REPORT AMENDMENT
Number 1

Chlormequat chloride:
Validation of a Residue Analytical Method for the Determination of Chlormequat Chloride in Drinking and Surface Water

21-Sep-2015 the legal entity Harlan Laboratories Ltd. was renamed as Envigo CRS (Switzerland) Limited. No other changes to the legal entity have occurred.

GENERAL INFORMATION

Reason for Amendment

On sponsor's request, the following details were added to the final report of study D96114:

- A schematic representation of the analytical method.
- A statement that the calibration curve covers the range between 30% of the LOQ to 20% above the highest fortification level, as required in Guideline SANCO/825/00 rev. 8.1, November 16, 2010
- The time required for the analysis (i.e. chromatographic run time)

Distribution List

The original report amendment will be retained in the study file. Copies of the amendment will be distributed as follows:

AMENDMENTS DETAILS

Parts of Report to be Altered

Page Number: 7

Concerning Section: **Summary**

Previous Wording: Quantitation was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) monitoring 2 ion transitions to satisfy the confirmatory analysis requirement.

Additional Wording: Quantitation was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) monitoring 2 ion transitions to satisfy the confirmatory analysis requirement.

The total chromatographic run time of the method was four minutes.

Purpose of Change:

The time to complete a sample set was stated to fulfil guideline requirements

Page Number: 15

Concerning Section: **4.1.4 Preparation of Calibration Solutions**

Previous Wording: The intermediate calibration solutions were further diluted using control drinking and surface water to obtain matrix matched standard solutions in the range of 1.5 ng/mL to 0.03 ng/mL. These solutions were freshly prepared before use.

Additional Wording: The intermediate calibration solutions were further diluted using control drinking and surface water to obtain matrix matched standard solutions in the range of 1.5 ng/mL to 0.03 ng/mL. These solutions were freshly prepared before use.

The concentration range of 1.5 ng/mL to 0.03 ng/mL covered the range required by the guideline (12*LOQ to 0.3*LOQ) for a LOQ of 0.1 µg/L.

Purpose of Change:

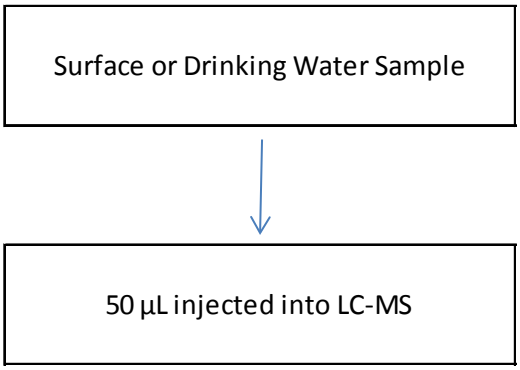
Clarification was needed that the calibration curve covered the range required by the guideline.

Page Number: 40

Concerning Section: **Figures**

Previous Wording: Not present

Additional Wording: Figure 12 Schematic Representation of the Sample Preparation



Purpose of Change:

A schematic representation was added as required by the guideline.

REPORT

Chlormequat chloride:
Validation of a Residue Analytical Method for the Determination of Chlormequat chloride in Drinking and Surface Water

GENERAL INFORMATION

Schedule

Experimental Starting Date:	23 September 2014
Experimental Completion Date:	09 October 2014

Additional Responsibilities

Study Monitor:	Dr. Mirana Verschuere Taminco BVBA

Archiving

Unless instructed otherwise by the Sponsor, the study plan, all raw data (paper and electronic) a sample of the test item and the final report will be retained in the Harlan Laboratories Ltd. archives (4414 Füllinsdorf, Switzerland) for at least ten years after which instructions will be sought as to further retention or disposal. Further retention or return of the data will be chargeable to the Sponsor.

No data will be discarded without contacting the Sponsor to obtain their written consent.

1 INTRODUCTION AND PURPOSE

The purpose of the study was to validate a residue analytical method in compliance with international guidelines (SANCO) and internal Harlan Laboratories Ltd. SOPs (standard operating procedure).

For Chlormequat chloride in drinking and surface water a limit of quantification (LOQ) of 0.1 µg/L and a working range from 0.1 µg/L to 1.0 µg/L was successfully validated.

1.1 Guidelines / Regulations

These study procedures indicated in this report meet or exceed the requirements of the following guidelines:

- "European Commission, Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8.1, November 16, 2010"

2 TEST AND REFERENCE ITEM

2.1 Test Item

The test item was supplied by the Sponsor and was also used as reference item (analytical standard). All information about the test item was provided by the Sponsor (see Appendix for CoA) or based on the e-Pesticide Manual (Thirteenth Edition, Version 3.1).

Identification:	Chlormequat chloride pestanal
CAS No.:	999-81-5
Formula:	$C_5H_{13}Cl_2N$
Molar Weight:	158.1 g/mol
Batch:	SZBD273XV
Purity:	97.2%
Physical State/Appearance:	Beige crystalline
Expiry date:	30 September 2018
Storage Conditions (as provided by the Supplier):	Room temperature
Storage Conditions (as handled by Harlan Laboratories Ltd.):	20 ± 5 °C

3 MATERIALS AND METHODS

Details of the materials and methods that are not specified in the subsequent sections of the present report are described in the appropriate standard operating procedures.

3.1.1 Definitions and Abbreviations

LC	Liquid Chromatography
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification

3.2 Test System

The test systems were selected by the Sponsor according to the guidelines.

3.2.1 Drinking Water

Source:	Harlan Laboratories canteen, 4452 Itingen / Switzerland
Blank Matrix:	Drinking water (freshly sampled tap water)
Storage Location:	Harlan Laboratories Ltd., 4452 Itingen / Switzerland
pH-Value:	6.87
Dissolved organic carbon (DOC):	0.86 mg C/L
Total hardness:	38.4 °fr.H

3.2.2 Surface Water

Source:	Ergolz, 4415 Lausen / Switzerland
Blank Matrix:	Surface water (river)
Storage Location:	Harlan Laboratories Ltd., 4452 Itingen / Switzerland
Storage temperature:	5 ± 3 °C
pH-Value:	8.23
Total organic carbon (TOC):	56.37 g/L
Total hardness:	39.6 °fr.H
Evaporation residue:	0.56 g/L
Filtration residue:	0.6 mg

3.3 Reagents

ELGA water:	Harlan Laboratories Ltd.
Acetonitrile:	J.T. Baker no. 9017
Formic acid:	Merck, 1-00264
Heptafluorobutyric acid (HFBA):	Fluka, 52411
Methanol:	J.T. Baker no. 8402

3.4 Equipment

Balance:	UMT-2 AX 205 DR	Mettler Toledo Mettler Toledo
Laboratory Material:	HPLC vials (2 mL, amber) Volumetric flasks	BGB Various
Pipettes:	Micro pipette	Eppendorf
Refrigerator:	5 ± 3 °C	Liebherr
Ultrasonic Bath:	RK 100	Bandelin Sonorex
Vortex:	Genie 2	Bender & Hobein AG

4 ANALYTICAL METHOD

Concentrations of Chlormequat chloride were determined by liquid chromatography (LC) coupled with tandem mass spectrometric detection (MS/MS). The method was developed at Harlan Laboratories Ltd. under non-GLP conditions.

4.1 Solutions for Fortification and Calibration

4.1.1 Preparation of Stock Solutions

Stock Solution A (1000 µg/mL):

An amount of 11.30 mg of Chlormequat chloride (see Section 2.1) was dissolved in methanol (10.984 mL) using an ultrasonic bath for about 5 minutes.

Stock Solution B (1000 µg/mL):

An amount of 10.79 mg of Chlormequat chloride (see Section 2.1) was dissolved in methanol (10.488 mL) using an ultrasonic bath for about 5 minutes.

The stock solutions were stored refrigerated (5 ± 3 °C) until completion of the analyses.

4.1.2 Preparation of Fortification Solutions

A defined volume of the stock solution A was diluted with methanol / water; 100 mM HFBA (50/50, v/v) to obtain a fortification solution with a concentration of 10 µg/mL (solution 1F). The latter solution was further diluted as described in the table below.

Fortification solution	Aliquot [µL]	Aliquot taken from	Final volume [mL]	Solvent	Final concentration [µg/mL]
1F	100	Stock solution A	10	Methanol / water /; 100 mM HFBA (50/50, v/v)	10
2F	100	Solution 1F	10		0.1
3F	1000	Solution 2F	10		0.01
4F	1000	Solution 3F	10		0.001

These solutions were kept in the refrigerator (5 ± 3 °C) until completion of the analyses.

4.1.3 Preparation of the Intermediate Solutions

A defined volume of the stock solution B was successively diluted with methanol / water; 100 mM HFBA (50/50, v/v) to obtain three intermediate solutions with concentrations of 10, 0.1 and 0.01 µg/mL. These solutions were kept in the refrigerator (5 ± 3 °C) until completion of the analyses.

4.1.4 Preparation of Calibration Solutions

Defined volumes of the intermediate solutions were diluted with methanol / water; 100 mM HFBA (50/50, v/v) to obtain additional intermediate calibration solutions in the range of 15 ng/mL to 0.3 ng/mL. These solutions were kept in the refrigerator (5 ± 3 °C) until completion of the analyses.

The intermediate calibration solutions were further diluted using control drinking and surface water to obtain matrix matched standard solutions in the range of 1.5 ng/mL to 0.03 ng/mL. These solutions were freshly prepared before use.

4.2 Fortification

Untreated drinking and surface water samples were fortified with defined volumes of the fortification solutions (see Section 4.1.2) prior to the extraction step and carried through the procedure as described in Section 4.3.

The following fortification levels were prepared:

Aliquot [µL]	Aliquot taken from fortification solution	Amount of untreated sample [mL]	Fortification level [µg/L]
1000	3F	9	1.0
1000	4F	9	0.1

4.3 Sample Work up

- 5 mL untreated drinking or surface water were pipetted into a 10 mL volumetric flask
- the fortification samples were spiked accordingly to the spike system
- the volumetric flask was filled up with untreated drinking or surface water to the mark
- the sample was mixed properly
- an aliquot was transferred into an amber HPLC-vial

Final solutions were measured by LC/MS/MS.

4.3.1 LC/MS/MS Conditions

Instrumentation

MS Detector: API 5000, MDS Sciex, Toronto/Canada
Software: *ANALYST*, version 1.4.1

LC Pumps: High pressure gradient system consisting of two Shimadzu LC-10AD pumps and a Shimadzu SCL System Controller
LC Injector: CTC PAL

Sample Injection

Wash Solvent: 1: water / methanol / formic acid (80+20+0.5, v/v/v)
2: water / acetonitrile / methanol (10+45+45, v/v/v)

Washing Procedure: 2 x syringe and 2 x injection port with each solvent

Injection Volume: 50 µL

Chromatographic Separation

Analytical Column: Inertsil ODS-3 (GL Sciences) [2.1 mm x 50 mm; 3.0 µm]

Mobile Phases A: water / methanol; 10 mM HFBA (95+5, v/v)
B: water / methanol; 10 mM HFBA (5+95, v/v)

Gradient Program:

Time [min]	**0**	**0.5**	**2.0**	**2.1**	**4.0**
A [%]	100	0	0	100	100
B [%]	0	100	100	0	0
Flow [µL/min]	300	300	300	300	300

Detection

Ionization: Pneumatically and thermally assisted electro spray ionization (ESI)
Source: Sciex Turbo-V-Source

Spray Voltage: 4500 V
Heater Gas Temperature: 550 °C
Gases: Nebulizer (air), heater (air), curtain (N_2), collision (N_2)

Scan Mode: Multiple reaction monitoring (MRM)

Analyte	Ion Polarity	*m/z* → *m/z*	Dwell time [ms]	CE [eV]
Chlormequat chloride (^{37} Cl)	$[M+H]^+$	124.1 → 58.0	300	40
Chlormequat chloride (^{35} Cl)	$[M+H]^+$	122.1 → 58.0	300	40

Resolution Q1: Unit resolution
Resolution Q3: Unit resolution

4.4 Data Acquisition, Calculation and Quantification

Acquisition and peak calculations were performed with the software ANALYST, version 1.4.1. Quantification of the analyte was performed using the regression model:

y = b * x + a, weighting 1/y (1)

y = Area [counts]
x = Final concentration of analyte in extract [ng/mL]
a = Intercept
b = Slope

4.4.1 Calculation

The results are calculated by external calibration using peak areas.

Individual residue levels in the specimen are calculated as shown in the following equation 2:

$$R = \frac{x \cdot V_F}{V_{sample}} \quad (2)$$

R = Recovered residue of analyte [µg/L]
x = Final concentration of analyte in extract [ng/mL] (calculated from equation 1)
V_F = Final sample volume [mL]
V_{sample} = Volume of sample [mL]

Recoveries are calculated as shown in the following equation 3:

$$Rec = \frac{R}{F} \cdot 100\% \qquad (3)$$

Rec = Recovery [%]
R = Residue of analyte [µg/L]
F = Fortification level [µg/L]

Note: The tabulated values represent rounded-off results obtained by calculations based on the exact data.

4.4.2 Example of Calculation

The calculation is exemplified with internal sample ID 15 (Chlormequat chloride in surface water, primary mass transition, see Table 3). Numerical data in the tables represent rounded-off results obtained by calculations based on the exact data. Therefore, manual recalculation may slightly differ in values.

For example, the correlation of the calibration row (Chlormequat chloride) of the recovery analysis of 23 September 2014 is calculated to be:

$$y = 498.25 + 160776 \cdot x \quad \text{or} \quad x = \frac{y - 498.25}{160776} \qquad (4)$$

For a peak area of y = 19480 (counts) for the internal sample ID 15 the correlation of Chlormequat chloride in sample solution is calculated to be 0.118 ng/mL.

The residue of Chlormequat chloride in the sample is calculated according to equation 2 using:

R = Recovered residue of analyte [µg/L]
x = Final concentration in sample solution (here: 0.118 ng/mL)
V_F = Final sample volume (here: 10 mL)
V_{sample} = Volume of sample (here: 10 mL)

$$R = \frac{0.118 \cdot 10}{10}$$

The residue of Chlormequat chloride in the sample is calculated to be 0.118 µg/L.

The recovery of Chlormequat chloride in the sample is calculated according to equation 3 using:

Rec = Recovery [%]
R = Calculated residue of Chlormequat chloride in the sample of 0.118 µg/L
F = Fortification level (here: 0.1 µg/L)

$$Rec = \frac{0.118}{0.1} \cdot 100\%$$

The recovery of Chlormequat chloride in this sample of drinking water is calculated to be 118%.

5 RESULTS AND DISCUSSION

5.1 Limit of Quantification (LOQ)

The limit of quantification is defined as the lowest fortification level with mean recoveries ranging from 70% to 120% at a relative standard deviation (RSD) of ≤20%. These criteria were fulfilled for Chlormequat chloride in drinking and surface water with a LOQ of 0.1 µg/L.

5.2 Limit of Detection (LOD)

The limit of detection was found to be 0.03 µg/L for Chlormequat chloride in drinking and surface water. The LOD was estimated from the lowest calibration standard concentration (0.03 ng/mL) by calculating with equation 2.

5.4 Linearity

For the LC/MS/MS analysis of Chlormequat chloride the correlation was performed using a least square fit of a linear function. The calibration was found to be linear in the concentration range of 0.03 ng/mL to 1.5 ng/mL

5.5 Specificity

The retention times of Chlormequat chloride signals in the specimen extracts match the retention time of the standard solution. No interferences above 30% of the LOQ at the retention time of Chlormequat chloride were detected in the untreated control samples. Thus, the acceptance criteria (according to the guidelines) are fulfilled for specificity.

5.6 Confirmation

Monitoring of a second mass transition (m/z 122.1 → m/z 58.0) demonstrates that the method is highly specific for the determination of Chlormequat chloride in drinking and surface water.

5.7 Analytical Sample Storage Period

5.7.1 Sample Extract Stability

The maximum time between starting the extraction and measurement was 8 days for drinking water and 6 days for surface water. Samples were stored in the refrigerator (5 ± 3 °C) before LC/MS/MS measurement.

5.7.2 Intermediate Calibration Solution Stability

The stability of intermediate calibration solutions was proven for 7 days when stored in the refrigerator (5 ± 3 °C). Comparison of peak areas of calibration solutions prepared from

intermediate calibration solutions stored for 7 days and intermediate calibration solutions/calibration solutions from a freshly prepared stock solution showed no significant differences (mean deviation ≤10%).

5.8 Matrix Effect

The final sample solution of an untreated control sample was fortified with Chlormequat chloride and analyzed by LC/MS/MS. The area counts were compared to an equivalent solution prepared without matrix.

No significant matrix influence (± 20%) for the determination of Chlormequat chloride in drinking and surface water (besides of 10x LOQ for the primary mass transition) using this LC/MS/MS method was observed However, matrix matched standard solutions were used for analysis.

Appendix 2 Study Plan

STUDY PLAN

Chlormequat chloride:
Validation of a Residue Analytical Method for the Determination of Chlormequat chloride in Drinking and Surface Water

1 INTRODUCTION AND PURPOSE

The purpose of the study will be to validate a residue analytical method in compliance with international guidelines (SANCO) and internal Harlan Laboratories Ltd. SOPs (standard operating procedure).

For Chlormequat chloride in Drinking and Surface Water a limit of quantification (LOQ) of 0.1 µg/L and a working range from 0.1 µg/L to 1.0 µg/L will be validated.

1.1 Guidelines / Regulations

This test methods described are designed to be compatible with the procedures indicated by the following internationally accepted guidelines and recommendations:

- "European Commission, Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8.1, November 16, 2010"

2 TEST AND REFERENCE ITEMS

2.1 Test Item

The test item was supplied by Sigma-Aldrich and will be also used as reference item (analytical standard). All information about the test item were provided by the Supplier or base on the e-Pesticide Manual (Thirteenth Edition, Version 3.1).

Information as provided by the Supplier.

Identification:	Chlormequate chloride pestanal
Chemical name:	2-chloro-N,N,N-trimethylethanaminium chloride
CAS No.:	999-81-5
Formula:	$C_5H_{13}Cl_2N$
Structure:	
Molar Weight:	158.1 g/mol
Batch:	SZBD273XV
Purity:	97.2%
Expiry Date:	30 September, 2018
Storage Conditions:	Room temperature
Safety Precautions:	Routine hygienic procedures (gloves, goggles, face mask).

3 MATERIALS AND METHODS

3.1 Definitions and Abbreviations

LC	Liquid Chromatography
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification

3.2 Test System

The Sponsor selected Drinking and Surface Water to be used as test system. Untreated Drinking and Surface Water will be used for the preparation of LOQ and 10xLOQ samples. The supplier of the test system and the blank Drinking and Surface Water will be mentioned in the raw data and the final report.

3.3 Analytical Method

The analytical method will be developed prior to its validation and will be described in detail in the final report.
The development of the method will not be performed according to the regulations of GLP and will be therefore excluded from the statement of compliance
However, the raw data or copies of the raw data will be archived under the study number of this study. Validation results will be reported only.
All procedures for sample preparation, analysis, analytical equipment and reagents will be documented in the raw data and in the final report.

3.4 Experimental Design

3.4.1 Calibration Procedure

The instrument calibration standards for this study will be prepared by making appropriate dilutions of the stock standard solution. The standard solution should bracket the working range. The analytical calibration should extend over a range appropriate to the lowest and highest nominal concentration of the analyte, at least 30% of the LOQ as well as 20% above the highest level.

Calibration solutions will be injected concurrently with sample injections for the determination of the retention time of Chlormequat chloride and the calculation of the calibration curves. Injections of calibration solutions will be distributed evenly over the analytical sequence. Each analytical run will begin and end with a standard injection. A maximum of three specimen injections will be made between standard injections. For single injections at least 5 different concentrations of standard solutions will be used for preparing the calibration curve.

Quantification will be performed using LC/MS/MS. Details will be given in the raw data and in the final report. Peak identity (specificity) will be described in the raw data and in the final report.

3.4.2 Linearity

The calibration curve will be constructed from the results of a minimum of 5 levels for single injection over the target concentration. The correlation will be performed using a least squares fit of a linear or non-linear function. The correlation coefficient (r) obtained should be $\geq$ 0.99 and will be given in the final report.

3.4.3 Fortification

The following procedure will be carried out:

Known amounts of fortification solutions of Chlormequat chloride will be added to untreated blank Drinking and Surface Water samples to achieve the following fortification levels:

0	µg/L	control
0.1	µg/L	LOQ
1.0	µg/L	10xLOQ

Two control specimens, five specimens fortified at LOQ (0.1 µg/L) and five specimens fortified at 10xLOQ (1.0 µg/L) will be analyzed for each matrix.

3.4.4 Recovery

The percent recovery of Chlormequat chloride will be determined as follows:

$$Recovery\ [\%] = \frac{found\ [\mu g/L]}{fortification\ amount\ [\mu g/L]}\ 100\%$$

Details will be given in the raw data and in the final report.

3.4.5 Specificity

The method must allow the determination of Chlormequat chloride in Drinking and Surface Water. There should be no interference with other substances observed at the retention times of Chlormequat chloride above 30% of the limit of quantification.
Details will be given in the raw data and in the final report.

3.4.6 Accuracy – Precision – Repeatability

The mean recoveries, range of recoveries and relative standard deviations / coefficients of variation will be given. The mean recovery should be within 70 and 120% of the nominal concentration (accuracy). Its relative standard deviation should be $\leq$ 20% (repeatability, precision). Details will be given in the raw data and in the final report.

3.4.7 Limit of Quantification

The lowest fortification level that will be validated within this study represents the limit of quantification (LOQ) of this analytical method. Here the limit of quantification will be 0.1 µg/L in Drinking and Surface Water.
Details will be given in the raw data and in the final report.

3.4.8 Limit of Detection

The limit of detection of this analytical method for Chlormequat chloride in Drinking and Surface Water represents the residue calculated from the lowest calibration concentration used.
Details will be given in the raw data and in the final report.

3.4.9 Peak Confirmation

For peak confirmation a second ion transition will be validated.
Details will be given in the raw data and in the final report.

3.4.10 Ion Selection

A justification for the ion selection and a representative mass spectrum of the product ion(s) will be presented in the final report.

3.4.11 Matrix Effect

Possible matrix effects will be tested and addressed.

3.4.12 Stability

Stability of fortified sample extracts and of standard solutions will be tested. Details will be given in the raw data and in the final report.