JRF AMERICA

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FINAL REPORT

TITLE

Method Development and Validation of Mancozeb and ETU Analysis in Soil

TEST GUIDELINE(S)

OCSPP (formerly OPPTS) 860.1340, SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1

ABBREVIATIONS AND SYMBOLS

Abbreviation	Definition
°C	degrees Celsius or Centigrade
CAS	Chemical Abstract Services
cm	centimeter
EPA	Environmental Protection Agency (U.S.)
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
g	gram
HPLC	High-performance liquid chromatography
i.d.	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
kg	kilogram
L	litre
LC-MS/MS	tandem liquid chromatography/mass spectrometry/mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m	meter
ACN	acetonitrile
MeOH	methanol
DCM	dichloromethane
µg	microgram
µL	microliter
µm	micrometer
mg	milligram
mL	milliliter
mm	millimeter
mmol	millimole
min	minute
mol	mole
ms	millisecond
MS/MS	tandem mass spectrometry
mV	millivolt
MW	molecular weight

ABBREVIATIONS AND SYMBOLS - (continued)

Abbreviation	Definition
m/z	mass to charge ratio
N/A	not applicable
ND or nd	not detectable (below limit of detection)
ng	nanogram
No.	number
OES	Occupational Exposure Standards
OECD	Organisation for Economic Co-operation and Development
OCSPP	Office of Chemical Safety and Pollution Prevention
OPPTS	Office of Prevention, Pesticides and Toxic Substances
pg	picogram
ppb	parts per billion or micrograms per kilogram or micrograms per liter
ppm	parts per million or milligrams per kilogram or milligrams per liter
PSA	Primary and secondary amine
R^2 (or r^2)	square of correlation coefficient
RSD	relative standard deviation
Rt	retention time
s	second
SD	standard deviation
SPE	Solid Phase Extraction
UPW	ultra pure water
V	volt
vol	volume

2.0 INTRODUCTION AND BACKGROUND

Mancozeb is a very unstable compound; when ionized by an ion-spray in high voltage, it provides poor reproducibility. Hence, it is required to transform the analyte into its methylated form, dimethyl ethylene bisdithiocarbamate (EBDC), for improved solubility, stability, and instrument sensitivity.

The metal atoms in mancozeb are decoupled using ethylenediaminetetraacetic acid (EDTA). L-cysteine is added to the matrix before the addition of fortification and EDTA in order to stabilize the EDTA complex. The complex is then methylated with methyl iodide (CH_3I) and dimethyl sulfate ($(CH_3)_2SO_4$) to form dimethyl EBDC (M.W. = 240.50) as follows (note: equation is not balanced):

$$\underset{\text{M.W. } 266.51}{\text{Mancozeb}} + \text{EDTA} + CH_3I + (CH_3)_2SO_4 \rightarrow \underset{\text{M.W. } 240.50}{(CH_2)_2(CH_3)_2(CS_2)_2(NH)_2}$$

The methylated species is analyzed using an LC/MS/MS with MRM quantitation *m/z 240.870* to *133.900* and confirmatory *m/z 240.922* to *193.000*.

3.0 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, reagents, glassware, and techniques may be substituted for those described in this method, except where specifically

stated otherwise. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

4.0 SAFETY

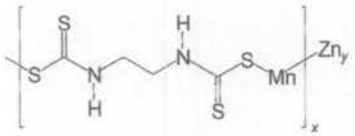
The chemicals used in this study should be treated as potential health hazards and exposure to these chemicals should be minimized. The analyst is responsible for maintaining awareness of OSHA (Occupational Safety and Health Administration) regulations regarding the safe handling of the chemicals used in this method. A reference file of safety data sheets (SDS) should be available to all personnel involved in the chemical analyses, as well as GHS (Globally Harmonized System) SDS training if required.

5.0 MATERIALS

5.1. Test and Reference Substance Identification

The chemical structures of mancozeb and ETU are summarized as follows:

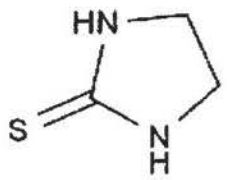
Common Name:	Mancozeb
Chemical Name (IUPAC):	Zinc Manganese Ethylenebisdithiocarbamate
CAS Registry No.:	8018-01-7
Molecular Weight:	266.51 $g \cdot mol^{-1}$
Batch No.:	SZBE225XV
Received Date:	July 26, 2016
Reassay Date:	July 20, 2019 (See Appendix X for COAs)
Purity:	97.5%
Storage Condition:	Refrigerated
Source:	Sigma-Aldrich
Structure:	



JRFA SAS-007-OOO

Common Name:	Ethylenethiourea (ETU)
Chemical Name (IUPAC):	2-Imidazolidinethione
CAS Registry No.:	96-45-7
Molecular Formula:	$C_3H_6N_2S$
Molecular Weight:	102.16 $g \cdot mol^{-1}$
Batch No.:	SZBC242XV
Received Date:	August 12, 2016
Reassay Date:	February 3, 2018

Purity:	99.7%
Storage Condition:	Ambient
Source:	Sigma-Aldrich
Structure:	



JRFA SAS-007-PPP

5.2. Test Matrix

The matrix was selected to be representative of typical soil, and was from a previous GLP study (collected as per guideline requirements). Information such as collection location, characteristics and storage are reported in the raw data. Soil samples were kept frozen. Matrix was tested to ensure no mancozeb or ETU was present prior to analysis.

6.0 APPARATUS AND EQUIPMENT

6.1. Laboratory Glassware

Vials and caps, autosampler, 2-mL, screw cap
Centrifuge tubes, 15 mL and 50 mL
Class A glassware (volumetric pipettes, flasks, graduated cylinders, etc.)

6.2. Laboratory Equipment

Pipettes, air-displacement, adjustable volume
Balance, top loading, capable of weighing to nearest 0.01 g
Balance, analytical, capable of weighing to the nearest 0.1 mg
Vortex mixer
Wrist action shaker
Centrifuge
See Appendix III for complete list of equipment and serial numbers.

6.3. Chromatographic System Options

Columns, Waters HSS T3 1.8 µm 2.1 mm x 100 mm, #015133305157
Phenomenex Kinetix C18 1.7 µm 2.1 mm x 100 mm, 504004-58 (for matrix matched standards test)
Liquid Chromatography System, Agilent 1290 UHPLC
Mass Spectrometer, Sciex 6500 Q-trap
Mass Spectrometer Data system, Analyst Software, version 1.6.2 or equivalent

6.4. Reagents

All solvents and other reagents are to be of high purity, e.g., LC-MS grade solvents and analytical grade reagents. See Appendix III for a complete list of reagents and lot numbers.

6.5. Reagents and Materials to be Prepared

Mobile Phase A – 5 mM ammonium formate/0.1% formic acid in water:

Weigh out approximately 0.3 g NH_4HCO_2 and 1.00 mL of formic acid, transfer to 1000-mL volumetric flask. Bring to volume with water.

Mobile Phase B – 0.1% formic acid in MeOH:

Transfer 1.00 mL of formic acid to a 1000-mL volumetric flask. Bring to volume with MeOH.

0.05 M Dimethyl Sulfate + 0.1 M Iodomethane in ACN:

Transfer 1.180 mL of dimethyl sulfate and 1.555 mL of iodomethane to a 250 mL volumetric flask and bring to volume with LC-MS ACN.

50:50 H_2O/MeOH:

100 mL water and 100 mL MeOH were measured in separate graduated cylinders and mixed in a flask.

6.6. Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves, protective eyewear, and other appropriate PPE.
3. Prevent inhalation and contact with skin.
4. Wash any contaminated areas.

Mancozeb stock standard solution was prepared in 50:50 isopropyl alcohol (IPA):water solution. The following is a description for preparing 250 mL of a 16,400 µg/L stock standard.

1. A mass of 0.0042 g of mancozeb reference standard is weighed (adjusted for purity of 97.5%) and transferred to a 0.250 L class A volumetric flask.
2. Fill the volumetric flask halfway with 50:50 IPA/H_2O and agitate gently (sonicate for no more than 15 minutes if necessary) until standard is completely dissolved.
3. Dilute to volume with 50:50 IPA/H_2O and mix by inverting several times.
4. Calculate the exact concentration using the exact weight and purity, for example:

$$\left(\frac{0.0042\text{g}*0.975}{0.250\text{ L}}\right)*\left(\frac{10^6\mu\text{g}}{\text{g}}\right) = 16{,}400\ \mu\text{g/L}$$

Dimethyl EBDC stock standard solution was also prepared, as mancozeb is derivatized for analysis. EBDC and ETU stock were prepared in acetonitrile. The preparation and calculation of these stocks follow a similar procedure as mancozeb. The table below lists the stock solutions prepared and used during this study.

Analyte ID	JRFA ID	Purity	Weight (g) [1]	Final Volume (mL) [2]	Concentration (µg/L)
Mancozeb	JRFA-501/9-1	97.5%	0.0041	250	16400
Dimethyl EBDC	JRFA-501/5-3	97.7%	0.0118	100	118000
Dimethyl EBDC	JRFA-501/11-2	97.7%	0.0102	100	102000
ETU	JRFA-501/5-4	99.7%	0.0115	100	115000
ETU	JRFA-501/12-1	99.7%	0.0105	100	105000

[1] Corrected for purity

[2] Mancozeb was prepared in 50:50 H_2O/IPA, dimethyl EBDC and ETU were prepared in acetonitrile.

6.7. Preparation of Fortification Solutions

Sample fortification solutions should be prepared by serial dilution of the stock standard in 50/50 isopropanol/water for mancozeb and acetonitrile for EBDC and ETU. The following concentrations are prepared for fortification standards: 10,000 µg/L, and 1,000 µg/L. The table bellows lists the fort solutions prepared and used during this study.

Analyte ID	JRFA ID Starting Solution	Starting Concentration (µg/L)	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	JRFA ID Final Solution
Mancozeb	JRFA-501/9-1	16400	30.488	50.0	10000	JRFA-501/9-3
Mancozeb	JRFA-501/9-3	10000	10.0	100.0	1000	JRFA-501/9-4
ETU	JRFA-501/5-4	115000	8.70	100.0	10000	JRFA-501/8-3
ETU	JRFA-501/8-3	10000	10.0	100.0	1000	JRFA-501/8-4
ETU	JRFA-501/12-1	105000	0.476	50.0	1000	JRFA-501/12-2
Dimethyl-EBDC	JRFA-501/5-3	118000	0.847	100.0	1000	JRFA-501/8-5
Dimethyl-EBDC	JRFA-501/11-2	102000	0.490	50.0	1000	JRFA-501/11-3

6.8. Standard Solution Storage and Expiration

EBDC and ETU stock and standard solutions should be stored in a refrigerator (~ 4° C) when not in use to prevent degradation and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of 6 months is recommended for the EBDC-dimethyl and ETU stock standard solutions, and 3 months for fortification standards and calibration standards, as per JRFA's SOPs.

Stock and fortification solutions of mancozeb should be prepared fresh and used immediately on the day of analysis, due to the fast hydrolysis of mancozeb.

6.9. Preparation of Calibration Standards for LC-MS/MS

Calibration solutions suitable for LC-MS/MS analysis should be prepared in solvent (95:5 H2O/ACN). At least four levels of external calibration standards should be prepared to develop calibration curves for calculation of sample residues. Dilution schemes used to prepare the LC-MS/MS calibration solutions are as follows:

Analyte	JRFA ID Starting Solution	Starting Concentration (µg/L)	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	JRFA ID Final Solution
Dimethyl -EBDC	JRFA-501/8-5	1000	1.00	50.0	20	JRFA-501/8-12
Dimethyl -EBDC	JRFA-501/8-12	20	25.0	50.0	10	JRFA-501/8-13
Dimethyl -EBDC	JRFA-501/8-13	10	25.0	50.0	5	JRFA-501/8-14
Dimethyl -EBDC	JRFA-501/8-14	5	25.0	50.0	2.5	JRFA-501/8-15
Dimethyl -EBDC	JRFA-501/8-15	2.5	20.0	50.0	1	JRFA-501/8-16
ETU	JRFA-501/8-6	20	25.0	50.0	10	JRFA-501/8-7
ETU	JRFA-501/8-7	10	25.0	50.0	5	JRFA-501/8-8
ETU	JRFA-501/8-8	5	25.0	50.0	2.5	JRFA-501/8-9
ETU	JRFA-501/8-9	2.5	20.0	50.0	1	JRFA-501/8-10

7.0 METHOD SUMMARY

7.1. Analytical Method for the Determination of Residues of Mancozeb in Soil by LC-MS/MS Analysis

Weigh 2 g ± 0.05 g of soil into a 50 mL centrifuge tube. Add 1-2 mL of water to the sample, and then add 0.1 g of L-cysteine + the appropriate fortification + 0.5 g of EDTA-4Na. The sample is then derivatized by adding 10 mL of acetonitrile containing 0.05 M dimethyl sulfate + 0.1 M iodomethane. Samples are vortexed for 1 minute and then shaken via wrist action shaker for 15 min. One packet of QuEChERS mix of 4 g anhydrous MgSO4 + 1 g NaCl is added to the samples and shaken for 1 min. Tubes are centrifuged for 10 min at 3500 rpm. A 3 mL aliquot of the supernatant is transferred to a new 15 mL centrifuge tube containing a QuEChERS mix of 150 mg Supelclean PSA, 150 mg Discovery DSC-18, and 900 mg $MgSO_4$. Tubes are vortexed for 1 min and centrifuged for 10 min at 3500 rpm. Samples are diluted if necessary and then vialed for analysis. Instrumental analysis is accomplished using a LC-MS/MS system.

7.2. Analytical Method for the Determination of Residues of ETU in Soil by LC-MS/MS Analysis

Weigh 2 g ± 0.05 g of soil into a 50 mL centrifuge tube and fortify the sample appropriately. Add 10 mL of extraction solvent (50/50 mix of methanol and water) and then vortex for 1

min followed by shaking via wrist action shaker for 15 min. Tubes are centrifuged for 10 min at 3500 rpm and 3 mL of the supernatant is transferred to a new 15 mL centrifuge tube containing QuEChERS mix of 150 mg Supelclean PSA, 150 mg Discovery DSC-18, and 900 mg $MgSO_4$. Tubes are vortexed for 1 min and then centrifuged for 10 min at 3500 rpm. Samples are diluted if necessary and then vialed for analysis. Instrumental analysis is accomplished using a LC-MS/MS system. This method is illustrated in Figure 48.

7.3. Linearity

A series of standards were prepared and analyzed to empirically determine the linearity of the detector response (1/x weighting was used as an option). The calibration range extended beyond (by at least 20%) the highest and lowest nominal concentration of the analyte in the relevant analytical solutions. Linearity was calculated as the correlation coefficient (r) resulting from a least squares equation that reflects the detector response as a function of the analyte concentrations.

7.4. Limits of Detection and Quantification

The lower limit of quantification (LOQ) for the method is 0.05 µg/g. The limit of detection (LOD) for the matrices is calculated from the data of the seven (7) LOQ recovery samples, as described in "Assigning Values to Non-detected/Non-quantified Pesticide Residues in Human Health Food Exposure Assessments, Item 6047, U.S. EPA, March 23, 2000." The LOD was determined to be 0.0120 µg/g for mancozeb and 0.0187 µg/g for ETU.

7.5. Validation of Confirmatory Techniques

Confirmation of the presence of the analyte was performed by using a primary quantitation transition ion and a confirmatory transition ion MRM with same retention time.

Calibration curve and linearity r values, recoveries for the fortified samples and precision data were calculated for the quantitation and confirmatory ions and are reported in the appendices.

7.6. Determination of Matrix Effects

Matrix matched standards were prepared for both dimethyl-EBDC and ETU, and analyzed to determine if any matrix interference was present. Matrix effects were not significant for either analyte, therefore neat solvent standards were used for both matrices; the results can be found in Table 10.

7.8. Dimethyl-EBDC and ETU Stability

Dimethyl-EBDC and ETU stock solutions were considered stable and can be used for up to 6 months as per JRFA SOPs. Calibration standard solutions, however, should be prepared fresh prior to instrument analysis to ensure accurate results.

7.10. Chromatographic Conditions

Typical Operating Conditions for Mancozeb and ETU:

The following LC-MS/MS parameters were used to determine the concentration of mancozeb and ETU residues in soil. The parameters may be modified to achieve adequate chromatographic resolution and/or detector sensitivity. The actual parameters used are documented with each HPLC-MS/MS analysis sequence in the raw data.

HPLC System:	Agilent 1290 HPLC System
MS Detector:	Sciex 6500 QTrap MS with Analyst™ software version 1.6.2
Mobile Phase A:	5 mM ammonium formate/0.1% formic acid in LC-MS grade water
Mobile Phase B:	0.1% formic acid in LC-MS grade MeOH
Flow Rate:	550 µL/min
Column:	HSS T3 waters 1.8µm 2.1 mm x 100 mm
Column Oven Temp:	Ambient
Injection Vol.	Dimethyl EBDC: 8 µL; ETU: 15 µL
Run Time:	8 minutes
Detector:	Sciex 6500 QTrap
Retention Time:	Dimethyl-EBDC: ~3.31 minutes, ETU: ~0.50-0.60 minutes

Mobile Phase Composition (linear gradient changes):

A gradient elution, using an increased percentage of organic solvent (methanol) in the mobile phase, is used to resolve interferences and improve separation. See the specific gradient listed below:

Time (Min)	A% (5 mM ammonium formate/ 0.1% formic acid in water)	B% (0.1% formic acid in MeOH)	Flow (µL/min)
0.00	90	10	550
1.00	90	10	550
2.00	50	50	550
3.00	10	90	550
5.50	10	90	550
6.00	90	10	550
8.00	90	10	550

Note: Retention times may differ depending upon the flow rate, column, and gradient used.

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition (*m/z*)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
EBDC (Quantitation)	*240.870→133.900*	60	31	25	12
EBDC (Confirmatory)	*240.922→193.000*	60	16	11	12
ETU (Quantitation)	*102.887→44.000*	160	51	19	6
ETU (Confirmatory)	*102.887→59.900*	160	51	45	8

Typical MS/MS Voltage Conditions Used:

Ionization Mode:	ESI
Scan Type	MRM
Polarity	Positive
Resolution Q1	unit
Resolution Q3	unit
Curtain gas (N_2, psi)	22
GS1 (psi)	90
GS2 (psi)	70
CAD gas (N_2)	High
Ion Spray (V)	5300
Temperature (°C)	520
EP (V)	10

7.11. Statistics and Sample Calculations: Mancozeb

Mancozeb, as dimethyl-EBDC residues, may be calculated in µg/g using a multi-point calibration procedure as follows:

1. Prepare standard solutions over a concentration range appropriate to the expected residues in the samples.
2. Make an injection of each standard solution and measure the areas under the peaks corresponding to dimethyl-EBDC. Calibration standard solutions should be interspersed throughout the analysis, after four injections of sample solutions.

3. Calibration standards and samples were analyzed using HPLC/MS-MS. Calibration curves and residue values were calculated using Analyst 1.6.2 data handling software using linear regression (1/x weighting is recommended).

   The standards were fit to the linear equation y = mx + b

   Where: x is the concentration of sample in final extract
   m is the calibration line slope
   b is the calibration line intercept
   y is the peak area

4. The following equation can be used to calculate residues as follows by accounting for the extraction volume and sample weight:

$$Mancozeb\ Found\left(^{\mu g}/_{g}\right) = \frac{Calc\ Conc\ \left(^{\mu g}/_{L}\right) * Final\ Volume\ (L) * Dilution\ Factor}{Sample\ Weight\ (g) * Conversion\ Factor * Efficiency\ yield\ factor}$$

   Where: Conversion factor = 1.11 (ratio of molecular weights of mancozeb and dimethyl-EBDC)

   Efficiency yield factor = 1.515 (1/0.66, as described in Reference 4)

Example: Soil sample UTC + PR LOQ R1 was analyzed
Sample weight = 2.01 g
Final volume = 0.010 L
Dilution factor = 2
Peak area in the quantitation transition was 2116234.6 counts
Calibration curve generated in the run was y = 222557.2447 * x + 240728.3697

$$x = \frac{y - b}{m}$$

$$x = \frac{2116234.6 - 240728.3697}{222557.2447}$$

$$x = 8.427\ ^{\mu g}/_{L}$$

$$^{\mu g}/_{g}\ Found = \frac{8.427\ ^{\mu g}/_{L} * 0.010\ L * 2}{2.01g * 1.11 * 1.515}$$

$$^{\mu g}/_{g}\ Found = 0.0499\ ^{\mu g}/_{g}$$

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/g) - Control\ concentration\ (\mu g/g)}{Theoretical\ concentration\ (\mu g/g)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Example: Soil sample for UTC+PR LOQ 1 was analyzed.

As no residues of the analyte were found in the control, the recovery was calculated as:

$$\%Recovery = \frac{0.0499\ \mu g/g - 0.00\ \mu g/g}{0.05\ \mu g/g} * 100\% = 100\%$$

The LOD was calculated using the seven LOQ sample data and the following equation:

$$LOD = Stdev(LOQ\ R1{:}\ LOQ\ R7) * t_{0.99}$$

The standard deviation is calculated using:

$$Stdev(LOQ\ R1{:}\ LOQ\ R7) = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Where: Stdev is the sample standard deviation of the calculated concentrations of the seven LOQ samples; n is number of samples, and $\bar{x}$ is the average calculated concentration

$t_{0.99}$ is the one-tailed t-statistic at the 99% confidence level for n-1 replicates and is equal to 3.143 for n=7 samples.

Example: Mancozeb in soil was analyzed.

$$LOD = Stdev(LOQ\ R1{:}\ LOQ\ R7) * 3.143$$

$$Stdev(LOQ\ R1{:}\ LOQ\ R7) = \sqrt{\frac{1}{7-1}\sum_{i=1}^{7}(x_i - 0.0478)^2} = 3.8\ \text{ng/g}$$

$$LOD = 3.8\frac{ng}{g} * 3.143 * \frac{1\ \mu g}{1000\ ng} = 0.0120\ \mu g/g$$

7.12. Statistics and Sample Calculations: ETU

ETU may be calculated in µg/g using a multi-point calibration procedure as follows:

1. Prepare standard solutions over a concentration range appropriate to the expected residues in the samples.
2. Make an injection of each standard solution and measure the areas under the peaks corresponding to ETU. Calibration standard solutions should be interspersed throughout the analysis, after four injections of sample solutions.
3. Calibration standards and samples were analyzed using HPLC/MS-MS. Calibration curves and residue values were calculated using Analyst 1.6.2 data handling software using linear regression (1/x weighting is recommended).

   The standards were fit to the linear equation y = mx + b

   Where: x is the concentration of sample in final extract
   m is the calibration line slope

b is the calibration line intercept

y is the peak area

4. The following equation can be used to calculate residues as follows by accounting for the final volume and sample weight:

$$ETU Found\ (^{\mu g}/_{g}) = \frac{Calc\ Conc\ (^{\mu g}/_{L}) * Final\ Volume\ (L) * Dilution\ Factor}{Sample\ Weight\ (g)}$$

No conversion or efficiency factor is used here because the ETU was not derivatized like mancozeb was.

Example: Soil sample UTC + PR LOQ R1 was analyzed.

Sample weight = 1.99 g

Final volume = 0.010 L

Dilution factor = 2

Peak area in the quantitation transition was 326515.525948 counts

Calibration curve generated in the run was y = 52465.51271 * x + 36211.28786

$$x = \frac{y - b}{m}$$

$$x = \frac{326515.525948 - 36211.28786}{52465.51271}$$

$$x = 5.533\ ^{\mu g}/_{L}$$

$$^{\mu g}/_{g}\ Found = \frac{5.533\ ^{\mu g}/_{L} * 0.010\ L * 2}{1.99\ g}$$

$$^{\mu g}/_{g}\ Found = 0.0556\ ^{\mu g}/_{g}$$

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/g) - Control\ concentration\ (\mu g/g)}{Theoretical\ concentration\ (\mu g/g)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Example: Soil sample UTC + PR LOQ R1 was analyzed.

As no residues of the analyte were found in the control, the recovery was calculated as:

$$\%Recovery = \frac{0.0556\ \mu g/g - 0.00\ \mu g/g}{0.050\ \mu g/g} * 100\% = 111\%$$

The LOD was calculated using the seven LOQ sample data and the following equation:

$$LOD = Stdev(LOQ\ R1{:}\ LOQ\ R7) * t_{0.99}$$

The standard deviation is calculated using:

$$Stdev(LOQ\ R1{:}\ LOQ\ R7) = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Where: Stdev is the sample standard deviation of the calculated concentrations of the seven LOQ samples; n is number of samples, and $\bar{x}$ is the average calculated concentration

$t_{0.99}$ is the one-tailed t-statistic at the 99% confidence level for n-1 replicates and is equal to 3.143 for n=7 samples.

Example: Soil was analyzed.

$$LOD = Stdev(LOQ\ R1{:}\ LOQ\ R7) * 3.143$$

$$Stdev(LOQ\ R1{:}\ LOQ\ R7) = \sqrt{\frac{1}{7-1}\sum_{i=1}^{7}(x_i - 0.0493)^2} = 5.94 \text{ ng/g}$$

$$LOD = 5.94\ ng/g * 3.143 * \frac{1\ \mu g}{1000\ ng} = 0.0187\ \mu g/g$$

8.2. Linearity

Linearity was confirmed through statistical analysis of the standard curve data including linear regression, and the least squares equation. The least squares equation is used as it reflects the detector response to the analyte with respect to concentration.

The raw data reflects all of these parameters.

8.3. Limit of Detection and Quantification

For this method, the LOD was determined to be 0.0120 µg/g for mancozeb and 0.0187 µg/g for ETU. The limit of quantification (LOQ) is set at 0.05 µg/g for both mancozeb and ETU.

The results are reported in the raw data and in the analytical method.

8.4. Sample Extract Stability

Validation trial samples were again analyzed approximately one week after initial instrument analysis. The results are summarized in Appendix I, Table 6 to Table 9.

8.5. Matrix Effects

No significant ion suppression was found for analysis of residues of mancozeb (as Dimethyl-EBDC) or ETU as determined through comparison of matrix-matched and solvent standards. Therefore, neat solvent standards were used for both analytes for soil. The results are summarized in Appendix I, Table 10.

Table 11 Soil Characteristics Data (See Appendix IX for Characterization Report)

Percent Sand	86	
Percent Silt	9	
Percent Clay	5	
USDA Textural Class (hydrometer method)	Loamy Sand	
Bulk Density (disturbed) (gm/cc)	1.27	
Cation Exchange Capacity (meq/100 g)	6.6	
Percent Moisture at 1/3 Bar	8.8	
% Organic Matter - Walkley Black	2.0	
pH in 1:1 soil:water ratio	6	
Base Saturation Data		
Cation	**Percent**	**ppm**
Calcium	31.2	415
Magnesium	9.0	72
Sodium	0.5	8
Potassium	3.9	100
Hydrogen	55.4	37

Figure 47 Analysis Flowchart – Mancozeb Conversion to Dimethyl-EBDC for Soil Analysis

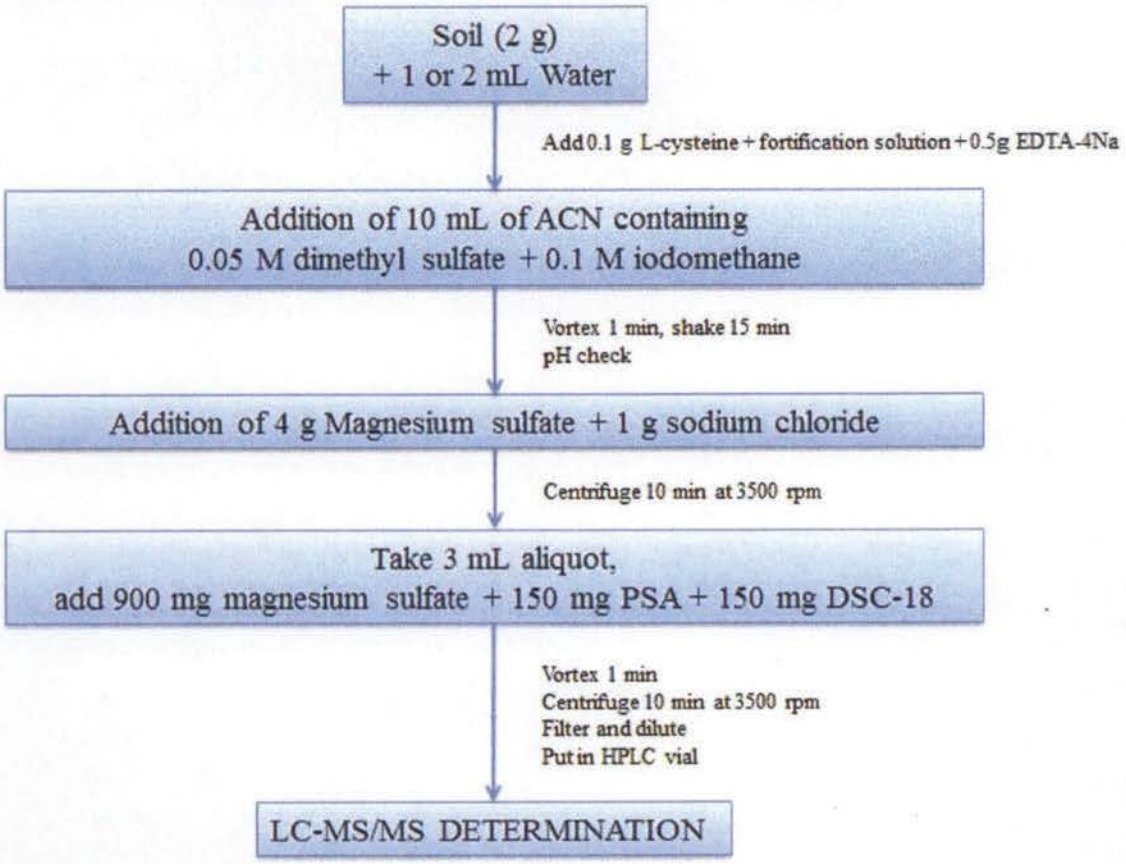
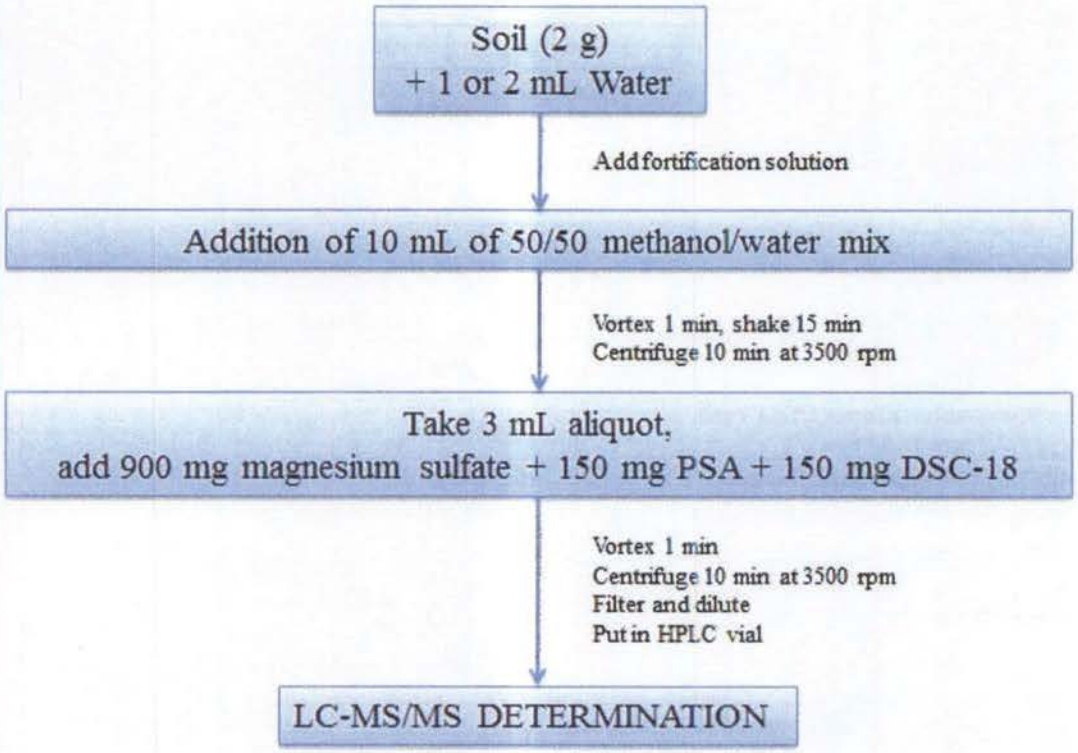


Figure 48 Analysis Flowchart – ETU Soil Analysis



13.3. Appendix III: Equipment and Materials

Equipment		
Electronic Pipettes – Eppendorf Xplorer	100 µL	R10117C
	1000 µL	117127A
	1000 µL	J20536C
	1000 µL	H73677B
	5 mL	R32413C
	5 mL	R32379C
	10 mL	3105819
Centrifuge – Beckham Coulter	Allegra 6R	ALROOJA3
Column: HSS T3 waters 1.8 um 2.1 mm x 100 mm	015133305157	
Column: Phenomenex Kinetix C18 1.7 µm 2.1 mm x 100 mm	504004-58	
Mettler PC2000 Top-loading Balance	A50795	
Mettler Toledo AT 200 Analytical Balance	K51405	
Wrist Action Shaker	Burnell model 75	
Benchmark Bench Mixer BV 1000	13091401	
	13112382	
	13112215	

Solvents/Liquid Reagents		
Water (LC-MS grade)	EMD	56190
		56253
		57153
		57156
		57126
Acetonitrile (LC-MS grade)	EMD	57121
		56110
		57090
Methanol (LC-MS grade)	EMD	56118
		57135
Formic Acid (LC-MS grade)	Fluka	BCBR2425V
		BCBS54853V
Iodomethane	Sigma-Aldrich	SHBH8999
Dimethyl Sulfate	Sigma-Aldrich	BCBN8964V
Isopropyl alcohol	Alfa Aesar	R25D002

13.3 Appendix III: Equipment and Materials – (continued)

QuEChERS	
Q1 ($MgSO_4$/NaCl) UCT; Cat No. ECMSSC-MP	108861-AV 023581-BA
Q2 ($MgSO_4$/PSA/C18) Agilent; Cat No. 5982-5022	8013601 8899301 8690401

Solid Reagents		
Ammonium formate	Fisher	157841
L-cysteine	Sigma-Aldrich	MKBQ7669V
		STBG4139V
EDTA-4Na	Beantown Chem	50002646

13.5. Appendix V: Protocol

JRF AMERICA

PROTOCOL/STUDY PLAN

STUDY TITLE: Method Development and Validation of Mancozeb and ETU Analysis in Soil

Protocol – (continued)

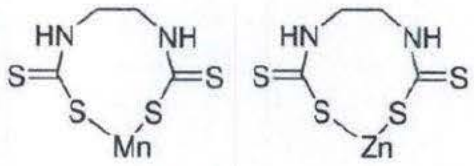
4.0 JUSTIFICATION OF TEST SYSTEM

The method AU-275R0 will be validated in soil. The matrix will be selected to be representative of typical soils, and collected as per guideline requirements. Information such as collection location, characteristics and storage will be reported in the raw data and final report.

5.0 TEST SUBSTANCE

The test substance will be mancozeb and ETU. The following sample information and chemical/physical properties have been provided with the test substance sample:

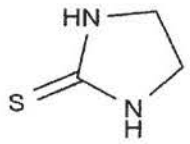
Common Name:	Mancozeb
IUPAC Chemical Name:	Zinc Manganese Ethylenebisdithiocarbamate
CAS Registry No.:	8018-01-7
Molecular Formula:	$(C_8H_{12}MnN_4S_8)_x(Zn)_y$, where x:y = 1:0.091
Molecular Weight:	266.51 $g \cdot mol^{-1}$
Batch No.:	SZBE225XV
Reassay Date:	August 1, 2017
Purity:	97.5%
Storage Condition:	Refrigerated
Source:	Sigma-Aldrich
Structure:	



JRFA SAS-007-OOO

Protocol – (continued)

Common Name:	Ethylenethiourea (ETU)
IUPAC Chemical Name:	2-Imidazolidinethione
CAS Registry No.:	96-45-7
Molecular Formula:	$C_3H_6N_2S$
Molecular Weight:	102.16 $g \cdot mol^{-1}$
Batch No.:	SZBC242XV
Expiration Date:	February 3, 2018
Purity:	99.7%
Storage Condition:	Ambient
Source:	Sigma-Aldrich
Structure:	



JRFA SAS-007-PPP

An archive sample of the substance mancozeb and ETU will be retained (under the same storage conditions as detailed above) at JRF America.

6.0 REAGENTS AND SOLVENTS

Materials such as reagents or solvents will be obtained by JRF America. Chemicals will be of reagent grade or higher as applicable to their use.

7.0 SAMPLE IDENTIFICATION

Soil matrix will be acquired by JRF America. Matrix will be tested to ensure no mancozeb or ETU is already present. The untreated control samples will be identified with unique sample numbers assigned by JRF America personnel during sample check-in. These numbers will be used to track the samples during receipt, storage, and analysis. Sample preparation sheets will be used to further describe and track the fortification of control samples during the method validation.

8.0 PROCEDURES

The accuracy and precision of the residue method AU-275R0 will be determined using freshly fortified untreated control samples. Accuracy will be calculated as the percent recovery while the precision will be calculated using the statistical treatments described in Section 15 below.

Recovery samples will be prepared by fortifying the untreated samples with the appropriate amounts of the analyte. At least one reagent blank and two unfortified control samples will be included in the validation sample set.

Protocol – (continued)

Sample ID	Number of Sample
Reagent Blank	1
Control	2
LOQ	7
10X LOQ	5
TOTAL	15

Additional control, reagent blank, and recovery samples may be analyzed (at the discretion of the study director) as needed to generate additional recovery data or verify the calculated limits of detection and quantitation as described in Section 10.

9.0 LINEARITY

A series of standards will be prepared and analysed to empirically determine the linearity of the detector response ($1/x$ or $1/x^2$ weighting can be used as an option). The calibration range will extend beyond (by at least 20%) the highest and lowest nominal concentration (30% of LOQ, if sensitivity allows for it) of the analyte in the relevant analytical solutions. Linearity will be calculated as the coefficient of determination (r^2) resulting from a least squares equation that reflects the detector response as a function of the analyte concentrations.

10.0 LIMITS OF DETECTION AND QUANTITATION

The limit of detection (LOD) for the matrices will be calculated from the data of the seven (7) LOQ recovery samples, as described in "Assigning Values to Non-detected/Non-quantified Pesticide Residues in Human Health Food Exposure Assessments, Item 6047, U.S. EPA, March 23, 2000". The limit of quantitation (LOQ) is set at 0.05 mg/kg for soil matrix as per toxicology and guideline requirements.

11.0 ACCEPTABILITY OF RECOVERIES AND METHOD RUGGEDNESS

For the method validation, the mean recoveries at each fortification level should fall within the range of 70-120%. The relative standard deviation (RSD) of replicate recovery measurements should not exceed the level of 20% at or above the LOQ, and any interference should be negligible (≤30% of the response found in a sample fortified at the LOQ). The ruggedness of the method will be demonstrated by the recovery over the seven replicate samples fortified at the LOQ and the five replicate samples fortified at 10X LOQ. A rugged method will show good recovery at both concentration levels and for multiple replicates.

12.0 VALIDATION OF CONFIRMATION TECHNIQUE

Confirmation of the presence of the analyte will be performed by, simultaneously with the primary transition, monitoring an additional MRM transition for the same precursor ion at the same retention time.

Protocol – (continued)

Calibration curve linearity r^2 value, recoveries for the fortified samples and precision data, as well as the results in blank samples, will be calculated for the confirmatory transition and reported in the final report. A parent ion spectrum will be presented in the final report.

13.0 DETERMINATION OF MATRIX EFFECTS

Matrix effects will be investigated by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions for each matrix type. Experiments should assess whether or not matrix effects are significant (i.e. > 20% enhancement or suppression). Matrix-effects for both the quantitation and the confirmatory transitions will be reported. If matrix effects are acceptable, standards prepared in neat solvent may be used for the duration of the study.

14.0 DETERMINATION OF SAMPLE EXTRACT AND WORKING SOLUTION STABILITY

The stability of the sample extracts will be evaluated based upon comparison of sample extract(s) injected after initial preparation and then injected a second time after storage for 7 ± 3 days. Sample extracts will be stored in a freezer at approximately -20° C. Stock, fortification, and calibration standard solutions stability will also be evaluated by storing aliquots under the same conditions and tested after storage for 7 ± 3 days.

15.0 STATISTICAL TREATMENT

Descriptive statistics such as coefficients of determination, means, standard deviations, and relative standard deviations will be used for this study. Additional statistical calculations may be used if necessary.

Protocol – (continued)

18.0 PROTOCOL AMENDMENTS AND DEVIATIONS

Changes to this protocol/study plan will be documented and the reason for the change stated, signed, and dated by the Study Director. A copy of each amendment will be retained with the protocol. Additionally, a copy of each amendment will be sent to the Sponsor/Study Monitor for signature.

In the event of a protocol/study plan deviation, a written description of the deviation including the reason for the deviation and any impact on the study as a result of the deviation will be signed and dated by the study director and maintained with the protocol/study plan.

19.0 REPORT

A draft, unsigned final report, and final report presenting the procedures and results of this study will be prepared and submitted to the Sponsor/Study Monitor for review. The format will be either the standard US EPA PRN 2011-3 format or a format supplied by the Sponsor/Study Monitor. The final report will include, but will not be limited to, the following:

1. GLP compliance statement signed by the Study Director;
2. Quality Assurance Statement showing the phases inspected, inspection dates, and dates inspection results were reported to the study director and management;
3. Study information containing the identification of testing facility, location of the raw data, study dates, and name of the Study Director;
4. Names of principal study personnel and management;
5. Information on the analytical standard. Method of preparation of standard solution, the amount and identity of any co-solvent used;
6. Description of experimental design and experimental procedures, and any deviations from the procedures stated in the protocol;
7. Example chromatograms and calculations;
8. Details of sample extraction, clean-up procedure and dilution;
9. Details of instrument analysis;
10. Specificity;
11. Linearity;
12. Accuracy;
13. Precision;

13.8. Appendix VIII: Analytical Method

JRF AMERICA

Mancozeb

Analytical Method for the Determination of Mancozeb and ETU in Soil

JRFA Method No. AU-275R0

Analytical Method – (continued)

ABBREVIATIONS AND SYMBOLS

Abbreviation	Definition
°C	degrees Celsius or Centigrade
CAS	Chemical Abstract Services
cm	centimeter
EPA	Environmental Protection Agency (U.S.)
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
g	gram
HPLC	high performance liquid chromatography
i.d.	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
kg	kilogram
L	litre
LC-MS/MS	tandem liquid chromatography/mass spectrometry/mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m	meter
ACN	acetonitrile
MeOH	methanol
DCM	dichloromethane
µg	microgram
µL	microliter
µm	micrometer
mg	milligram
mL	milliliter
mm	millimeter
mmol	millimole
min	minute
mol	mole
ms	millisecond
MS/MS	tandem mass spectrometry
mV	millivolt
MW	molecular weight

Analytical Method – (continued)

Abbreviations and Symbols (continued)

Abbreviation	Definition
m/z	mass to charge ratio
N/A	not applicable
ND or nd	not detectable (below limit of detection)
ng	nanogram
No.	number
OES	Occupational Exposure Standards
OECD	Organisation for Economic Co-operation and Development
OCSPP	Office of Chemical Safety and Pollution Prevention
pg	picogram
ppb	parts per billion or micrograms per kilogram or micrograms per liter
ppm	parts per million or milligrams per kilogram or milligrams per liter
R^2 (or r^2)	square of correlation coefficient
RSD	relative standard deviation
Rt	retention time
s	second
SD	standard deviation
SPE	Solid Phase Extraction
PSA	Primary and secondary amine
UPW	ultra pure water
V	volt
vol	volume

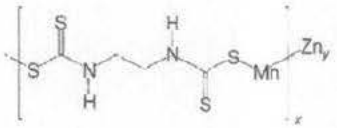
Analytical Method – (continued)

1.0 INTRODUCTION

The chemical structures of mancozeb and ethylenethiourea (ETU) are summarized as follows:

Common Name:	**Mancozeb**
Chemical Name (IUPAC):	Zinc Manganese Ethylenebisdithiocarbamate
CAS Registry No.:	8018-01-7
Molecular Formula:	$(C_8H_{12}MnN_4S_8)_x(Zn)_y$
Molecular Weight:	266.51 $g \cdot mol^{-1}$
Batch No.:	SZBE225XV
Reassay Date:	July 20, 2019
Purity:	97.5%
Storage Condition:	Refrigerated
Source:	Sigma-Aldrich

Structure:



JRFA SAS-007-OOO

Common Name:	**Ethylenethiourea (ETU)**
Chemical Name (IUPAC):	2-Imidazolidinethione
CAS Registry No.:	96-45-7
Molecular Formula:	$C_3H_6N_2S$
Molecular Weight:	102.16 $g \cdot mol^{-1}$
Batch No.:	SZBC242XV
Reassay Date:	February 3, 2018

Analytical Method – (continued)

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in **APPENDIX 1**. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents are to be of high purity, e.g., glass distilled/HPLC grade solvents and analytical grade reagents. Water must be deionized prior to use or purchased HPLC grade water utilized. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in **APPENDIX 2**.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation
2. Wear gloves, protective eyewear and lab coat
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated areas

2.3.1 Stock Solutions

Mancozeb stock standard solution was prepared in 50:50 isopropyl alcohol (IPA):water solution. The following is the description for preparing 250 mL of a 16,400 µg/L stock standard.

1. A mass of 0.0042 g of mancozeb reference standard is weighed (adjusted for purity of 97.5%) and transferred to a 0.250 L class A volumetric flask.
2. Fill the volumetric flask halfway with 50:50 IPA/H_2O and agitate gently (sonicate if necessary) until standard is completely dissolved.
3. Dilute to volume with 50:50 IPA/H_2O and mix by inverting several times.
4. Calculate the exact concentration using the exact weight and purity, for example:

Analytical Method – (continued)

$$\left(\frac{0.0042g*0.975}{0.250\,L}\right)*\left(\frac{10^6\mu g}{g}\right) = 16{,}400\ \mu g/L$$

Dimethyl EBDC stock standard solution was also prepared, as mancozeb is derivatized for analysis. EBDC and ETU stock was prepared in acetonitrile. The preparation and calculation of these stocks follow a similar procedure as mancozeb. The table below lists the stock solutions prepared and used during this study.

Analyte ID	JRFA ID	Purity	Weight (g) [1]	Final Volume (mL) [2]	Concentration (µg/L)
Mancozeb	JRFA-501/9-1	97.5%	0.0041	250	16400
Dimethyl EBDC	JRFA-501/5-3	97.7%	0.0118	100	118000
Dimethyl EBDC	JRFA-501/11-2	97.7%	0.0102	100	102000
ETU	JRFA-501/5-4	99.7%	0.0115	100	115000
ETU	JRFA-501/12-1	99.7%	0.0105	100	105000

[1] Corrected for purity

2.3.2 Preparation of Fortification Solutions

Sample fortification solutions should be prepared by serial dilution of the stock standard in 50/50 isopropanol/water for mancozeb and acetonitrile for EBDC and ETU. The following concentrations are prepared for fortification standards: 10,000 µg/L, and 1,000 µg/L. The table bellows lists the stock solutions prepared and used during the validation.

Analyte ID	JRFA ID Starting Solution	Starting Concentration (µg/L)	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	JRFA ID Final Solution
Mancozeb	JRFA-501/9-1	16400	30.488	50.0	10000	JRFA-501/9-3
Mancozeb	JRFA-501/9-3	10000	10.0	100.0	1000	JRFA-501/9-4
ETU	JRFA-501/5-4	115000	8.70	100.0	10000	JRFA-501/8-3
ETU	JRFA-501/8-3	10000	10.0	100.0	1000	JRFA-501/8-4
ETU	JRFA-501/12-1	105000	0.476	50.0	1000	JRFA-501/12-2
Dimethyl-EBDC	JRFA-501/5-3	118000	0.847	100.0	1000	JRFA-501/8-5
Dimethyl-EBDC	JRFA-501/11-2	102000	0.490	50.0	1000	JRFA-501/11-3

2.3.3 Preparation of Calibration Standards for LC-MS/MS

Calibration solutions suitable for LC-MS/MS analysis should be prepared in solvent (95:5 water:acetonitrile). At least four levels of external calibration standards should be prepared to develop calibration curves for calculation of sample residues. Typical dilution schemes used to prepare the LC-MS/MS calibration solutions for each analyte are as follows:

Analytical Method – (continued)

Analyte	JRFA ID Starting Solution	Starting Concentration (µg/L)	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	JRFA ID Final Solution
Dimethyl -EBDC	JRFA-501/8-5	1000	1.00	50.0	20	JRFA-501/8-12
Dimethyl -EBDC	JRFA-501/8-12	20	25.0	50.0	10	JRFA-501/8-13
Dimethyl -EBDC	JRFA-501/8-13	10	25.0	50.0	5	JRFA-501/8-14
Dimethyl -EBDC	JRFA-501/8-14	5	25.0	50.0	2.5	JRFA-501/8-15
Dimethyl -EBDC	JRFA-501/8-15	2.5	20.0	50.0	1	JRFA-501/8-16
ETU	JRFA-501/8-6	20	25.0	50.0	10	JRFA-501/8-7
ETU	JRFA-501/8-7	10	25.0	50.0	5	JRFA-501/8-8
ETU	JRFA-501/8-8	5	25.0	50.0	2.5	JRFA-501/8-9
ETU	JRFA-501/8-9	2.5	20.0	50.0	1	JRFA-501/8-10

2.3.4 Standard Solution Storage and Expiration

EBDC and ETU stock and standard solutions should be stored in a refrigerator (~ 4° C) when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use. An expiration date of 6 months is recommended for the EBDC-dimethyl and ETU stock standard solutions, and 3 months for fortification standards and calibration standards as per JRFA SOPs.

Stock and fort solutions of mancozeb should be prepared fresh and used immediately on the day of analysis, due to fast hydrolysis of mancozeb.

2.4 Safety Precautions and Hazards

All caution should be exercised when handling pure material or concentrated stock solutions. Avoid skin contact and inhalation. See Safety Data Sheet (SDS) documentation accompanying standard shipment. All personnel should be familiar with all solvents and equipment precautions and hazards prior to use.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples should be prepared using an approved method for sample preparation for residue analysis. Soil samples should be kept frozen.

Analytical Method – (continued)

Mancozeb Sample Preparation

1. Measure 2 g ± 0.05 g of soil into 50 mL centrifuge tube.
2. Add 1-2 mL of water to each sample. Add 0.1 g of L-cysteine, fortify samples with the proper amount (if necessary), then add 0.5 g of EDTA-4Na.
3. Add 10 mL of acetonitrile containing 0.05 M dimethyl sulfate + 0.1 M iodomethane. Vortex for 1 min, then shake via wrist action shaker for 15 min.
4. One packet of QuEChERS mix of 4 g anhydrous MgSO4 + 1 g NaCl is added to the samples and shaken for 1 min.
5. Centrifuge samples at 3500 rpm for 10 min.
6. 3 mL of supernatant is transferred to a new 15 mL centrifuge tube containing QuEChERS mix of 150 mg Supelclean PSA, 150 mg Discovery DSC-18, and 900 mg $MgSO_4$.
7. Tubes are vortexed for 1 min and centrifuged for at 3500 rpm 10 min.
8. Supernatant is diluted as necessary and vialed.
9. Analyze via LC-MS/MS.

ETU Sample Preparation

1. Measure 2 g ± 0.05 g of soil into 50 mL centrifuge tube.
2. Fortify samples with the proper amount (if necessary).
3. Add 10 mL of 50/50 methanol/water extraction solvent. Vortex for 1 min, then shake for 15 min via wrist action shaker.
4. Centrifuge at 3500 rpm for 10 min.
5. 3 mL of supernatant is transferred to a new 15 mL centrifuge tube containing QuEChERS mix of 150 mg Supelclean PSA, 150 mg Discovery DSC-18, and 900 mg $MgSO_4$.
6. Tubes are vortexed for 1 min and centrifuged at 3500 rpm for 10 min.
7. Supernatant is diluted as necessary and vialed.
8. Analyze via LC-MS/MS.

3.2 Time Required for Analysis

The methodology is normally performed with a batch of 15 samples. On average, one chemist can complete the analysis of one batch of 15 samples including instrument analysis and data processing in a period of 8 working hours.

3.3 Method Stopping Points

No stop point is considered necessary.

3.4 Modifications and Potential Problems

Mancozeb samples should be analyzed as soon as possible after extraction due to fast hydrolysis

Analytical Method – (continued)

of mancozeb. ETU extracts remain stable one week after extraction, as determined by stability testing. An expiration date of 6 months is recommended for the EBDC stock standard solution, and 3 months for EBDC calibration standards as per JRFA SOPs. Standard solutions should be made fresh, if consistent decrease of response is noticed. Stock and fort solutions of mancozeb should be prepared fresh and used immediately on the day of analysis. Note that degradation occurs greatly as concentration increases.

4.0 FINAL DETERMINATION

The method has been developed for use on a Sciex 6500 QTrap MS and Agilent 1290 system. The following instrumentation and conditions can be used as a general guidance. Other instrumentation, column and mobile phases can also be used, though optimization may be required to achieve the desired separation and sensitivity.

4.1 Instrument Description

HPLC System:	Agilent 1290 HPLC System
Detector:	Sciex 6500 QTrap MS with Analyst™ software version 1.6.2

4.2 Chromatography Conditions for Mancozeb Analysis

Mobile Phase A:	5 mM ammonium formate/0.1% formic acid in LC-MS H_2O
Mobile Phase B:	0.1% formic acid in LC-MS grade MeOH
Flow Rate:	550 µL/min
Column:	HSS T3 waters 1.8µm 2.1 mm x 100 mm
Column Oven Temp:	Ambient
Injection Vol.	EBDC: 8 µL; ETU: 15 µL
Run Time:	8 minutes
Detector:	Sciex 6500 QTrap
Retention Time:	EBDC: ~3.31; ETU: ~0.50-0.60 min

Mobile Phase Composition (linear gradient changes):

A gradient elution, using an increased percentage of organic solvent (methanol) in the mobile phase, is used to resolve interferences and improve separation. See the specific gradient listed below:

Analytical Method – (continued)

Time (Min)	A% (5 mM ammonium formate/0.1% formic acid in water)	B% (0.1% Formic Acid in MeOH)	Flow (µL/min)
0.00	90	10	550
1.00	90	10	550
2.00	50	50	550
3.00	10	90	550
5.50	10	90	550
6.00	90	10	550
8.00	90	10	550

Note: Retention times may differ depending upon the flow rate, column, and gradient used.

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition (*m/z*)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
EBDC (Quantitation)	*240.870→133.900*	60	31	25	12
EBDC (Confirmatory)	*240.922→193.000*	60	16	11	12
ETU (Quantitation)	*102.887→44.000*	160	51	19	6
ETU (Confirmatory)	*102.887→59.900*	160	51	45	8

Typical MS/MS Voltage Conditions Used:

Ionization Mode:	ESI
Scan Type	MRM
Polarity	Positive
Resolution Q1	unit
Resolution Q3	unit
Curtain gas (N_2, psi)	22
GS1 (psi)	90
GS2 (psi)	70
CAD gas (N_2)	High
Ion Spray (V)	5300
Temperature (°C)	520
EP (V)	10

Initial and Final Q1 and Product Scans can be found in **APPENDIX 6**.

Note: The MS settings as provided above should be used as guidelines only. For optimal results, compound and source optimization should be performed by the analyst.

Analytical Method – (continued)

5.0 CALCULATION OF RESULTS: DIMETHYL EBDC

5.1 Multi Point Calibration Procedure

Mancozeb, as EBDC residues, may be calculated in µg/g using a multi-point calibration procedure as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples. An appropriate number of different concentrations within this range should be prepared (at least four).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to EBDC. Calibration standard solutions should be interspersed throughout the analysis, after approximately five injections of sample solutions.

c) Calibration standards and samples were analyzed using HPLC/MS-MS. Calibration curves and residue values were calculated using Analyst 1.6.2 data handling software using linear regression (1/x weighting is recommended).

The standards were fit to the linear equation $y = mx + b$

Where: x is the concentration of sample in final extract
m is the calibration line slope
b is the calibration line intercept
y is the peak area

d) The following equation can be rearranged and used to calculate residues as follows by accounting for the extraction volume and sample mass:

$$Mancozeb\ Found\ \left(^{\mu g}/_{g}\right) = \frac{Calc\ Conc\ \left(^{\mu g}/_{L}\right) * Final\ Volume\ (L) * Dilution\ Factor}{Sample\ Weight\ (g) * Conversion\ Factor * Efficiency\ yield\ factor}$$

Where: Conversion factor = 1.11 (ratio of molecular weights of mancozeb and dimethyl-EBDC)
Efficiency yield factor = 1.515 (1/0.66, as described in Reference 4)

Note: Efficiency yield factor is determined/established as per matrix.

5.2 Example Calculation

Soil sample UTC + PR LOQ R1 was analyzed

Sample weight = 2.01 g

Final volume = 0.010 L

Dilution factor = 2

Peak area in the quantitation transition was 2116234.6 counts

Analytical Method – (continued)

Calibration curve generated in the run was y = 222557.2447 * x + 240728.3697

$$x = \frac{y - b}{m}$$

$$x = \frac{2116234.6 - 240728.3697}{222557.2447}$$

$$x = 8.427\ {}^{\mu g}/_{L}$$

$${}^{\mu g}/_{g}\ Found = \frac{8.427\ {}^{\mu g}/_{L} * 0.010\ L * 2}{2.01 g * 1.11 * 1.515}$$

$${}^{\mu g}/_{g}\ Found = 0.0499\ {}^{\mu g}/_{g}$$

5.3 Recovery Calculation

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/g) - Control\ concentration\ (\mu g/g)}{Theoretical\ concentration\ (\mu g/g)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

5.4 Example Calculation

Soil sample for UTC+PR LOQ 1 was analyzed.

As no residues of the analyte were found in the control, the recovery was calculated as:

$$\%Recovery = \frac{0.0499\ \mu g/g - 0.00\ \mu g/g}{0.05\ \mu g/g} * 100\% = 100\%$$

6.0 CALCULATION OF RESULTS: ETU

6.1 Multi Point Calibration Procedure

ETU residues may be calculated in µg/g using a multi-point calibration procedure as follows.

Analytical Method – (continued)

e) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples. An appropriate number of different concentrations within this range should be prepared (at least four).

f) Make an injection of each sample solution and measure the areas of the peaks corresponding to ETU. Calibration standard solutions should be interspersed throughout the analysis, after approximately five injections of sample solutions.

g) Calibration standards and samples were analyzed using HPLC/MS-MS. Calibration curves and residue values were calculated using Analyst 1.6.2 data handling software using linear regression (1/x weighting is recommended).

The standards were fit to the linear equation y = mx + b

Where: x is the concentration of sample in final extract
m is the calibration line slope
b is the calibration line intercept
y is the peak area

h) The following equation can be rearranged and used to calculate residues as follows by accounting for the extraction volume and sample mass:

$$ETU\ Found\ (^{\mu g}/_{g}) = \frac{Calc\ Conc\ (^{\mu g}/_{L}) * Final\ Volume\ (L) * Dilution\ Factor}{Sample\ Mass\ (g)}$$

No conversion or efficiency factor is used here because the ETU was not derivatized like mancozeb was.

6.2 Example Calculation

Soil sample UTC + PR LOQ R1 was analyzed.
Sample weight = 1.99 g
Final volume = 0.010 L
Dilution factor = 2

Peak area in the quantitation transition was 326515.525948 counts
Calibration curve generated in the run was y = 52465.51271 * x + 36211.28786

$$x = \frac{y - b}{m}$$

$$x = \frac{326515.525948 - 36211.28786}{52465.51271}$$

Analytical Method – (continued)

$$x = 5.533\,{}^{\mu g}/_{L}$$

$${}^{\mu g}/_{g}\ Found = \frac{5.533\,{}^{\mu g}/_{L} * 0.010\ L * 2}{1.99\ g}$$

$${}^{\mu g}/_{g}\ Found = 0.0556\,{}^{\mu g}/_{g}$$

6.3 Recovery Calculation

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/g) - Control\ concentration\ (\mu g/g)}{Theoretical\ concentration\ (\mu g/g)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

6.4 Example Calculation

As no residues of the analyte were found in the control, the recovery was calculated as:

$$\%Recovery = \frac{0.0556\ \mu g/g - 0.00\ \mu g/g}{0.050\ \mu g/g} * 100\% = 111\%$$

7.0 UNTREATED CONTROL AND RECOVERY SAMPLES

If untreated control samples are available, untreated control samples should be analyzed for each set of samples analyzed to verify that samples are free from analyte contamination. A minimum of one control should be analyzed with each batch of samples.

A total of two recovery samples, which are untreated samples accurately fortified with a known amount of mancozeb, should also be analyzed in each analytical set. The recovery levels should be run at the LOQ and a higher level to encompass the treated sample results.

8.0 SPECIFICITY

8.1 Labware Interference

All reusable glassware is suggested to be detergent washed in hot water and then rinsed with deionized water and acetone prior to use.

Analytical Method – (continued)

8.2 Reagent and Solvent Interference

None.

9.0 METHOD VALIDATION

Method validations have been carried out on the procedures described in this method for analysis of mancozeb in soil. The following discussion is based on the validation data.

9.2 Matrix Effect

No significant ion suppression was found for analysis of residues of mancozeb (as Dimethyl-EBDC) or ETU as determined through comparison of matrix-matched and solvent standards. Therefore, solvent standards were used for both validation sets.

9.3 Limit of Quantification (LOQ)

The limit of quantification (LOQ) is defined as the lowest analyte concentration in a sample at which the methodology has been validated. Generally, for accurate quantitation, the response for an analyte peak should be no lower than four times the mean amplitude of the background noise in an untreated sample at the corresponding retention time. The LOQ for the analysis of mancozeb has been set at 0.05 µg/g.

9.4 Limit of Detection (LOD)

The limit of detection of the method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample at the corresponding retention time. The limit of detection (LOD) for the matrices is calculated from the data of the seven (7) LOQ recovery samples, as described in "Assigning Values to Non-detected/Non-

Analytical Method – (continued)

quantified Pesticide Residues in Human Health Food Exposure Assessments, Item 6047, U.S. EPA, March 23, 2000". For this method, the LOD was determined to be 0.0120 µg/g for mancozeb and 0.0187 µg/g for ETU.

9.5 Detector Linearity

For accurate quantification of residue concentrations, analyses should be carried out within the linear range of the MS-MS detector response. Linearity of the MS-MS detector is assured by the development of a calibration curve with each batch injected. It has been shown that the HPLC/MS-MS detector responses are generally linear in the range from 1 µg/L to 10 µg/L.

9.6 Stability of Analytes in Sample Extracts and in Final Solutions

Samples should be analyzed within one week of receipt or preparation. Mancozeb stock and fort solutions should be prepared and used immediately on the day of analysis.

10.0 LIMITATIONS

The method has been tested on soil. It can reasonably be assumed that the method can be applied to other types of soil. Test experiments for the recovery, matrix effects, interferences and sensitivity, etc. are strongly suggested prior to sample analysis.

Analytical Method – (continued)

APPENDIX 2 REAGENTS

A. Solvents and Reagents

1. LC/MS grade solvents or better should be utilized. Other brands and grades of solvents may be substituted as long as they do not produce interferences with the chromatography.

   a. Acetonitrile, EMD Millipore Corporation, Billerica, MA
   b. Water, EMD Millipore Corporation, Billerica, MA
   c. Isopropyl Alcohol, Alfa Aesar, Ward Hill, MA
   d. Methanol, EMD Millipore Coporation, Billerica, MA

2. Working Solutions

   a. Mobile Phase A – 5 mM ammonium formate/0.1% formic acid in water:

      Weigh out 0.3041 g NH_4HCO_2 and 1.00 mL of formic acid, transfer to 1000-mL volumetric flask. Bring to volume with water.

   b. Mobile Phase B – 0.1% formic acid in MeOH:

      Transfer 1.00 mL of formic acid to a 1000-mL volumetric flask. Bring to volume with MeOH.

   c. 0.05 M Dimethyl Sulfate + 0.1 M Iodomethane in ACN:

      Transfer 2.36 mL of dimethyl sulfate and 3.11 mL of iodomethane to a 500 mL volumetric flask and bring to volume with LC-MS ACN.

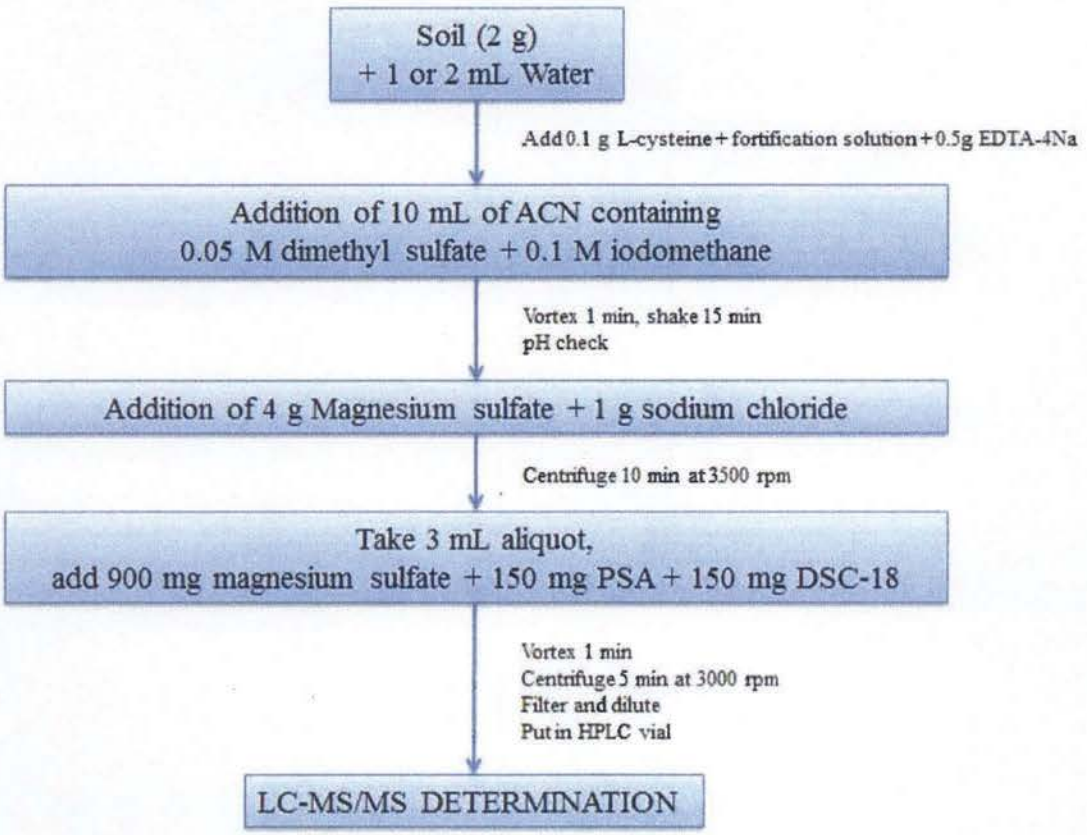
   d. 50:50 H_2O/MeOH:

      100 mL water and 100 mL MeOH were measured in separate graduated cylinders and mixed in a flask.

Analytical Method – (continued)

APPENDIX 5 ANALYSIS FLOWCHARTS

Figure 41 Mancozeb conversion to Dimethyl-EBDC method for soil analysis



Analytical Method – (continued)

Figure 42 ETU method for soil analysis