JRFAMERICA 50452901

FINAL REPORT

TITLE

Method Validation for Analysis of Mancozeb in Surface and Drinking Waters

TEST GUIDELINE(S)
OCSPP 860.1340, SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1

ABBREVIATIONS AND SYMBOLS

Abbreviation	Definition
oc	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
mlz	mass to charge ratio
N*IA*	not applicable
NDornd	not detectable (below limit of detection)
ng	nanogram
No.	number
OBS	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 mmigrams 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 jmproved solubility, stability, and instrument sensitivity.

The metal atoms in mancozeb are decoupled using ethylenedjaminetetraacetic acid (EDTA), and the resulting complex is further stabilized by ammonium fonnate. The complex is then methylated with methyl iodide (CH3I) and dimethyl sulfate ($(CH_3)_2SO_4$) to form dimethyl EBDC (M.W. =240.50) as follows (note: equation is not balanced):

Mancozeb + EOTA + NH. + CH3I -+ (CH2)i(CH3)z(CS2h(NH)2
M.W. 266.51 M.W. 240.50

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

3.0 EQUIVALENCESTATEMENT

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

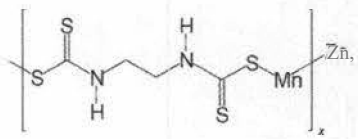
The chemicals used in this study shouJd be treated as potential health hazards and exposur1♦ to these chemicaJs should be m[nimized. The analyst is responsible for maintaining awareness of OSHA (Occupational Safety and Health Administration) regulations regarding the safe handling of the chemicals used 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

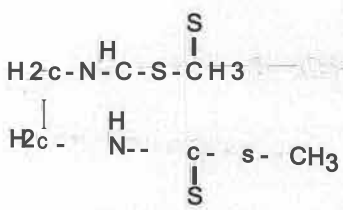
The chemical structures of mancozeb and dimethyl-EBDC are summarized as follows:

Common Name:	**Mancozeb**
Chemical Name (IUPAC):	Zinc Manganese Ethylenebisdithiocarbamate
CAS Registry No.:	8018-01-7
Molecular Weight:	266.5} g·mol^{-1}
Batch No.:	SZBE22SXV
Reassay Date:	August 1, 2017
Purity:	97.5%
Storage Condition:	Refrigerated
Source:	Sigma-Aldrich
Structure:	



JRFA SAS-007-0OO

Common Name:	**Dimethyl-EBDC**
Chemical Name (IUPAC):	Dimethyl Ethylenebisdithiocarbamate
CAS Registry No.:	20721-48-6
Molecular Formula:	(CH2)2(CH3)2(CS2n(NH)2
Molecular Weight:	240.50 g·mol^{-1}
Batch No.:	JRF-001-058
Reassay Date:	February 16, 2018
Purity:	97.7%
Storage Condition:	Ambient
Source:	Sponsor
Structure:	



JRFA SAS-007-YYY

5.2. Test Matrices

The matrices were selected to be representative of typical surface and drinking waters, and were collected as per guideline requirements. Information such as collection location, characteristics and storage are reported in the raw data. The test matrices are drinking water (Aquafina) and surface water from Skippack Creek in Collegeville, PA (coordinates

40.150077, -75.447406.) Water samples should be kept refrigerated. Matrices were tested to ensure no mancozeb was present prior to analysis.

6.0 APPARATUS AND EQUIPMENT

6.1. Laboratory Glassware

Test tubes, glass, 20-mL
Vials and caps, autosampler, 2-m.L, screw cap
Screw cap test tubes, 10, 15, and 20 mL
Graduated cylinders, 25, 50, 100 mL
Class A glassware (volumetric pipettes, flasks, graduated cylinders class A, etc.)
10 µL glass syringe

6.2. Laboratory Equipment

Pipettes, air-displacement, adjustable volume
Balance, analytical, capable of weighing to the nearest 0.1 mg
Platform shaker, orbital action shaker, and vorteX;
Syringe filters, polytetrafluoroethylene (PTFE), 0.45-µm, Agilent
Heating block

6.3. Chromatographic System Options

Column, Kinetex Phenomenex 100 x 2.1mm, 1.7 µm C18
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 solvents and analytical grade reagents.

Methanol (MeOH), LC/MS grade Omni Millipore 56182
Water, LC-MS grade Omni Millipore 56253
EDTA acid disodium salt. Alfa Aesar J25Y017
Iodomethane, Alfa Aesar, 10153144, Sigma Aldrich SHBG8296V
Formic acid, Fluka BCBR2425V
Hydrochloric acid (HCl), JT Baker 107033
Acetonitrile, LC/MS grade Omni Millipore 55140
Ammonium formate, Fluka MKBV0601V
pH Strips

6.5. Reagents and Materials to be Prepared

6.5.1. 2.0 mM EDTA and 10 mM ammonium Formate Solution

Weigh 0.755 g EDTA and 0.63 g of ammonium formate, transfer to a 1000-mL volumetric flask, and bring to approximately 100 mL with DI water. Verify that pH is between 8 and 10, and if so, bring to volume with DI water. Otherwise, adjust pH with HCl solution, before bringing to volume.

6.5.2. 10% HCl Solution for Adjusting pH

Transfer 20 mL of concentrated HCl (12M) to a 200-mL volumetric flask. Add approximately 100 mL of DI water. Mix well and bring to a volume of 200 mL with DI water.

6.5.3. Mobile Phase A - 5 mM Ammonium Formate/0.1 % Formic Acid in Water

Weigh out 0.3271 g NH_4HCO_2 and 1.00 mL of fomlic acid, transfer to 1000-mL volumetric flask. Bring to volume with water.

6.5.4. 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.

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 lab coat
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated areas

6.7. Stock Solutions

Mancozeb stock standard solution was prepared in 50:50 isopropyl alcohol (IPA):water solution. Typical concentration for a stock standard is 10,000 µg/L. The following is an example for preparing 250 mL of a 9,750 µg/L stock standard.

1. A mass of 0.0025 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:

$$\frac{(0.002 \text{ g} \times 0.975)}{0.250 \text{ L}} \times \left(10^{6} \frac{\mu\text{g}}{\text{g}}\right) = 9750 \ \mu\text{g/L}$$

Dimethyl EBDC stock standard solution was also prepared, as mancozeb is derivatized for analysis. EBDC stock was prepared in acetonitrile and typical concentration is 150,000 µg/L. The preparation and calculation of these stocks follow a similar procedure as mancozeb. The table below lists the stock solutions prepared and used dwing this study.

Analyte fD	Purity	Weight (mg)[1]	Final Volume (mL)	Concentration (µg/L)
Mancozeb	97.5%	2.4	250	9750
Dimethyl EBDC	97.7%	6.4	100	64480

[1] Corrected for purity

6.8. Preparation of Fortification Solotions

Sample fortification solutions should be prepared by serial dilution of the stock standard in 50/50 isopropanol/water for mancozeb and acetonitrile for EBDC. The following concentrations are prepared for fortification standards: IO µf/IL, and l,000 µg/L.

6.9. Standard Solution Storage and Expiration

Mancozeb stock and fortification solutions should be stored in a freezer (~ -20 ° C) and EBOC 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 EBOC–dimethyl stock standard solution, 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 anaJysis, due to the instability of mancoz.eb.

Calibration solutions suitable for LC-MS/MS analysis should be prepared in matrix (surface or drinking waters). At least five 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 are as follows:

Starting Concentration (µg/L)	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)
1000	0.0625	25.0	2.50
2.50	10.0	25.0	1.00
1.00	12.5	25.0	0.500
0.500	5.00	25.0	0.100
0.100	12.5	25.0	0.0500

7.0 METHOD SUMMARY

7.1. Analytical Method for the Determination of Residues of Mancozeb in Drinking and Surface Waters by LC-MS/MS Analysis

5.00 mL of each water sample was fortified and 0.25 mL of 2.0 mM EOTA/10 mM ammonium formate solution were added and then vortexed for 45 seconds. The pH was adjusted to between 8 and JO if necessary. 10 µL of iodomethane and 10 µL dimethyl sulfate were added via glass syringe to the sample and vortexed for 45 seconds again. Samples were then placed in a heating block set to 50-60° C for I hour for refluxing to occur. Samples were cooled and filtered, then analyzed by LC/MS/MS. This method is illustrated in Appendix II, Figure 56. Instrumental analysis is accomplished using a LC-MS/MS system. Separation is achieved using a reversed phase column. The molecuJar ions formed in positive ion mode are fragmented by collision with neutral gas. The fragment ions generated are filtered, and one ion is selected for quantification and another product ion for confirmation.

7.2. 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) resuJting from a least squares equation that reflects the detector response as a function of the anaJyte concentrations.

7.3. Limits of Detection and Quantification

The lower limit of quantification (LOQ) for the method is 0.1 µg/L. The limit of detection (LOO) 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 LOO was determined to be 0.0193 µg/L in drinking water and O.Ql 32 µlefl, in surface water.

7.4. Validation of Confirmatory Techniques

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

Calibration curve and linearity r^2 values, recoveries for the fortified samples and precision data as weJl as results in blank samples were calculated for the quantitation and confirmatory ions and are reported.

7.5. Determination of Matrix Effects

Matrix matched standards were prepared and analyzed to determine if any matrix interference was present by direct comparison with solvent prepared standards. Calibration standards were prepared as described in Section 6.10. Although matrix effects were not significant, matrix-matched standards were used for both matrices as per SANCO guidelines.

7.6. Mancozeb Stability

Mancozeb stock and fort solutions should be prepared and used immediately on the day of analysis.

7.7. Dimetbyl-EBDC Stability

Calibration standard solutions should be prepared fresh prior to instrument analysis.

7.8. Extract Stability

Samples should be analyzed within one week of receipt or preparation. It was noted that stability of solutions and samples is related to concentration in that the higher the concentration, the faster the solution/sample degrades.

7.9. Chromatographic Conditions

Typical Operating Conditions for Mancozeb:

The following LC-MS/MS parameters were used to detennine the concentration of mancozeb residues in water matrices. 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:	Kinetex Phenomenex 100 x 2.1 mm, 1.7 µm C 18
Column Oven Temp:	Ambient
Injection Vol.:	8 µL
Run Time:	8 minutes
Detector:	Sciex API 6500
Retention Time:	Dimethyl-EBDC: ~3.20

Mobile Phase Composition (Linear gradient changes):

A gradient elution, using an increased percentage of organic solvent (acetonitrile) 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 (µUmin)
0.00	90	10	550
1.00	90	10	550
2.00	SO	50	550
3.00	IO	90	550
5.50	IO	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 (Confirmato ry)	240.922-+193. 000	60	16	11	12

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
GSI (psi)	90
GS2 (psi)	70
CAD gas CN2	High
Ion Spray (V)	5300
Temperature {°C)	520
EP(V)	10

7.10. Statistics and Sample Calculations

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

I. 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 anaJysis, 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 rearranged and used to calculate residues as follows by accounting for the extraction volume and sample mass:

$$\text{Mancozeb Found}\left(\frac{\mu g}{IL}\right) = \frac{\text{Cale Cone}\,(\mu g IL) \bullet \text{Final Volume}\,(ml)}{\text{Sample Volume}\,(ml) \bullet \text{Conversion Factor} \bullet \text{Efficiency yield factor}}$$

Where: Conversion factor = 1.11

Efficiency yield factor = 1.515 (I/0.66, as described in Reference 4)
Note: Efficiency yield factor is detennined/established as per matrix.

Example: Drinking water sample UTC + LOQ R3 was analyzed.

Sample volume = 0.00500 L

Final volume = 0.00527 L

Peak area in the quantitation transition was 89884.0 counts

Calibration curve generated in the run was y = 644549.1667 x + 1699.683723

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

$$x = \frac{89884.0 - 1699.683723}{644549.1667}$$

$$x = 0.137\ \mu g/i$$

$$\mu g\ IL\ \text{Found} = \frac{0.137\ \mu g/L \cdot 0.00527\ L}{0.00500\ L * 1.11 * 1.515}$$

$$\mu g\ IL\ \text{Found} = 0.08587\ \mu g/i$$

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

$$\%\ \text{Recovery} = \frac{\text{Measured concentration}\,(\mu g/L) - \text{Control concentration}\,(\mu g/L)}{\text{Theoretical concentration}\,(\mu g/L)} \bullet 100\%$$

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: Drinking water sample UTC + LOQ R3 was analyzed.

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

$$\%Recovery = \frac{0.08587\ \mu g/L - 0.00\ \mu g/L}{0.100\ \mu g/L} \times 100\% = 85.9\%$$

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

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

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 x is the average calcuJated concentration

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

Example: Surface water was analyzed.

$$LOD = Stdev(LOQ\ R1: LOQ\ R7) \cdot 3.143$$

$$Stdev(LOQ\ R1: WQ\ R7) = \sqrt{\frac{1}{7-1}\sum_{i=1}^{7}(x_i - 0.09109)^2} = 0.00421$$

$$LOD = 0.00421 * 3.143 = 0.0132$$

8.2. Linearity

The linearity was demonstrated by the standard linear regression curve and statistical data available, in conj unction with the data provided in the tables.

The linearity was also demonstrated by the least square equation which reflects the detector response of the analyte vs. concentration.

8.3. Limit of Detection and Quantification

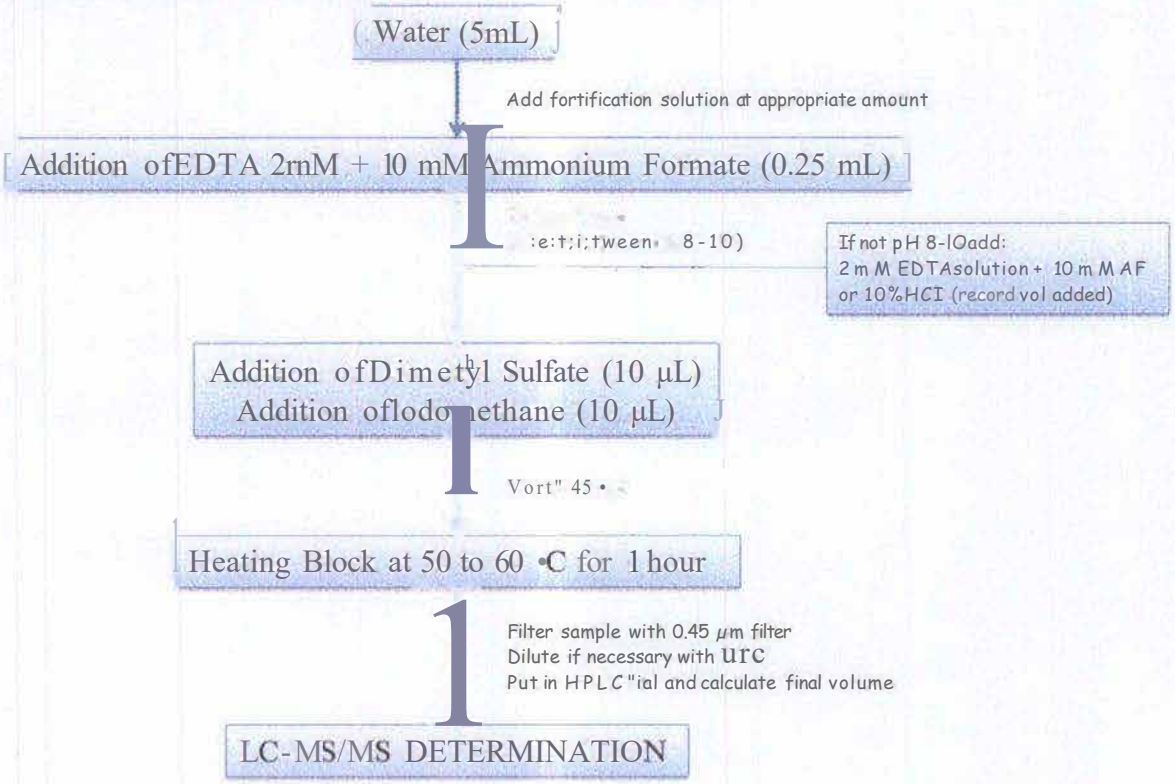
For this method, the LOD was determined to 0.0132 µg/L for surface water and 0.0193 µg/L for drinking water matrices. The limit of quantification (LOQ) is set at 0.1 µg/L for botb drinking and surface waters.

8.4. Sample Extract Stability

Validation trial samples were again analyzed one week after initial instrument analysis. AJl of the surface water samples (LOQ and 1Ox LOQ) experienced reduced recoveries. Six of the seven drinking water samples fortified at LOQ reported increased recoveries whereas 1Ox LOQ sample all underwent degradation.

Figure 56 Analysis Flowchart

Mancozeb Conversion to Dimetbyl-EBDC for Water Analysis



Protocol-(continued)

1.0 SUMMARY

The analytical method AU-274R0 will be developed at JRF America for determination of mancozeb in drinking and surface waters. This validation study is needed to define important method parameters such as accuracy, precision, ruggedness, linearity, specificity. and limits of detection and quantitation. The validation smdy will be conducted to comply with the requirements of OPPTS 860.1340 (1). SANCO/825/00 rev. 8.1 (2) and SANCO/3029/99 rev. 4 (3).

2.0 INTRODUCTION

2.1 Study P11rpose

Th,s protocol has been prepared to meet the requirements of OPPTS 860.1340 (IJ. SANCO/825/00 1ev. 8.1 (2) and SANCO/3029/99 rev. 4 (3) for the analysis of surface and drinking waters.

2.2 Study Objectives

The objective of !his study is to provide residue method validation da1a for the quantitation and continnation of mancozeb in drinking and surface waters. The method AU-274R0 will be validated at a target limit of quantitation of 0.1 µ&'L for both matrices. wilh a range of LOQ (O.I µg/L) to IOx LOQ (1.0 µg/L).

3.0 SPONSOR AND TEST FACILITY

3.1 Sponsor

Mancozeb Task Force
c/o McDcnnott, Will and Emery,
500 North Capitol Street, N.W.
Washington D.C., 20001
U.S.A.

Protocol - (continued)

An archive sample of the substance mancozeb 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

Water matrices will be acquired by JRF America, with source infonnation documented in the study fiJe. Matrices wlll be tested to ensure no mancozeb is already present. The untreated control samples will be identified with unique sample numbers assii:,'lled by JRF Ame1ica 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 fo11ification of control samples during the method validation.

8.0 PROCEDURES

The accuracy and precision of the residue method AU-274R0 will be detennined 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.

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

Sample ID	Number of Sample
Reagent Blank	I
Control	2
LOQ	7
10XLOQ	5
TOTAL	15

Additional control, reagent blank. and recove1y 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 LIM1:ARITY

A series of matrix matched standards will be prepared and analyzed to empi1foally detennine the linearity of the detector response pix or $1/x^2$ weighting cai1 be used as an option). The calibration range will be 0.05 ro 2.5 µg/L for the analyte in both matrices, with the limit of detection (LOO) determined through data calculations. Linearity will be calculated as the coefficient of dete1mination (r^2) resulting from a least squares equation that reflects the detector response as a fi.mction of the aoalyte concentrations.

Protocol-(continued)

10.0 LIMITS OF DETECTION AND QUANTITATION

TIle limit of detection (LOD) for the matrices will be calculated f'om the data of the seven (7) LOQ recovery samples, as described in "Assigning VaJues to Non-detected/Non-quantified Pesticide Residues in Hwnan Health Food Exposure Assessments. Item 6047. U.S. EPA. March 23, 2000". The limit of quantitation (LOQ) is set at 0.1 µg/L for aJI matrices as per toxicology and guideline requirements.

11.0 ACCEPTABILITY OF RECOVERIES AND METHOD RUGGEDNESS

For the metbod 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 f01titied 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 !OX LOQ. A rugged method will show good recovery at both concentration levels and for multiple·replicates.

12.0 VALIDATION OF CONFIRMATION TECHNIQUE

Confi'mation of the presence of the analyte will be perfom1ed by, simultaneously with the primary transition, monitoring an additional MRM transition for the same precursor ion.

Calibration curve linearity r value, recoveries for the fortified samples and precision data. as well as tbe results in blank samples will be calculated for the contin11atory transition and reported in the final report. A parent ion spectrum will be presented in the final report.

13.0 DETERMINATION OF MATRIX EFFECTS

The matrix effects of the sample extracts upon the analyre during analysis will be investigated by preparation of matrix-matched standards (for each matrix type) for comparison with standards prepared in a neat solvent. The details regarding the determination of matrix effects will be recorded iu the raw data file.

14.0 DETERMINATION OF SAMPLE EXTRACT STABTLITI'

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 :l: 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 lUlder tbe 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 de, ations will be used for this study. Additional statistical calculations may be used if necessary.

Protocol - (continued)

16.0 GLPCOMPLIANCE

This study will be conducted and reported in compliance with the U.S EPA Good Laboratory Practice Standards (40 CFR Part 160). which are compatible with the OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/Cl-fEM (98)l 7. OECD. Paris 1998 and EC Commission Directive 2004/10/E(' of Feb 2004 (Official Journal No L50/44).

A statement of compliance. signed by the Study Di.rector, will be included in the final report.

17.0 QUALITY ASSURANCE

The *JRF*A Quality Assurance Unit will inspect one or more critica.J events to assure that equipment. personnel, procedures. and records confonn to the guidelines listed in this protocol. A signed statement will be included in tl1e report specifying types of inspections made. the dates that inspections were made, and the dates inspectioos were reported to the Study Director and Sructy Dtrector Management.

The draft and final reo rt will be audited. Quality Assurance audiv'i:uspection reports including observations, comments of critical nature, and findings shaJl be provided to the Sn1dy Director for responses.

These inspections and audits will be can-ied out by Quality Assurance personnel independeot of the staff involved in the study.

18.0 PROTOCOL AMENDN1ENTS AND D EVIATIONS

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 wi ll be retafoed with the protocol. Additionally, a copy of each amendment will be sent to the Sponsor/Study Monitor for signature.

In the even1 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 deYiation will be signed aod dated by the study director and maintained with the protocol/study plan.