REPORT TITLE

Independent Laboratory Validation (ILV) of an Analytical Method for the Determination of Metamitron in Drinking Water

DATA REQUIREMENTS

EC Guidance document on pesticide residue analytical methods SANCO/825/00 rev. 8.1, 16/11/2010

OECD Principles of Good Laboratory Practice

German GLP Standards "Grundsätze der Guten Laborpraxis (GLP)"

US EPA OCSPP 860.1340 and 850.6100

1. INTRODUCTION

Background and Objective:

The objective of this study was to independently validate an analytical method[2] for the determination of metamitron in water, exemplified for drinking water, and the limit of quantitation (LOQ) of 0.05 µg/L.

2. EXPERIMENTAL

2.1 Test System

2.1.1 Source of Specimens

Drinking water was obtained from the tap at PTRL Europe. The analysis data sheet is depicted in Appendix 2.

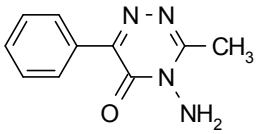
2.1.2 Preparation of Specimens

Aliquots of drinking water were directly used for HPLC-MS/MS analysis. The samples were stored refrigerated until analysis.

2.2 Analytical Test and Reference Item

The analytical standard of metamitron obtained by Sigma-Aldrich Laborchemikalien GmbH (see Appendix 1 for Certificate of Analysis) was used as test / reference item:

Metamitron



IUPAC name:	4-amino-4,5-dihydro-3-methyl-6-phenyl-1,2,4-triazin-5-one		
Empirical formula:	$C_{10}H_{10}N_4O$	Molecular mass:	202.2 g/mol
Batch/Lot no.:	SZBF232XV	Purity:	99.9 %
Expiry date:	20-Aug-2020		

[2] Mende, P. 11-Apr-07. " Validation of an Analytical Method for the Determination of Metamitron and Desaminometamitron in Drinking and Surface Water" eurofins-GAB Study Code 20071119/01-RVW

2.3 Analytical Methods

2.3.1 Apparatus

2.3.1.1 Laboratory Equipment

- Assorted labware (all glassware rinsed with de-ionized water to remove detergents and dried before use), volumetric pipettes and typical laboratory equipment
- Shaker: Vortex mixer REAX top (Heidolph)
- Analytical Balance: Mettler-Toledo XP205DR

2.3.1.2 LC-MS/MS System

Agilent 1290 infinity series LC system (vacuum solvent degasser, binary LC pump, column oven), CTC Analytics HTC PAL Autosampler. Applied Biosystems API5500 Q-Trap LC-MS/MS system with TurboIonspray (ESI) source, Analyst 1.6.2 Instrument control and data acquisition software.

Column:
Phenomenex Synergi Fusion C_{18}, 4 µm particle size, 50 mm length, 2.0 mm i.d.
Pre-column: Phenomenex C_{18}, 4 mm length, 3.0 mm i.d.

2.3.2 Solvents and Chemicals

- Acetic acid (LC-MS grade, Sigma Aldrich)
- Acetonitrile (≥ 99.9 %, Sigma Aldrich)
- Methanol (HPLC grade, Promochem)
- Water (LC-MS grade, Sigma Aldrich)
- Millipore Water (supply at PTRL Europe)

2.3.3 Preparation of Standard Solutions

Stock solutions of metamitron were prepared in methanol as exemplified:

Substance name	Weighed [mg]	Dissolved in [mL]	Obtained [mg/mL]
Metamitron (purity 99.9 %) (*)	10.01	10.0	1.0

(*): Purity taken into account.

Fortification solutions of metamitron with concentrations of 10, 0.50 and 0.050 µg/mL were prepared in methanol* by accurate dilution of the stock solution.

Intermediate calibration solutions of metamitron were prepared by volumetric dilution of the stock solution in methanol/water (1/1, v/v) to obtain concentrations of 10 and 0.10 µg/mL.

* Original method used methanol/water (1/1, v/v).

The intermediate solution with a concentration of 0.10 µg/mL was further diluted in HPLC grade water to obtain calibration solutions with concentrations of 10, 1.0, 0.75, 0.50, 0.25, 0.10, 0.050, 0.025, 0.010 and 0.005 ng/mL metamitron.

For preparation of matrix-matched standards untreated drinking water was dosed with calibration solutions in solvent to obtain concentrations of 1.0, 0.75, 0.50, 0.25, 0.10, 0.050, 0.025, 0.010 and 0.005 ng/mL used for evaluation of the specimens.

All standard solutions were stored refrigerated when not in use.

2.3.4 Stability of Solutions and Extracts

Metamitron was stable when stored refrigerated as stock solution in methanol for at least 11 days (Table 3).

2.3.5 Effects of Matrix on Analyte Response

A significant matrix effect (> 40 % enhancement) on LC-MS/MS responses was observed, thus drinking water samples were evaluated with multi-point calibrations based on calibration standards in matrix (Table 2).

2.3.6 Sample Preparation

From drinking water sample aliquots of 1.0 mL were filled into autosampler vials. For fortification experiments an aliquot of 10 mL of the homogenous drinking water sample was treated with 10 µL of spike solution containing 0.05 µg/mL, respectively 0.5 µg/mL, of metamitron.

2.4 LC-MS/MS Analysis

The drinking water samples were analysed by liquid chromatography with mass spectrometric detection (LC-MS/MS). LC conditions were adapted for present HPLC equipment as follows:

LC Conditions

LC System	Agilent 1290 infinity series LC system (vacuum solvent degasser, binary LC pump, column oven), CTC Analytics HTC PAL Autosampler.
LC Column	Phenomenex Synergi Fusion C_{18}, 4 µm particle size, 50 mm length, 2.0 mm i.d., column oven temperature 40 °C.
LC Injection Volume	50 µL.

LC Method	Solvent A: Water containing 0.05 % of acetic acid Solvent B: Acetonitrile containing 0.05 % of acetic acid Mobile Phase Composition:			
	Time (min)	Flow rate (mL/min)	% A	% B
	0.00	0.50	95	5
	0.50	0.50	95	5
	5.00	0.50	40	60
	5.01	0.50	95	5
	7.00	0.50	95	5
Retention Time	Metamitron: approx. 2.8 min			

MS Conditions

The $[M+H]^+$ ion of metamitron at 203 m/z was used as parent ion for MS/MS detection. The MS/MS transition to the daughter ion at 175 m/z was used for quantification of the analyte. A 2nd MS/MS transition (203 m/z -> 104 m/z) was used for quantitative confirmation.

MS System	Applied Biosystems API5500 Q-Trap LC-MS/MS system with TurboIonspray (ESI) source.	
Ion Source Conditions ESI Positive Polarity	Source temperature: Curtain gas: CAD gas: Entrance potential (EP): IonSpray voltage: Declustering potential (DP): Resolution Q1: Resolution Q3:	400 °C 30 (arbitrary units) medium (arbitrary units) 10 V 5000 V 106 V unit unit
MS/MS Conditions	MS/MS transition for quantification: Collision energy (CE): Cell exit potential (CXP): Dwell time: MS/MS transition for confirmation: Collision energy (CE): Cell exit potential (CXP): Dwell time:	203 m/z → 175 m/z 21 V 16 V 500 ms 203 m/z → 104 m/z 29 V 12 V 500 ms

Figure 1 shows the product ion spectrum of metamitron.

The quantitative determination for drinking water was carried out by external standardization using calibration solutions in matrix Calibration functions ranging from 0.005 to 1.0 ng/mL were used to evaluate the extracts. Exemplary LC-MS/MS calibration diagrams with linear regression equations are shown in Figure 2. Calibration data and a diagram plotting response factors over analyte concentrations are shown in Figure 3.

2.5 Calculations

Results derived from direct injection LC-MS/MS and calculations are shown in detail in Table 1. The following equation was used to calculate the individual residues R in µg/L:

$R = C_{End} \times [V_{End} / V_{Sample}]$

$= C_{End} \times M$

R: Residue in µg/L.

C_{End}: Final concentration of analyte in sample in ng/mL.
(where multiple injections were evaluated: mean).

V_{End} Final volume: 10 mL.

V_{Sample}: 10 mL.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

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

$R_{fortified}$: Residue fortified, in µg/L.

The calculation is exemplified with the drinking water specimen P3863-24 (see Table 1) fortified at 0.05 µg/L (LOQ). The final volume was examined by LC-MS/MS in run file P3863API5500#011 (Figure 6) to give a final concentration C_{End} of 0.0460 ng/mL for 203 m/z -> 175 m/z and of 0.0478 ng/mL for 203 m/z -> 104 m/z. The following calculation is demonstrated for metamitron with the ion transition m/z 203 → 175:

$R = C_{End} \times [V_{End} \times V_{Sample}]$

$= C_{End} \times M$

$= 0.0460$ ng/mL x [10 mL / 10 mL]

$= 0.0460$ ng/mL x 1 = 0.0460 µg/L

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

$= (0.0460$ µg/L / 0.05 µg/L) x 100 % = 92 %

Calculations were performed in excel with full precision, thus small discrepancies may arise when recalculated with a pocket calculator.

3. RESULTS AND DISCUSSION

The objective of this study was to independently validate an analytical method for the determination of metamitron in water, exemplified for drinking water, and the limit of quantitation (LOQ) of 0.05 µg/L.

3.1 Sensitivity, Calibration, Specificity

The highly specific LC-MS/MS method uses two mass transitions for quantitation and quantitative confirmation.

LC-MS/MS allows the detection of the analyte at concentrations as low as 0.005 ng/mL with 50 µL injections, therefore providing sufficient sensitivity to determine and to confirm residues of metamitron in the final volume. Calibration functions obtained from injections of calibration

solutions in matrix containing at least 5 different concentrations ranging from 0.0050 to 1.0 ng/mL (10 % to 200 % of LOQ) were used to evaluate the extracts and the response factor (see Figure 2 and Figure 3).

Linear calibration functions were calculated and plotted by regression analysis.

As demonstrated by the method validation results, the method allows the determination of metamitron with a limit of quantification (LOQ) of 0.05 µg/L. The limit of detection (LOD) of the method was < 0.01 µg/L (< 20 % of the LOQ).

No detectable residues in two blank control specimens were observed.

3.3 Time Required for Analysis

The time required for a set with 12 specimens and the preparation of sufficient calibration standards is approx. 2 hours. LC-MS/MS analyses can be performed unattended overnight followed by evaluation of the LC-MS/MS results (about 3 hours per specimen set). Thus, the time span from the initiation until completion of instrumental analysis including evaluation is about one working day.

3.4 Storage Stability of Solutions and Extracts

Metamitron was stable when stored refrigerated as stock solution (in methanol) for at least 11 days (see Table 3). To demonstrate stability a dilution of a stored and freshly prepared stock solution were injected into LC-MS/MS and the resulting peak areas were compared (see Table 3 for details).

3.6 Minor Modifications

For LC-MS/MS analysis the Eurofins-GAB method was slightly modified as a column with a different inner diameter (2.0 mm instead of 2.1 mm, Phenomenex Synergi Fusion C_{18}, 4 µm particle size, 50 mm length) was used. As mobile phase a binary mixture of water containing 0.05 % of acetic acid and acetonitrile containing 0.05 % of acetic acid was used instead of a ternary mixture of water, acetonitrile and water containing 1 % of acetic acid. Finally a different HPLC system (Agilent 1290 infinity series LC system with vacuum solvent degasser, binary LC pump and column oven, CTC Analytics HTC PAL Autosampler) was used instead of a MS Surveyor pump with autosampler.

Since retention time, sensitivity and selectivity are comparable, these minor modifications have no impact on the results obtained for metamitron in the tested drinking water.

4. CONCLUSION

PTRL Europe performed successfully the independent laboratory validation (ILV) for the determination of residues of metamitron in drinking water by LC-MS/MS, demonstrating the LOQ of 0.05 µg/L and the limit of detection (LOD) of < 0.01 µg/L.

It is concluded that the residue analytical method fulfils the reproducibility requirements as defined in the EC Guidance document on residue analytical methods SANCO/825/00 rev. 8.1, 16/11/2010), the OECD Principles of Good Laboratory Practice as revised in 1997 (ENV/MC/CHEM (98) 17, Paris, France, 1998) and is, therefore, applicable as an enforcement method.