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JAI RESEARCH FOUNDATION

Original Final Report 2 of 2

STUDY TITLE

INDEPENDENT LABORATORY VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF MANCOZEB IN SURFACE AND DRINKING WATER BY LC-MS/MS ANALYSIS

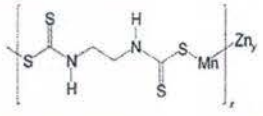
GUIDELINES AND DATA REQUIREMENT

OCSPP 860.1340, SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1;
Commission Regulation (EU) No 283/2013 setting out the data requirements for active substances, in accordance with Regulation (EC) NO 1107/2009

2. EXPERIMENTAL PROCEDURE

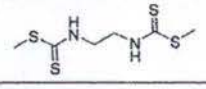
2.1 Test Item

Reference Standard of Mancozeb was used as the test item.

Test Item	Mancozeb
IUPAC Name	Manganese ethylenebis (dithiocarbamate) (polymeric) complex with zinc salt
CAS N°	8018-01-7
Molecular Weight	266.51 g/mol
Molecular Structure	
Batch N°	SZBE225XV
Manufacturing Date	August 13, 2014
Expiry Date	August 13, 2019
Purity	97.5 % (Refer Certificate of Analysis in APPENDIX 3)
Source	Sigma-Aldrich
Storage Condition	Refrigerator
JRF Entry Number	PC-3616

Source of IUPAC Name, C.A. Name and Molecular Structure: e-Pesticide Manual, 15th Edition, 2010.

Reference Standard of Dimethyl-EBDC

Reference Standard	Dimethyl-EBDC
IUPAC Name	Dimethyl Ethylenebisdithiocarbamate
CAS N°	20721-48-6
Molecular Formula	$(CH_2)_2(CH_3)_2(CS_2)_2(NH)_2$
Molecular Weight	240.42 g/mol
Molecular Structure	
Batch N°	JRF-001-058
Re-test Date	May 27, 2018 (Refer Certificate of Analysis in APPENDIX 4)
Purity	97.705 % (Refer Certificate of Analysis in APPENDIX 5)
Source	JRF
Storage Condition	Refrigerator
JRF Entry Number	PC-3854

Source of CAS N°, Molecular Structure and Molecular weight: Certificate of Analysis

2.2 Equipments/Instruments

Sr. N°	Instrument/Apparatus	Model	Make / Supplier
1	Mass Spectrometer	Q Trap 6500	AB Sciex
		LC-MS 8050	Shimadzu
2	HPLC	Nexera X2	Shimadzu
3	Analytical Balance	GR 202	Adair Dutt
4	Microbalance	MYA-5/2Y	Radwag
5	Refrigerator	Eon	Godrej
6	Deep Freezer	Forma 900 series	Thermo scientific
7	Ultrasonicator	UCB 70	Spectralab
8	Laboratory Oven	-	Laboratory Instruments
9	Micropipette	Research Plus	eppendorf
		Research	
10	Vortexer	Spinix	Tarsons

2.3 Solvents and Chemicals

Sr. N°	Name	Grade	Source
1	2-propanol	HPLC	Finar
2	Acetonitrile	HPLC	J.T baker
3	Methanol	HPLC	J.T baker
4	Formic Acid	LC-MS	Fluka
5	Milli-Q water	Type-I	Millipore Gradient System (Milli-Q)
6	Distilled water	Type-II	Millipore Gradient System (Elix-10)
7	Ammonium Formate	HPLC	Fluka
8	EDTA	ExcelaR	Fisher scientific
9	Iodomethane	LR	SDFCL
10	Dimethyl Sulphate	LR	SDFCL

2.4 Principle

The residue of mancozeb in surface and drinking water was determined using LC-MS-MS.

2.5 Test System

A] Surface Water

Sampling Location : Daman Ganga River, Near N.H. No.8, Jai Research Foundation, Vapi, Gujarat, India.

B] Drinking Water

Sampling Location : Jai Research Foundation, Vapi, Gujarat, India.

Test System	Physico-Chemical Properties	Observation
Surface Water	pH	8.19
	Conductivity	173.8 µs/cm
	Total Hardness as CaCO3	122 mg/L
	TOC	<1.0 ppm
Drinking Water	pH	7.37
	Conductivity	70.6 µs/cm
	Total Hardness as CaCO3	12 mg/L
	TOC	8.849 ppm

Note: Water samples has been analyzed under JRF S. N°: RES-2-14-19682. Above water analysis results are complied from the exact data taken from JRF S. N°: RES-2-14-19682.

2.6 Independent Laboratory Validation of an Analytical Method for Residue of Mancozeb in Surface and Drinking Water by LC-MS/MS

The analytical method for the determination of residue of Mancozeb in surface water and drinking water was validated. The validation covered the aspects viz., specificity, linearity, precision (% RSD) and accuracy (% Recovery) at LOQ and 10 x LOQ level.

2.6.1 Characterization of Dimethyl EBDC Reference Standard

The dimethyl EBDC was characterized by analyzing the 99.171 µg/L concentration of dimethyl EBDC in three replicates, determining the area percent and identification at mass range 100 to 500 amu.

2.6.1.1 Preparation of Dimethyl EBDC Reference Standard Stock Solution

Name of Reference Standard	Purity (%)	Weight of Reference Standard (mg)	Capacity of Volumetric flask (mL)	Volume made up with	Concentration obtained (µg/L)	Stock Solution ID
Dimethyl EBDC	97.705	10.15	100	Acetonitrile	99170.575	D1

2.6.1.2 Preparation of Dimethyl EBDC Reference Standard Working Solution

Stock Solution ID (µg/L)	Solution Taken (mL)	Capacity of Volumetric Flask (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Working Solution ID
D1 (99170.575)	0.010	10	Acetonitrile	99.171	CD02

Dimethyl EBDC Reference Standard Working Solution (CD02) was injected in three replications along with one sample of acetonitrile onto LCMSMS in accordance with **section 2.6.9**.

2.6.2 Specificity

2.6.2.1 Preparation of Dimethyl EBDC Reference Standard Working Solution (For Surface water)

Stock / Working Solution ID (µg/L)	Solution Taken (mL)	Capacity of Volumetric Flask (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Working Solution ID
D1 (99170.575)	0.010	1	Surface water	991.706	D06
D06 (991.706)	0.100	10	Surface water	9.917	D07

2.6.2.2 Preparation of Dimethyl EBDC Reference Standard Working Solution (For Drinking water)

Stock / Working Solution ID (µg/L)	Solution Taken (mL)	Capacity of Volumetric Flask (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Working Solution ID
D1 (99170.575)	0.010	1	Drinking water	991.706	D08
D08 (991.706)	0.100	10	Drinking water	9.917	D09

2.6.2.3 Preparation of Mancozeb Reference Standard Stock Solution

Name of Reference Standard	Purity (%)	Weight of Reference Standard (mg)	Capacity of Volumetric flask (mL)	Volume made up with	Concentration obtained (µg/L)	Stock Solution ID
Mancozeb	97.5	2.4	250	50:50 IPA: Milli-Q water	9360.000	M1

2.6.2.4 Preparation of Mancozeb Reference Standard Fortification Solution (For Surface water)

Stock Solution ID (µg/L)	Solution Taken (mL)	Capacity of Volumetric Flask (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Fortification Solution ID
M1 (9360.000)	0.100	1	Surface water	936.000	MD02 (Surface water)

2.6.2.5 Preparation of Mancozeb Reference Standard Fortification Solution (For Drinking water)

Stock Solution ID (µg/L)	Solution Taken (mL)	Capacity of Volumetric Flask (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Fortification Solution ID
M1 (9360.000)	0.100	1	Drinking water	936.000	MD02 (Drinking Water)

0.050 mL of MD02 (Surface water) and MD02 (Drinking water) fortification solution was taken and fortified to 5 mL of surface water and drinking water respectively to prepare fortification level concentration 9.36 µg/L for both test systems. The fortified samples along with surface and drinking water blanks were extracted as per procedure mentioned in **section 2.6.6**. The solvent (acetonitrile and methanol), blank (surface water), blank (drinking water), extracted blank (surface water), extracted blank (drinking water), extracted mancozeb (surface water), extracted mancozeb (drinking water), dimethyl EBDC (surface water) (D07), dimethyl EBDC (drinking water) (D09) were injected onto LC-MS/MS in accordance with **section 2.6.8**.

2.6.3 Linearity

2.6.3.1 Preparation of Dimethyl EBDC Reference Standard Working Solutions in Surface Water

Working Solution ID (µg/L)	Solution Taken (mL)	Final volume (mL)	Obtained Concentration (µg/L)	Sample ID
D07 (9.917)	0.250	1	2.479	SWL5
SWL5 (2.479)	0.400	1	0.992	SWL4
SWL4 (0.992)	0.500	1	0.496	SWL3
SWL3 (0.496)	0.200	1	0.099	SWL2
SWL2 (0.099)	0.500	1	0.050	SWL1

The reference standard working solutions SWL1, SWL2, SWL3, SWL4 and SWL5 for surface water were injected onto the LC-MS-MS accordance with **section 2.6.8** and peak area was plotted against concentration (µg/L). The correlation coefficient (r), slope (b) and intercept (a) were calculated.

2.6.3.2 Preparation of Dimethyl EBDC Reference Standard Working Solutions in Drinking Water

Working Solution ID (µg/L)	Solution Taken (mL)	Final volume (mL)	Obtained Concentration (µg/L)	Sample ID
D09 (9.917)	0.250	1	2.479	DWL5
DWL5 (2.479)	0.400	1	0.992	DWL4
DWL4 (0.992)	0.500	1	0.496	DWL3
DWL3 (0.496)	0.200	1	0.099	DWL2
DWL2 (0.099)	0.500	1	0.050	DWL1

The reference standard working solutions DWL1, DWL2, DWL3, DWL4 and DWL5 for drinking water were injected onto the LC-MS-MS accordance with **section 2.6.8** and peak area was plotted against concentration (µg/L). The correlation coefficient (r), slope (b) and intercept (a) were calculated.

2.6.4 Precision (% RSD) and Accuracy (% Recovery) in Surface Water

2.6.4.1 Preparation of Mancozeb Reference Standard Stock Solution

Name of Reference Standard	Purity (%)	Weight of Reference Standard (mg)	Capacity of Volumetric flask (mL)	Volume made up with	Concentration obtained (µg/L)	Stock Solution ID
Mancozeb	97.5	2.5	250	50:50 IPA: Milli-Q water	9750.000	M2

2.6.4.2 Preparation of Mancozeb Reference Standard Working Solution

Stock / Working Solution ID (µg/L)	Solution Taken (mL)	Final volume (mL)	Obtained Concentration (µg/L)	Sample ID
M2 (9750.000)	1.025	10	999.375	M04
M04 (999.375)	1.000	10	99.938	M05
M05 (99.938)	1.000	10	9.994	M06

Mancozeb reference standard working solution M04, M05 and M06 were prepared in 50:50 IPA: Milli-Q water.

2.6.4.3 Fortification of Mancozeb Reference Standard at LOQ and 10 x LOQ in Surface Water

Fortification level	Replication	Concentration of Reference Standard working Solution (µg/L)	Volume (mL) taken	Final Volume (Surface Water) (mL)	Sample ID
LOQ (0.1 µg/L)	R1	M06 (9.994)	0.050	5	LOQ_1
	R2		0.050	5	LOQ_2
	R3		0.050	5	LOQ_3
	R4		0.050	5	LOQ_4
	R5		0.050	5	LOQ_5
	R6		0.050	5	LOQ_6
	R7		0.050	5	LOQ_7
10 X LOQ (1 µg/L)	R1	M04 (999.375)	0.005	5	10LOQ_1
	R2		0.005	5	10LOQ_2
	R3		0.005	5	10LOQ_3
	R4		0.005	5	10LOQ_4
	R5		0.005	5	10LOQ_5

The solutions at LOQ and 10 x LOQ level along with two blank extract samples were extracted as per **section 2.6.6** and injected onto LC-MS/MS in accordance with the parameters described in **section 2.6.8**.

2.6.5 Precision (% RSD) and Accuracy (% Recovery) in Drinking Water

2.6.5.1 Preparation of Mancozeb Reference Standard Stock Solution

Name of Reference Standard	Purity (%)	Weight of Reference Standard (mg)	Capacity of Volumetric flask (mL)	Volume made up with	Concentration obtained (µg/L)	Stock Solution Identification
Mancozeb	97.5	2.4	250	50:50 IPA: Milli-Q water	9360.000	M1

2.6.5.2 Preparation of Mancozeb Reference Standard Working Solution

Stock/Working Solution ID (µg/L)	Solution Taken (mL)	Final volume (mL)	Obtained Concentration (µg/L)	Sample Identification
M1 (9360.000)	1.068	10	999.648	M01
M01 (999.375)	1.000	10	99.965	M02
M02 (99.938)	1.000	10	9.997	M03

Mancozeb reference standard working solution M01, M02 and M03 were prepared in 50:50 IPA: Milli-Q water.

2.6.5.3 Fortification of Mancozeb Reference Standard at LOQ and 10 x LOQ in Drinking Water

Fortification level	Replication	Concentration of Reference Standard working Solution (µg/L)	Volume (mL) taken	Final Volume (Drinking Water) (mL)	Sample ID
LOQ (0.1 µg/L)	R1	**M03 (9.997)**	0.050	5	LOQ_1
	R2		0.050	5	LOQ_2
	R3		0.050	5	LOQ_3
	R4		0.050	5	LOQ_4
	R5		0.050	5	LOQ_5
	R6		0.050	5	LOQ_6
	R7		0.050	5	LOQ_7
10 X LOQ (1 µg/L)	R1	**M01 (999.648)**	0.005	5	10LOQ_1
	R2		0.005	5	10LOQ_2
	R3		0.005	5	10LOQ_3
	R4		0.005	5	10LOQ_4
	R5		0.005	5	10LOQ_5

The solutions at LOQ and 10 x LOQ level along with two blank extract samples were extracted as per **section 2.6.6** and injected onto LC-MS/MS in accordance with the parameters described in **section 2.6.8**.

2.6.6 Extraction Procedure

To the 5 mL samples of Surface and drinking water fortified with mancozeb at different concentration level (**Section 2.6.2.4, 2.6.2.5, 2.6.4.3, 2.6.5.3**), a volume of 0.250 mL of 2 mM EDTA prepared in 10mM ammonium formate solution was added and shook for 45 seconds on hand vortexer. The pH of above samples were checked and found within 8 to 10. pH Adjusted (if necessary) to between 8 – 10 with either 10% HCl or 2.0 mM Na4EDTA/NH4HCO2 or NaOH (record volume added for final volume calculation). A volume of 10 µL of dimethyl sulphate was added to all the sample followed by the addition of 10 µL of iodomethane. All the samples were vortexed for 45 seconds and kept at 55°C for 1h. Samples were allowed to cool down at room temperature, mixed well and filtered with 0.45 µm filter. The filtered solution was injected onto LC-MS/MS in accordance with the parameters described in **section 2.6.6**. The blank extract of surface and drinking water for matrix match samples were prepared by following the same extraction procedure without mancozeb fortification.

2.6.7 Calculation

Mancozeb (µg/L):

$$= \frac{\text{Calculated Concentration of Dimethyl EBDC (µg/L) x Final volume (L)}}{\text{Sample volume (mL) x Conversion factor x Efficiency yield factor}}$$

Where: Conversion factor = 1.11

Efficiency yield factor = 1.515

The residue of Mancozeb in media and regression equation was established by analyst® software version 1.6.2.

2.6.8 Instrumental Parameters

Instrument	: LC-MS/MS (HPLC, Shimadzu Nexera X2 coupled with Qtrap 6500 mass spectrometer)
Column	: Kinetex Phenomenex, 2.1 x 100 mm, 1.7 µm, C18
Flow Rate	: 550 µL/min
Injection Volume	: 8 µL
Oven Temperature	: Ambient
Run Time	: 8 minutes
Mobile phase	: A) 5mM ammonium formate/0.1% formic acid in Milli-Q water B) 0.1% Formic Acid in Methanol

Gradient Program :

Time (min)	A%	B%
0.00	90	10
1.00	90	10
2.00	50	50
3.00	10	90
5.50	10	90
6.00	90	10
8.00	90	10

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition (m/z)	Dwell (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
EBDC (Quantitation)	241.1/134.1	200	20	10	25	10
EBDC (Confirmatory)	241.1/193.2	200	28	9	11	15

Mass Spectrometer Conditions:

Ionization Mode	ESI
Scan Type	MRM
Polarity	Positive
Resolution – Q1	Unit
Resolution – Q3	Unit
Curtain Gas (N_2)	40
GS1	70
GS2	60
CAD gas (N_2)	High
Ion Spray (V)	5300
Temperature (°C)	520

2.6.9 Instrument Parameters for Characterization of Dimethyl EBDC Reference Standard

Instrument : LC-MS/MS (HPLC, Shimadzu Nexera X2 coupled with mass spectrometer, Shimadzu LCMS-8050)

Column : Thermo BDS Hypersil, 4.6 x 100 mm, 5µm, C18

Flow Rate : 600 µL/min

Injection Volume : 8 µL

Oven Temperature : Ambient

Run Time : 3.5 minutes

Mobile phase : 0.1% Formic Acid in Methanol : 5mM ammonium formate/0.1% formic acid in Milli-Q water (80:20,v/v)

Typical MS/MS Voltage Conditions:

Ionization Mode	ESI	Nebulizing Gas Flow (L/min)	3.00
Scan Type	Q1 Scan	Heating Gas Flow (L/min)	12.00
Scan Range (amu, m/z)	100 to 500	Heat block Temperature (°C)	400
Polarity	Positive	Drying Gas Flow (L/min)	12.00
Interface Temperature (°C)	300	CID Gas (kPa)	230
DL Temperature (°C)	250		

3. RESULTS

3.1 Validation of Analytical Method

3.1.1 Specificity

The specificity of the method for the determination of mancozeb residue in surface water and drinking water was studied by injecting acetonitrile, methanol, blank (surface and drinking water), extracted blank, extracted mancozeb and dimethyl EBDC solution, and the method was considered to be specific for the analyte.

3.1.2 Linearity

The linearity of the method for precision and accuracy in surface and drinking water at LOQ and 10 times LOQ levels was established by injecting five different concentrations viz., (0.050, 0.099, 0.496, 0.992, 2.479 µg/L) of dimethyl EBDC reference standard solutions respectively.

3.1.3 Precision (% RSD)

Precision of the analytical method was determined by analysing test media at the LOQ and 10 x LOQ levels.

3.1.4 Accuracy (% Recovery)

Accuracy of the analytical method was determined by analysing test media at LOQ and 10 times LOQ level.

3.1.5 Characterization of Dimethyl EBDC Reference Standard

The mean % purity obtained for dimethyl EBDC reference standard was 97.70 **(TABLE 9)**.

Independent Laboratory Validation of an Analytical Method for the Determination of Mancozeb in Surface and Drinking water by LC-MS/MS Analysis

TABLE 9: Characterization of Dimethyl EBDC Reference Standard

Replicates	Peak Area of Dimethyl EBDC	Area percent of Dimethyl EBDC
I	1905349	98.723
II	1401546	98.031
III	1594968	96.345
Mean % Purity	-	**97.70**