CE: 23 V CXP: 14.0 V CAD: 5 Transition 203 m/z > 104 m/z (Confirmation) CE: 31 V CXP: 7.0 V CAD: 5 *Desaminometamitron:* Transition 188 m/z > 160 m/z CE: 24 V CXP: 11.0 V CAD: 5 Transition 188 m/z > 104 m/z (Confirmation) CE: 31 V CXP: 7.0 V CAD: 5 Dwell time per transition: 250 ms

3.3.2 Calibration and Evaluation Procedures

The quantitative determination was carried out by external standardisation using calibration standards ranging from 0.10 ng/mL to 10 ng/mL at 6 different levels.

3.4 Calculation of Results

3.4.1 Calculation

Calculation of results is based on peak area measurements and external calibration lines.

The individual residues in the specimens [in mg/kg] are calculated as shown in the following equation:

$$R = C_{End} \times (V_{Ex} \times V_{End}) / (V_1 \times W) / 1000 \text{ ng/µg}$$

$$= C_{End} \times \text{Multiplier M}$$

Where :

R: Analyte residue in mg/kg or ppm.

C_{End}: Concentration of analyte in final extract, in ng/mL.
(where multiple injections were evaluated: average).

V_{Ex}: Volume of raw extract: 200 mL

V_1: Volume aliquot of raw extract used for preparation of final extract: 0.10 mL

V_{End}: Volume of final extract: 1.0 mL

W: Weight of soil specimen, 25 g.

1000: Divisor used to adjust for dimensions, converts ng to µg.

Recoveries (in %) are calculated as follows:

Rec. = $(R / R_{fortified}) \times 100$ %.

3.4.2 Example for Calculation

The calculation is exemplified for the standard soil specimen 2.2 (P1355-21) fortified at 0.05 mg/kg (LOQ) per analyte. The final extract was examined by LC/MS/MS in run file P1355api#026 to give (using the MRM transition 203 m/z → 175 m/z) a final concentration C_{End} of 0.606 ng/mL (see Table 1 and Figure 7).

The residue R was thus calculated with:

R = $C_{End} \times (V_{Ex} \times V_{End}) / (V_1 \times W) / 1000$ ng/µg
= C_{End} x Multiplier M
= 0.606 ng/mL x (200 mL x 1.0 mL) / (0.10 mL x 25 g) / 1000 ng/µg
= 0.606 x 0.080 mg/kg
= 0.0485 mg/kg

The recovery is calculated with:

Rec. = $(R / R_{fortified}) \times 100$ %
= (0.0485 mg/kg / 0.050 mg/kg) x 100 % = 97 %.

4. RESULTS AND DISCUSSION

The objective of the study was to develop and validate an analytical method for the determination of residues of metamitron and desaminometamitron in/on soil, exemplified by two standard soils. The target limit of quantitation (LOQ) is 0.05 mg/kg per analyte.

After acidification with acetic acid, a 25-g soil specimen is extracted with 50 mL of methanol using a mechanical shaker. After centrifugation and filtration of the supernatant the filtrate is re-extracted twice with methanol. The combined raw extract is adjusted to a final extract volume of 200 mL using methanol. Finally the raw extract is diluted with water by a factor of 10 and subsequently analyzed by LC/MS/MS using two MRM transitions.

Two different soil types (LUFA Speyer loamy sand 2.2 and sandy loam 2.3) were used for method validation. The analytical method was validated successfully by analyzing for each soil type 2 blank control specimens, 5 replicate specimens fortified at LOQ (0.05 mg/kg per analyte), and 5 replicate specimens fortified at 10xLOQ (0.50 mg/kg per analyte).

4.1 LC/MS/MS Selectivity, Specificity and Sensitivity

LC/MS/MS employed electron spray ionization with positive source polarity and used the pseudomolecular ions of the analytes $[M+H]^+$ (203 m/z for metamitron, 188 m/z for desaminometamitron) as parent ions for MS/MS detection (MRM mode). The characteristic daughter ions at 175 m/z and 104 m/z (metamitron) or 160 m/z and 104 m/z (desaminometamitron) are used for highly specific quantitation of the analytes.

LC/MS/MS using the Multi Reaction Monitoring (MRM) mode allows to detect metamitron and desaminometamitron at concentrations of as low as 0.10 ng/mL with 20-µL injections, therefore providing sufficient sensitivity to determine and to confirm residues of the analytes in the final extracts.

The quantitative determination was carried out by external standardization. The LC/MS/MS calibration functions were ranging from 0.10 ng/mL to 10 ng/mL at 6 different levels.

Linear calibration functions were calculated and plotted by regression analysis, using the Analyst 1.4.2 software (Figure 1 and Figure 2).

As demonstrated by the validation results, the method allows determination of metamitron and desaminometamitron with a limit of quantification (LOQ) of 0.05 mg/kg for soil.

No interfering signals in blank control specimens were detected, resulting in a limit of detection (LOD) of 0.01 mg/kg.

4.3 Time required for Analysis

Sets of 12 soil specimens were processed by one person during one work day (8 hours). This was followed by sets of LC/MS/MS injections (approximately 11 minutes per LC/MS run, i.e. approx. 4 instrument hours including an appropriate number of standard injections) and evaluation of results (approx. 1 hour).

Thus a set of 12 specimens requires at total of approx. 1.5 calendar days.

5. CONCLUSIONS

An analytical method for the determination of metamitron and desaminometamitron in soil was developed using methanol extraction, filtration, dilution and LC/MS/MS.

The method was successfully validated for two standard soils, and thus demonstrated to be applicable for enforcement and monitoring purposes.

The use of LC/MS/MS is considered specific so that no additional confirmatory method is required.

The analytical method fulfills the requirements of the Council Directive 91/414/EEC Annex II (Part A, Section 4.2.2), detailed in the EC Guidance document on residue analytical methods (SANCO/825/00 rev. 7 (17/03/2004).