Summary

The purpose of this study was to perform the validation of the method for the determination of pethoxamid air by LC-MS/MS.

This method is referenced as AGR/MOA/PTX-10 at Eurofins Agroscience Services Chem SAS. This method involved the extraction from the sorbent material (TENAX) samples with methanol/ultra-pure water (75/25, v/v). The extract was then diluted in acetonitrile/ultra-pure water (20/80, v/v) prior to quantification by LC-MS/MS.

The limit of quantification (LOQ) is 6 µg/m^3, equivalent to 1.08 µg/tube.

The method was validated in compliance with European guidelines for residue analytical methods SANCO/825/00 rev.8.1 (16/11/2010). A full validation set was performed to demonstrate that the method allows accurate determination of pethoxamid in air matrix.

For method validation, after fortification with the analyte, the following specimens were analysed by LC-MS/MS:

- 5 specimens fortified at LOQ level: 6 µg/m^3 (1.08 µg/tube),
- 5 specimens fortified at 60 µg/m^3 (10.8 µg/tube) (10 × LOQ),
- 2 unfortified specimens,
- 1 reagent blank.

1. Introduction

The purpose of this study was to perform the validation of the method for the determination of pethoxamid air by LC-MS/MS.

The limit of quantification (LOQ) for pethoxamid was 6 µg/m^3, equivalent to 1.08 µg/tube.

2. Analytical method for pethoxamid

2.1. Reference of the method

The method is referenced at Eurofins Agroscience Services Chem SAS under the number AGR/MOA/PTX-10.

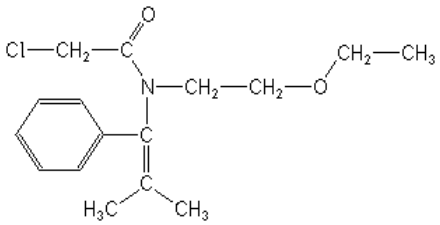
2.2. Principle of method AGR/MOA/PTX-10

This analytical method involved the extraction from the sorbent material (TENAX) samples with methanol/ultra-pure water (75/25, v/v). The extract was then diluted in acetonitrile/ultra-pure water (20/80, v/v) prior to quantification by LC-MS/MS.

The residues of pethoxamid were analysed by LC-MS/MS using two mass transitions.

2.3. Reference item

Common name:	Pethoxamid
Chemical name (CAS):	2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide
CAS-Registry-No.:	[106700-29-2]
Supplier:	Cheminova
Batch:	P1351-BKA-89
Purity:	99.8%
Storage condition:	Temperature set at -20°C
Expiry date:	12 Nov 2014



The certificate of analysis is located in appendix 1. The reference item was stored at a nominal temperature -20°C whereas the certificate of analysis indicates a temperature <-20°C. This was considered to have no impact on the study. The sponsor confirms that <-20°C means frozen state and not deep frozen state.

2.4. Test systems

The validation study was carried out using control air monitoring tubes.
Air samples were standardised to 180 L (air sampling during 6 hours with pump calibrated at 0.5 L/min). Air was monitored at 35°C and with relative humidity greater than 80% (mean value). The air temperature and relative humidity characteristics throughout the experimental phase are detailed in the following table.

Time	Temperature (T°C)	Relative humidity (%)
9h20	34.6	95.2
10h20	35.9	86
11h20	35.2	81.3
13h45	36.2	88.3
15h30	34.1	86.7
Mean	**35.2**	**87.5**

2.5. Detailed description of method AGR/MOA/PTX-10

2.5.1. Preparation and use of the standard solutions

2.5.1.1. Pethoxamid primary stock solution

- Between 2 and 50 mg of pethoxamid were accurately weighed into a brown flask.
- Adequate volume of acetone for pethoxamid was added in order to obtain stock solution at 1000 µg/mL taking into account the purity. This solution is sonicated until total dissolution.

The standard solutions of pethoxamid were stored at a temperature set at 4°C. The standard solution of pethoxamid was proven to be stable for 10 days.

2.5.1.2. Fortification solutions

For fortifications, appropriate dilutions of the pethoxamid primary stock solution were performed in acetone to obtain solution at 54 µg/mL and 540 µg/mL.

These solutions were freshly prepared.

2.5.1.3. Calibration solutions

Appropriate dilutions of pethoxamid stock solution were performed in acetonitrile/ultra-pure water (20/80, v/v) to obtain solution at 0.1 µg/mL and 1.0 µg/mL. Then, appropriate dilutions of these solutions were performed in acetonitrile/ultra-pure water (20/80, v/v) to obtain solutions at:

0.0005 – 0.001 – 0.0025– 0.005 – 0.01 – 0.025 – 0.05 and 0.1 µg/mL

These solutions were freshly prepared.

Preparation of calibration standards

Standards mentioned above were 10-fold diluted in acetonitrile/ultra-pure water (20/80, v/v) as matrix effect was not observed.

The following calibration solutions were prepared for analysis:

0.00005 - 0.0001 – 0.00025 – 0.0005 – 0.001 – 0.0025 – 0.005 and 0.01 µg/mL

These solutions were freshly prepared.

2.5.2. Preparation of reagents

- Methanol/ ultra-pure water (75/25, v/v)

  Methanol (750 mL) and ultra-pure water (250 mL) were mixed.

- Acetonitrile / ultra-pure water (20/80, v/v)

  Acetonitrile (200 mL) and ultra-pure water (800 mL) were mixed.

- Acetonitrile / ultra-pure water (90/10, v/v)

  Acetonitrile (900 mL) and ultra-pure water (100 mL) were mixed.

2.5.3. Analytical supplies and apparatus

2.5.3.1. Apparatus

- HPLC pump (Shimadzu LC20AD (XR))
- HPLC injector (Shimadzu SIL20AC (XR) or CTC Analytics HTC Pal)
- HPLC oven (Shimadzu CTO-20AC)
- LC-MS/MS: API 4000(Sciex)
- HPLC column ACE5 C18, 50 x 3 mm – 5 µm (AIT France, art. ACE -121-0503).
- ORBO™ 402 Tenax® air sampling tubes 100/50mg (Supelco ref. 20832-U)
- Pumps Gilair-3 (Panametrics SA, ref. 810-0202-02) (Panametrics SA, ref. 800518)
- Polypropylene centrifugation tubes
- Precision balance (Mettler)
- Standard laboratory glassware (volumetric flasks, measuring cylinders)
- Ultrasonic bath (Bioblock)
- Ultra-turrax (Fisher Bioblock)
- Various pipettes (Thermo Scientific)

2.5.3.2. Reagents and chemical compounds used

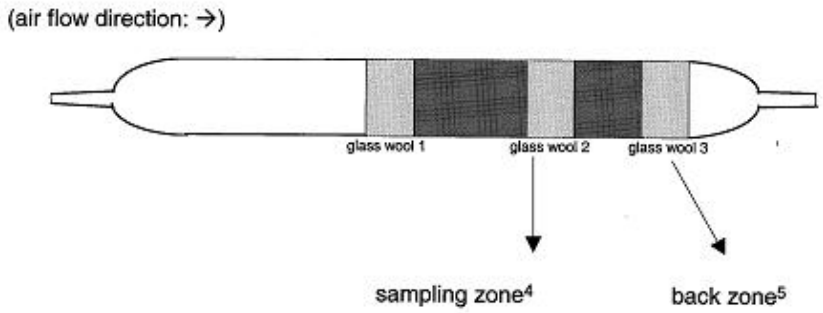
All solvents were HPLC-grade.

- Acetone (Sigma ref 34850-2.5l)
- Acetic acid (VWR, ref. 100063.1000)
- Acetonitrile (VWR, ref. 83640.320)
- Methanol (Sigma, ref. 34860-2.5L-R)
- Ultra-pure water (Eurofins Agroscience Services Chem SAS)

2.5.4. Analytical procedure

2.5.4.1. Preparation of ORBO™ 402 Tenax® air sampling tubes

- The end caps were cut from the ORBO™ 402 Tenax® air sampling tubes, as required.
- A suitable electronic flow meter was used to calibrate an appropriate motorised pump to draw air through the ORBO™ 402 Tenax® air sampling tubes at a rate of 0.5 L/min.



2.5.4.2. Air sampling

Recovery samples were fortified as required with pethoxamid. Fortifications were carried out as described in the table below on glass wool 1.

Fortification level (µg/m^3)	Fortification level (µg/tube)	Volume to use (µL)	Solution to use (µg/mL)
6	**1.08**	20	54
60	**10.8**	20	540

- The ORBO™ 402 Tenax® air sampling tubes was connected to the pre-calibrated motorised pump using plastic tubing. The tube was connected with the end containing the lower adsorbent layer attached to the pump via the plastic tubing.
- The pump was set to run for up to 6 hours at a rate of 0.5 L/min.
- After the sampling period, the pump was turned off and the tube was disconnected.

2.5.4.3. Desorption of pethoxamid from ORBO™ 402 Tenax® air sampling tubes (upper adsorbent layer)

- The glass fibre filter was removed and transferred into a 50 mL polypropylene centrifuge tube. Carefully the glass wool 1 and 2 filter and the upper 402 Tenax® air sampling tubes adsorbent layer were transferred into a 50 mL tube.
- Methanol/ultra-pure water (75/25, v/v) (20 mL) was added to the tube containing the ORBO™ 402 Tenax® air sampling tubes adsorbent and the glass wool 1 and 2 filter. The tube was capped and contents were ultra-sonicated for 30 minutes to desorb the pethoxamid from the adsorbent and glass fibre filter.
- The sample was vortexed for 1 minute.

- The contents of the tube were allowed to settle.
- The supernatant (500 µL) was transferred into a polypropylene flask (15 mL) and 9.5 mL of acetonitrile/ultra-pure water (20/80, v/v) was added.
- An aliquot (100 µL) was transferred into a suitable autosampler vial containing 900 µL of acetonitrile/ultra-pure water (20/80, v/v).
- Final determination was achieved by LC-MS/MS.

2.5.4.4. Desorption of pethoxamid from ORBO™ 402 Tenax® air sampling tubes (lower adsorbent layer)

- The glass wool 3 filter and the lower 402 Tenax® air sampling tubes adsorbent layer was carefully transferred into a 50 mL tube containing the glass fibre filter.
- Methanol/ultra-pure water (75/25, v/v) (20 mL) was added to the tube containing the ORBO™ 402 Tenax® air sampling tubes adsorbent and the glass wool 3 filter. The tube was capped and contents were ultra-sonicated for 30 minutes to desorb the pethoxamid from the adsorbent and glass fibre filter.
- The sample was vortexed for 1 minute.
- The contents of the tube were allowed to settle.
- The supernatant (500 µL) was transferred into a polypropylene flask (15 mL) and 9.5 mL of acetonitrile/ultra-pure water (20/80, v/v) was added.
- An aliquot (100 µL) was transferred into a suitable autosampler vial containing 900 µL of acetonitrile/ultra-pure water (20/80, v/v).
- Final determination was achieved by LC-MS/MS.

Parameters for chromatographic analysis

2.5.4.5. Operating conditions

The following parameters were used during the study.

- Pump + Autosampler: LC20AD, Shimadzu + HTC Pal, CTC Analytics
- Oven: CTO-20AC, Shimadzu
- Detector: API 4000 (Sciex)
- Data Acquisition: Analyst 1.4.2, Sciex
- Column HPLC: ACE5 C18, 50x3 mm - 5 µm
- Column temperature: 40 °C
- Retention time: approximately 1.9 minutes for pethoxamid
- Injection volume: 10 µL
- Flow: 0.8 mL/minute
- Mobile phase: Solvent A: Acetonitrile
  Solvent B: 0.2 % acetic acid in ultra-pure water
- Gradient:

Time (minute)	% A	% B
0.0	50	50
2.0	50	50
2.1	80	20
3.1	80	20
3.2	50	50
5.2	50	50

- Switch

Time (minute)	Position
0.0	waste
1.0	mass spectrometer

- Ionisation mode: ESI^+
- Scan Type: MRM

Analyte	Parent ion (m/z)	Daughter ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	Dwell (ms)
Pethoxamid	296.2	131.2 (primary)	45	10	9	29	500
		250.2 (confirmatory)	45	10	18	18	500

DP : declustering potential CE : collision energy CXP : collision cell exit potential EP: entrance potential

CAD (collision gas)	4	**TEM (°C)**	550
CUR (curtain gas)	30	**RESOLUTION Q1**	unit
GS1 (ion source gas 1)	50	**RESOLUTION Q3**	unit
GS1 (ion source gas 2)	60		
IS (ion spray voltage)	5500		

Integration and calibration parameters

Response	Area	**Type of regression**	Linear
Type of response	External	**Weighting**	1 / X
Unit	µg/mL	**Intercept**	No

2.5.4.6. Calibration

A calibration curve was injected prior to analysis. Besides, at least one quality control was injected every four injections to check the absence of signal deviation.

The determination coefficient R^2 was found to be ≥ 0.990.

Typical calibration curves and chromatograms for LC-MS/MS are presented in Appendix 2.

2.5.4.7. Result calculation

The chromatographic system was calibrated using a calibration curve of pethoxamid standards.

A linear calibration curve was calculated using the method of least squares (1/x weighting):

$$Y = A \times C + B$$

Y = detector response (as peak area)

A = slope of the linear least squares fit of the calibration curve

C = Concentration determined from standard curve (µg/mL)

B = Y-intercept of the linear least squares fit of the calibration curve

The concentration determined from standard curve is $C = \frac{(Y - B)}{A}$

The residue of pethoxamid in each test sample is calculated as follows:

$$\text{Residue}\ (\mu g / tube) = \frac{V_1 \times V_4 \times V_f}{V_2 \times V_3} \times extract\ concentration\ (\mu g / mL) \times dilution$$

Where:

V_1 (mL)	=	total extraction volume (20 mL)
V_f (mL)	=	final volume (1 mL)
V_2 (mL)	=	aliquot 1 volume (0.5 mL)
V_3 (mL)	=	aliquot 2 volume (0.1 mL)
V_4 (mL)	=	intermediary volume (10 mL)
Extract concentration	=	calculated concentration in final extract
Dilution	=	to be considered if dilution of final extract were made before analysis

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}\ (\%) = \frac{A}{S} \times 100$$

Where:

A = concentration of pethoxamid found in spiked sample (µg/tube).

S = concentration of pethoxamid added in spiked sample (µg/tube).