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JAI RESEARCH
FOUNDATION

JRF Study Number: 228-2-14-18970 (Final Report)

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STUDY TITLE

**INDEPENDENT LABORATORY VALIDATION OF AN ANALYTICAL METHOD
FOR THE DETERMINATION OF MANCOZEB AND ETU IN SOIL
BY LC-MS/MS ANALYSIS**

GUIDELINES AND DATA REQUIREMENT

OPPTS 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

1. INTRODUCTION

1.1 Study Objective

This study was performed to independently validate the LC-MS-MS analytical method for the determination of Mancozeb and ETU in soil as a part of ILV. The study was conducted in compliance with OECD Principles of GLP (1998).

1.2 Study Guidelines

The present study was conducted according to:

US EPA, "Residue Chemistry Test Guidelines OPPTS 860.1340 Residue Analytical Method", OPPTS, (EPA 712-C-96-174), (August 1996).

Commission Regulation (EU) No 283/2013 setting out the data requirement for active substances, in accordance with Regulation (EC) No 1107/2009. SANCO/825/00 rev. 8.1 (November 16, 2010), Guidance Document on Pesticide Residue Analytical Methods (Post-registration Requirements for Annex II and Annex III).

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 4)
Source	Sigma-Aldrich
Storage Condition	Refrigerator
JRF Entry Number	PC-3616

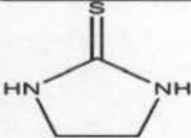
Source of IUPAC Name, C.A. Name and Molecular Structure: e-Pesticide Manual, 15th Edition, 2010.

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	240.50 g/mol
Molecular Structure	
Batch N°	JRF-001-058
Re-test Date	May 27, 2018 (Refer JRF Study N° 228-2-14-18969)
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

Reference Standard of Ethylenethiourea (ETU)

Reference Standard	Ethylenethiourea (ETU)
IUPAC Name	2-Imidazolidinethione
CAS N°	96-45-7
Molecular Formula	C ₃ H ₆ N ₂ S
Molecular Weight	102.16 g/mol
Molecular Structure	
Batch N°	BCBV4514
Quality release date	June 21, 2017
Expiry Date	May 2022
Purity	100.0 % (Refer Certificate of Analysis in APPENDIX 6)
Source	Sigma-Aldrich
Storage Condition	Refrigerator
JRF Entry Number	PC-4139

Source of CAS N°, IUPAC Name, Molecular Formula 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
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	Refrigerator Centrifuge	Servall Legend XTR	Thermo Scientific
9	Cyclomixer	Elektrote Chink	Remi
10	Orbital Shaker	-	Orbitek
11	Micropipette	Research Plus	Eppendorf
		Research	

2.3 Solvents and Chemicals

Sr. N ^o	Name	Grade	Source
1	2-propanol	HPLC	Finar
2	Acetonitrile	HPLC	J.T baker
3	Methanol	LC-MS	Merck
4	Formic Acid	LC-MS	Fluka
5	Milli-Q water	Type-I	Millipore
6	Distilled water	Type-II	Millipore Gradient System (Elix-10)
7	Ammonium Formate	HPLC	Fluka
8	EDTA 4Na	AR	Sigma Aldrich
9	Iodomethane	LR	SDFCL
10	Dimethyl Sulphate	LR	SDFCL
11	L-Cysteine	-	Sigma Aldrich
12	Supel QuE Non buffered Tube1	-	Supelco
13	Supel QuE PSA/C18 (EN) Tubes	-	Supelco

2.4 Principle

The residue of Mancozeb and ETU in soil was determined using LC-MS/MS as a part of ILV.

2.5 Test System

Soil

Place : Nani Vahiya village in Dharampur Tehsil, Valsad, Gujarat, India.
 Collection Site : Bank of Par River.
 Date of Collection : January 23, 2018
 Date of Expiry : April 22, 2018.
 Depth of Collection : 0 - 25 cm

Soil Analysis Results		
pH	1:2.5 in 0.01M CaCl ₂ (solution)	5.96
	1:2.5 in Distilled water	6.87
Organic Carbon Content (%)		0.76
Cation Exchange Capacity (meq/100g of soil)		22.72
Particle Size Distribution	Coarse Sand (%)	35.06
	Fine Sand (%)	35.11
	Silt (%)	3.68
	Clay (%)	16.43
Water / Moisture Content (%)		9.41
Oven Dry Weight of Soil (g)		18.28
Water Holding Capacity (%)		57.14
Total Nitrogen Content (%)		0.049
Initial Microbial Biomass	Optimum Glucose Concentration (mg/kg dry soil)	4000
	Minimum Microbial Biomass (mg/kg dry soil)	536.91
Initial Microbial Biomass Percentage of Total Organic Carbon of Soil (%)		7.06
Initial Nitrate Content (mg/kg soil)		59.47

2.6 Independent Laboratory Validation of an Analytical Method for Residue of Mancozeb and ETU in Soil by LC-MS/MS

The analytical method for the determination of residue of Mancozeb in soil 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 Specificity

2.6.1.1 Preparation of Mancozeb, Dimethyl EBDC and ETU 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	4.1	250	50:50 IPA: Milli-Q water	15990.000	M
Dimethyl EBDC	97.705	10.12	100	Acetonitrile	98877.460	D
ETU	100.0	10.10	100	Acetonitrile	101000.000	E

2.6.1.2 Preparation of Mancozeb Reference Standard Fortification Solution

Stock Solution ID (µg/L)	Solution Taken (mL)	Final Volume (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Fortification Solution ID
M (15990.000)	0.063	1	50:50 IPA: Milli-Q water	1007.370	MD01

2.6.1.3 Preparation of Dimethyl EBDC Reference Standard Working Solution

Stock / Working Solution ID (µg/L)	Solution Taken (mL)	Final Volume (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Working Solution ID
D (98877.460)	0.010	10	95:5 (Milli-Q Water : Acetonitrile, v/v)	98.877	D01
D01 (98.877)	1	10	95:5 (Milli-Q Water : Acetonitrile, v/v)	9.888	D02

2.6.1.4 Preparation of ETU Reference Standard Fortification Solution

Stock Solution ID (µg/L)	Solution Taken (mL)	Final Volume (mL)	Volume made upto mark with	Obtained Concentration Solution (µg/L)	Working Solution ID
E (101000.000)	0.010	1	95:5 (Milli-Q Water : Acetonitrile, v/v)	1010.000	ED01

0.200 mL of MD01 (For Mancozeb) and 0.200 mL of ED01 (For ETU) fortification solution were fortified in 2.00 g of soil each to prepare fortification level concentration 0.1 µg/g for both Mancozeb and ETU. The fortified samples along with soil blanks were extracted as per procedure mentioned in section 2.6.5. The solvent (acetonitrile and methanol), extracted blank of soil, extracted mancozeb and dimethyl EBDC (D02) were injected onto LC-MS/MS in accordance with section 2.6.8.

2.6.2 Linearity

2.6.2.1 Preparation of Dimethyl EBDC Reference Standard Working Solutions

Working Solution ID ($\mu\text{g/L}$)	Solution Taken (mL)	Final volume (mL)	Obtained Concentration ($\mu\text{g/L}$)	Sample ID
D01 (98.877)	0.102	1	10.085	S5
S5 (10.085)	0.700	1	7.060	S4
S4 (7.060)	0.714	1	5.040	S3
S3 (5.040)	0.500	1	2.520	S2
S2 (2.520)	0.400	1	1.008	S1

The reference standard working solutions S1, S2, S3, S4 and S5 (For Dimethyl EBDC) were prepared using 95:5 (Milli-Q Water : Acetonitrile, v/v) and injected onto the LC-MS-MS accordance with **section 2.6.8** and peak area was plotted against concentration ($\mu\text{g/L}$). The correlation coefficient (r), slope (b) and intercept (a) were calculated.

2.6.2.2 Preparation of ETU Reference Standard Working Solutions

Stock / Working Solution ID ($\mu\text{g/L}$)	Solution Taken (mL)	Final volume (mL)	Obtained Concentration ($\mu\text{g/L}$)	Sample ID
E (101000.000)	0.100	10	1010.000	E01
E01 (1010.000)	0.100	10	10.100	S5
S5 (10.100)	0.700	1	7.070	S4
S4 (7.070)	0.714	1	5.048	S3
S3 (5.048)	0.500	1	2.524	S2
S2 (2.524)	0.400	1	1.010	S1

The reference standard working solutions S1, S2, S3, S4 and S5 (For ETU) were prepared using 95:5 (Milli-Q Water: Acetonitrile, v/v) and injected onto the LC-MS-MS accordance with **section 2.6.8** and peak area was plotted against concentration ($\mu\text{g/L}$). The correlation coefficient (r), slope (b) and intercept (a) were calculated.

2.6.3.3 Fortification of Mancozeb Reference Standard at LOQ and 10 x LOQ

Fortification level	Replication	Concentration of Reference Standard working Solution ($\mu\text{g/L}$)	Volume (mL) taken	Soil Weight (g)	Sample ID
LOQ (0.05 $\mu\text{g/g}$)	R1	M01 (999.375)	0.100	2.01	LOQ 1
	R2		0.100	2.00	LOQ 2
	R3		0.100	2.02	LOQ 3
	R4		0.100	1.99	LOQ 4
	R5		0.100	2.01	LOQ 5
	R6		0.100	1.99	LOQ 6
	R7		0.100	2.03	LOQ 7
10 X LOQ (0.5 $\mu\text{g/g}$)	R1	M01 (999.375)	1.000	2.02	10 LOQ 1
	R2		1.000	2.00	10LOQ 2
	R3		1.000	2.01	10LOQ 3
	R4		1.000	2.03	10LOQ 4
	R5		1.000	1.99	10LOQ 5

The solutions at LOQ and 10 x LOQ level along with two blank extract samples were extracted as per section 2.6.5.1, diluted as per section 2.6.6 and injected onto LC-MS/MS in accordance with the parameters described in section 2.6.8.

2.6.4.2 Fortification of ETU Reference Standard at LOQ and 10 x LOQ

Fortification level	Replication	Concentration of Reference Standard working Solution ($\mu\text{g/L}$)	Volume (mL) taken	Soil Weight (g)	Sample ID
LOQ (0.05 $\mu\text{g/g}$)	R1	E02 (999.900)	0.100	2.01	LOQ 1
	R2		0.100	2.00	LOQ 2
	R3		0.100	2.02	LOQ 3
	R4		0.100	2.03	LOQ 4
	R5		0.100	1.99	LOQ 5
	R6		0.100	2.01	LOQ 6
	R7		0.100	2.02	LOQ 7
10 X LOQ (0.5 $\mu\text{g/g}$)	R1	E02 (999.900)	1.000	1.99	10LOQ 1
	R2		1.000	2.02	10LOQ 2
	R3		1.000	2.01	10LOQ 3
	R4		1.000	1.99	10LOQ 4
	R5		1.000	2.00	10LOQ 5

The solutions at LOQ and 10 x LOQ level along with two blank extract samples were extracted as per section 2.6.5.2, diluted 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 Extraction Procedure

2.6.5.1 Extraction Procedure for Mancozeb

Accurately weighted 2.00 g of blank soil was transferred in to pre-labelled extraction bottles. To each bottle different concentrations of Mancozeb solution was fortified as per experiment requirement. A volume of 2 mL of milli Q water was added to each fortified soil sample followed by the addition of 100 mg of L-cysteine and 500 mg of EDTA-4Na to it. A volume of 10 mL Acetonitrile containing 0.05 M dimethyl sulphate and 0.1 M iodomethane was added to the soil samples. All samples were vortexed for 1 minute on hand vortexer and shook for 15 minutes on orbital shaker. An amount of 4g Magnesium sulfate and 1g sodium chloride mixture was added to above samples. Samples were centrifuged for 10 minutes at 3500 rpm. A volume of 3 mL supernatant from each sample was taken out and transferred into pre-labelled extraction bottles. To the supernatant solution a powder mixture containing 900 mg magnesium sulphate, 150 mg PSA and 150 mg DSC-18 was added. All samples were vortexed for 1 minute on hand vortexer followed by centrifugation for 10 minutes at 3500 rpm. The supernatant of centrifuged samples then filtered with 0.45 μ m filters and the filtrate was diluted with diluent (Milli-Q Water: Acetonitrile (95:5) v/v) as per section 2.6.6 (Sample Dilution). The diluted sample solution then transferred into HPLC vials and injected onto LC-MS/MS in accordance with the parameters described in section 2.6.8. The blank extract for matrix match samples were prepared by following the same extraction procedure without mancozeb fortification.

2.6.5.2 Extraction Procedure for ETU

Accurately weighted 2.00 g of blank soil was transferred in to pre-labelled extraction bottles. To each bottle different concentrations of ETU solution was fortified as per experiment requirement. A volume of 2 mL of milli Q water was added to each fortified soil sample. A volume of 10 mL 50% methanol in Milli-Q water (% v/v) was added to it. All samples were vortexed for 1 minutes on hand vortexer and shook for 15 minutes on orbital shaker. Samples were centrifuged for 10 minutes at 3500 rpm. A volume of 3 mL supernatant from each sample was taken out and transferred into pre-labelled extraction bottles. To the supernatant solution a powder mixture containing 900 mg magnesium sulphate, 150 mg PSA and 150 mg DSC-18 was added. All samples were vortexed for 1 minute on hand vortexer followed by centrifugation for 10 minutes at 3500 rpm. The supernatant of centrifuged samples then filtered with 0.45 μ m filters and the filtrate was diluted with diluent (Milli-Q Water:Acetonitrile (95:5), v/v) as per section 2.6.6 (Sample Dilution). The diluted sample solution then transferred into HPLC vials and injected onto LC-MS/MS in accordance with the parameters described in section 2.6.8. The blank extract for matrix match samples were prepared by following the same extraction procedure without ETU fortification.

2.6.6 Sample Dilution

Samples	Sample ID (Concentration, $\mu\text{g/g}$)	Solution Taken (mL)	Final volume (mL)	Dilution Factor
Mancozeb	LOQ (0.05)	0.500	1	2
	10 LOQ (0.5)	0.050	1	20
ETU	LOQ (0.05)	0.500	1	2
	10 LOQ (0.5)	0.050	1	20

2.6.7 Calculation

Mancozeb ($\mu\text{g/g}$):

Calculated concentration of Dimethyl EBDC ($\mu\text{g/L}$) x Final volume (L) x Dilution factor

= -----

Sample weight (g) x Conversion factor x Efficiency yield factor

Where: Conversion factor = 1.11

Efficiency yield factor = 1.515

Conversion factor and Efficiency yield factor were applied as per JRFA method AU-275 R0 mentioned in **section 4 (Reference)**.

ETU ($\mu\text{g/g}$):

Calculated concentration ($\mu\text{g/L}$) x Final volume (L) x Dilution factor

= -----

Sample weight (g)

The residue of Mancozeb and ETU in soil 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 : HSS T3 waters, 2.1 x 100mm, 1.8 μm

Flow Rate : 550 $\mu\text{L}/\text{min}$

Injection Volume : 8 μL (EBDC), 15 μL (ETU)

Oven Temperature : Ambient

Detector : Sciex 6500 Qtrap

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 (minute)	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
ETU (Quantitation)	103.4/44.0	200	50	10	20	5
ETU (Confirmatory)	103.4/60.0	200	50	10	45	10

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