ABSTRACT

The purpose of this study was to demonstrate that the ISK Analytical Method MFT03817E: "Validation of an analytical method for the determination of DCC-3825 (Tiafenacil) and its metabolites (M-01, M-12, M-13, M-36, M-53) in Sediment" using LC-MS/MS determination could be performed successfully at an outside facility with no prior experience with the method.

Principle of the method
Residues of DCC-3825 and its metabolites were extracted from homogenized sediment sample by shaking twice with acidified acetonitrile/water (80/20, v/v). Sample extracts were combined after each extraction. An SPE cartridge (Oasis HLB VAC RC 60 mg) was conditioned using methanol followed by water:acetic acid (100:1, v/v). A 5 mL aliquot of the extract was mixed with water:acetic acid (100:1, v/v) solution, and eluted through the SPE column. The eluates were discarded. The residues of DCC-3825 and its metabolites were eluted using methanol:water (70:30, v/v) in a clean polypropylene test tube and were vialed for LC-MS/MS analysis.

Test conditions
For validation, an untreated sediment sample was fortified with DCC-3825 and its metabolites and analyzed according to the established method validation guidelines. The analytical set consisted of a reagent blank, two untreated controls, five replicates fortified at the method's limit of quantitation (LOQ), and five replicates fortified at 10xLOQ.

Two mass transitions were evaluated for DCC-3825 and its metabolites and are listed below:

	Quantitation (*m/z*)	Confirmation (*m/z*)
DCC-3825	512 → 381	512 → 152
M-01	498 → 381	498 → 59
M-12	427 → 381	427 → 152
M-13	426 → 381	426 → 152
M-36	443 → 218	443 → 353
M-53	445 → 371	445 → 355

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The LOQ for DCC-3825 and its metabolites in sediment was 0.01 ppm as stated in the method and the LOD was set to 30% of the LOQ or 0.003 ppm.

Selectivity
The method determines DCC-3825 and its metabolites residues in sediment by LC-MS/MS. No interfering peaks were found at the retention time for DCC-3825 and its metabolites.

Linearity
Acceptable linearity was observed for the standard range and the mass transitions tested. The method-detector response was linear over the 0.1 – 10.0 ng/mL range ($r = 0.9970$) or greater.

1. INTRODUCTION

1.1 Purpose of the Study

Analytical Method MFT03817E was developed by ISK Biosciences Corporation to determine the residues of DCC-3825 and its metabolites M-01, M-12, M-13, M-36, and M-53 in sediment using LC-MS/MS. The purpose of this study was to demonstrate that the Method MFT03817E can be performed with acceptable recoveries at an outside facility. This final report represents the method's validation by an independent laboratory, ADPEN Laboratories, Inc. in Jacksonville, Florida.

The independent laboratory validation was conducted at two fortification levels, the limit of quantitation (LOQ), which is 0.01 ppm, and 10xLOQ, which is 0.1 ppm, in sediment. Each analytical set contained five replicates at each fortification level, one reagent blank, and two unfortified control samples.

1.2 Analytical Procedure

Residues of DCC-3825 and its metabolites were extracted from a 20 g of homogenized sediment sample by shaking twice with 90 mL acetonitrile/water (80/20, v/v) and with 0.9 mL of formic acid. The mixture was agitated on a mechanical shaker, then centrifuged at 3000 rpm for 5 minutes and the extracts were combined after each extraction. The extract was brought to 200 mL with acetonitrile:water (80:20, v/v). An SPE cartridge (Oasis HLB VAC RC 60 mg) was placed on a vacuum manifold and conditioned using 5 mL of methanol followed by water:acetic acid (100:1, v/v). A 5 mL aliquot of the extract was mixed with 30 mL of water:acetic acid (100:1, v/v) solution, and eluted through the SPE cartridge. The eluates were discarded. The residues of DCC-3825 and its metabolites were eluted using 9.5 mL of methanol:water (70:30, v/v) in a clean polypropylene test tube; were brought to 10 mL volume using the same methanol and water solution, then vialed for LC-MS/MS analysis.

Detailed validation results for DCC-3825 and its metabolites are presented in **Tables 1 through 12**.

Detailed residue reports representing the analytical data are presented in **Appendix D**.

1.3 Specificity

To demonstrate the specificity of the analytical method, a confirmation mass transition was monitored simultaneously with the quantitation transition for analysis of DCC-3825 and its metabolites as described below.

	Quantitation (*m/z*)	Confirmation (*m/z*)
DCC-3825	512 → 381	512 → 152
M-01	498 → 381	498 → 59
M-12	427 → 381	427 → 152
M-13	426 → 381	426 → 152
M-36	443 → 218	443 → 353
M-53	445 → 371	445 → 355

The method was able to accurately determine residues of DCC-3825 and its metabolites. No interferences were observed at the retention time of the analyte peaks.

2. REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test System

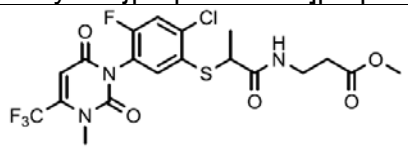
The test systems used in this study was sediment.

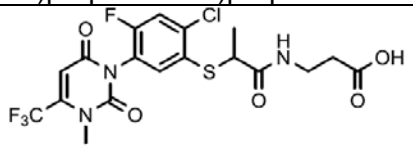
The sediment control sample was collected on July 6, 2015 from California soil and was received at ADPEN Laboratories, Inc. on July 20, 2015. The sample was stored in refrigerator E122, which maintained an average temperature of 5 ºC. The sample was removed from the refrigerator on July 13, 2017 to be processed for this study and then stored in the freezer E16 during the course of this study. The freezer E16 operates at an average temperature of -22 ºC. The sample was inspected, and logged into the Laboratory Information Management System (LIMS) in Order Identification Number 170815001. Unique laboratory code was assigned to the sample by the LIMS (e.g. 170815001-001). The sediment characterization report from Agvise Laboratories is presented in **Appendix B.**

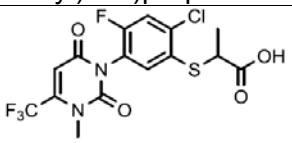
2.2 Test and Reference Substances

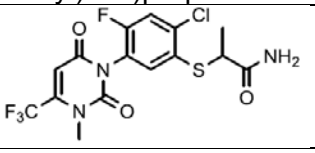
Analytical standards (reference substances) of DCC-3825 and its metabolites (M-01, M-12, M-13, M-36, M-53) were received at ADPEN Laboratories, Inc. on May 26, 2017 frozen and in good condition. The reference substances were stored frozen in freezer E-119, which maintained an average temperature of -23 ºC until use. All calibration and fortification standards were prepared using the reference substances and kept frozen in E- 109, which operated at an average temperature of -23 ºC during the course of the study. ISK Biosciences has retained a reserve sample of these chemicals, and has documentation specifying the location of the synthesis and characterization information.

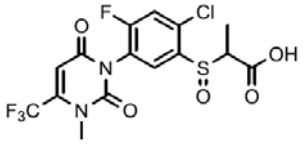
The certificates of analysis for all reference substances are presented in **Appendix A**. A summary of the reference substances is presented below.

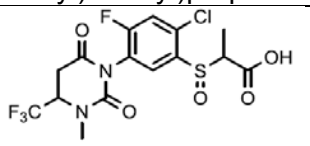
Identity:	DCC-3825
Common Name:	Tiafenacil
Lot Number:	KILOLAB-140109
Storage Condition:	Frozen
Molecular Weight:	511.87 g/mol
Purity:	98.9% (w/w)
Expiration Date:	December 16, 2018
Chemical Name:	methyl 3-[(2RS)-2-{2-chloro-4-fluoro-5-[1,2,3,6-tetrahydro-3-methyl-2,6-dioxo-4-(trifluoromethyl)pyrimidin-1(6H)-yl] phenylthio}propionamido]propionate
Chemical Structure:	

Identity:	M-01
Lot Number:	K20066-01
Storage Condition:	Keep frozen
Molecular Weight:	497.85 g/mol
Purity:	97.9% (w/w)
Expiration Date:	January 18, 2020
Chemical Name:	3-(2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6H)-yl)phenyl) thio)propanamido)propanoic acid
Chemical Structure:	

Identity:	M-12
Lot Number:	KM02478-01
Storage Condition:	Keep frozen
Molecular Weight:	426.77 g/mol
Purity:	98.6% (w/w)
Expiration Date:	January 18, 2020
Chemical Name:	2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6H)-yl) phenyl)thio)propanoic acid
Chemical Structure:	

Identity:	M-13
Lot Number:	K20067-01
Storage Condition:	Keep frozen
Molecular Weight:	425.79 g/mol
Purity:	99.5% (w/w)
Expiration Date:	January 18, 2020
Chemical Name:	2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6H)-yl) phenyl)thio)propanamide
Chemical Structure:	

Identity:	M-36
Lot Number:	K20268-02
Storage Condition:	Keep frozen
Molecular Weight:	442.77 g/mol
Purity:	99.2 % (w/w)
Expiration Date:	February 25, 2019
Chemical Name:	2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6H)-yl) Phenyl sulfinyl)propanoic acid
Chemical Structure:	

Identity:	M-53
Lot Number:	DB15-1210-01
Storage Condition:	Keep frozen
Molecular Weight:	444.78 g/mol
Purity:	93.9% (w/w)
Expiration Date:	March 29, 2019
Chemical Name:	2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)tetrahydropyrimidin-1(2H)-yl) phenyl)sulfinyl)propanoic acid
Chemical Structure:	

3. ANALYTICAL METHOD

Residues of DCC-3825 and its metabolites were determined using the extraction procedure and instrument conditions as described in the ISK Biosciences Analytical Method MFT03817E entitled, “Validation of an analytical method for the determination of DCC-3825 (Tiafenacil) and its metabolites (M-01, M-12, M-13, M-36, M-53) in Sediment” using LC-MS/MS, which is attached in **Appendix C**. Instrument parameters used for analysis are described in **Table 14**. No major modifications were made to the analytical method.

5. STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included statistics, such as determinations of averages, standard deviation and relative standard deviation (RSD) for the procedural recoveries and area counts. Calculation of the calibration curve and correlation coefficient (r) by linear regression of the instrument responses for the reference standards was performed within ABSciex Analyst® (version 1.6.2) software rather than what was suggested in the analytical method. The statistical calculations throughout this report were performed using an automated spreadsheet (Microsoft Excel®) from data entered into a Laboratory Information Management System (LIMS). All calculation results were rounded for presentation purposes. Slight differences may be noted in hand calculations using the recoveries presented in the tables. These differences are due to rounding and have no effect on the

scientific conclusions presented in this report. The detailed analytical data reports may be reviewed for confirmation of the calculated results.

Several measures were taken to ensure the quality of the study results. The quality assurance unit at ADPEN Laboratories, Inc. inspected analytical procedures for compliance with Good Laboratory Practices which included adherence to the protocol. The dates inspected are shown in the quality assurance statement. Study samples and test and reference items were maintained in a secured laboratory with limited access.

Example Recovery Calculations

Peak integration and quantitation were performed within ABSciex Analyst® 1.6.2 software; using the calibration curve equation to determine the amount of analyte found (ng) during sample analysis. Residue found in ppm and recovery results were calculated within the LIMS and reported in Microsoft® Excel data reports, which are presented in Appendix C.

The following equations are used for residue and recovery calculations for DCC-3825 and its metabolites.

Calculated within Analyst

a) Calibration curve: $y = mx + b$ Solving for X: $X = \frac{y - b}{m}$

Where, y = Peak area
m = Slope
x = Amount found (ng)

Calculated within LIMS/Excel

b) Residue found (ppm) = $\frac{X \times VF \times D \times CF}{VI \times VS}$

Where, X = Concentration of extract (mg/kg), solving for "x" from Equation "a" above)
VF = Final volume (10 mL)
D = Dilution Factor (1)
CF = Conversion factor
VI = Injection Volume (40 µL)
VS = aliquot of sediment sample (0.5 g)
20 g sample diluted to 200 mL and took 5 mL aliquot
= 20 g x 5 mL/200 mL = 0.5 g

As an example, calculations to obtain recovery results are shown below for a control sediment sample fortified with DCC-3825:

Lab Code: 17081503-Recovery1-1
Sample ID: Control + 0.01 ppm
Set Name: WO-17081503
Peak Area: 2153816

Calibration curve: y = mx + b

$$y = (9.6386e+007)\,x + 56870$$

c) Solving for X: $X = \frac{y - b}{m}$

$$= (2153816-56870)/9.6386e+007$$
$$= 0.021756 \text{ ng}$$

$$\text{Residue Found (ppm)} = \frac{0.021756 \text{ ng} \times 10 \text{ mL} \times 1 \times 1 \text{ g}}{0.040 \text{ mL} \times 0.5 \text{ g} \times 1000 \text{ mg}}$$

$$= 0.010878 \text{ ng/mg}$$

c) $\text{Recovery (\%)} = \frac{0.010878 \text{ ppm}}{0.010 \text{ ppm}} \times 100\% = 108.8\%$

Statistical treatment of the data included calculation of means, standard deviations (SD), and percent relative standard deviations (%RSD), and were performed in Microsoft® Excel. Results above were rounded only for reporting purposes. No calculations were made with rounded numbers.

6. SUMMARY OF METHOD

Type of Method LC-MS/MS

Test System Sediment

Selected mass transitions (m/z)

	Quantitation (*m/z*)	Confirmation (*m/z*)
DCC-3825	512 → 381	512 → 152
M-01	498 → 381	498 → 59
M-12	427 → 381	427 → 152
M-13	426 → 381	426 → 152
M-36	443 → 218	443 → 353
M-53	445 → 371	445 → 355

Analytical Procedure ISK Biosciences analytical method number MFT03817E: "Validation of an analytical method for the determination of DCC-3825 (Tiafenacil) and its metabolites (M-01, M-12, M-13, M-36, M-53) in Sediment"

Confirmatory Technique A secondary MRM transition was used for confirmation.

Method of Quantitation The quantitation is based on the monitoring of two mass transitions for DCC-3825 and its metabolites. Recovery data was reported for each mass transition considered.

LOD 0.003 ppm

LOQ 0.01 ppm (lowest fortification level)

Levels of Fortification 0.01 ppm and 0.1 ppm

Time Required A set of 13 samples requires approximately 24 hours of work (the calculation of the results included).

7. DISCUSSION

Recovery Findings
Method MFT03817E proved to be suitable to determine residues of DCC-3825 and its metabolites in sediment to an LOQ of 0.01 ppm. The mean recovery values of the validation experiments were within 70-120%, which fulfills the guideline requirements for mean recovery values.

Linearity
Good linearity ($r \geq 0.9970$) was observed in the range of 0.1 to 10.0 ng/mL for DCC-3825 and its metabolites.

Specificity
Method MFT03817E determines residues of DCC-3825 and its metabolites in sediment. No interfering peaks were found at the retention time for DCC-3825 and its metabolites in sediment.

Limit of Quantification and Limit of Detection
The LOQ for DCC-3825 and its metabolites in sediment was 0.01 ppm as stated in the method. The limit of detection (LOD) for DCC-3825 and its metabolites was shown to be detectable as the absolute amount of analyte injected into the LC-MS/MS when the lowest calibration standard was analyzed, which was 0.1 ng/mL. Furthermore, DCC-3825 and its metabolites demonstrated acceptable signal to noise ratio (S/N is >3:1) at the LOD.

Repeatability
The overall percent relative standard deviation, (% RSD) for all fortification levels were below 20%.

Method MFT03817E was demonstrated to fulfill the requirements with regards to specificity, repeatability, limit of quantification, limit of detection, linearity and recoveries. Therefore, it is applicable to correctly determine residues of DCC-3825 and its metabolites in sediment.

8. RECOMMENDATIONS/CONCLUSIONS FROM ILV

This independent laboratory validation was successfully completed on the first trial at ADPEN Laboratories, Inc. Recovery results and statistical data demonstrate Analytical Method MFT03817E can be performed successfully for quantitation of DCC-3825 and its metabolites in sediment. The method is thorough and contains sufficient guidance to aid the analyst through the procedure for the first time.

9. PROTOCOL, AMENDMENTS, AND DEVIATIONS

The study protocol was followed as written with one amendment and no deviations noted during this study.

Amendment 1: Included the matrix effect and final volume storage stability testing details that were not in the original protocol.

10. COMMUNICATION

There were no communications between the Study Director and the Study Monitor and personnel necessary to complete the study. At no time during the course of the study was anyone from allowed to visit the testing facility or communication made to discuss the analytical method or its procedures. The first trial results were sent to the study director via e mail attachment on August 24, 2017.

Table 14 Instrument Conditions and Parameters for the Analysis of DCC-3825 and its metabolites

HPLC Conditions			
Chromatographic System:	Agilent 1290 UHPLC		
Column:	Kinetex Biphenyl 150 x 2.1 mm, 2.6 µm		
Temperature:	40 ºC		
Flow rate (µL/min)	400		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	25	75
	3.00	25	75
Mobile Phase A:	0.1% formic acid in water (Optima Grade)		
Mobile Phase B:	0.1% formic acid in methanol		
Injection Volume (µL):	40		

MS/MS Conditions						
Detection System:	ABSciex 6500 Mass Spectrometer					
Ionization:	ESI					
Polarity:	Positive					
Curtain gas (CUR):	20					
Temperature (TEM):	500 °C					
Collision gas setting (CAD):	11					
GS1:	60					
GS2:	45					
Entrance potential (EP):	10					
Scan type:	MRM					
MRM Conditions	Transition (m/z)	Dwell (msec)	DP	CE	CXP	Expected Retention Time (min)
DCC-3825	512 → 381	30	86	37	20	~2.15
	512 → 152	50		55	18	
M-01	498 → 381	50	91	35	20	~1.63
	498 → 59	100		111	8	
M-12	427 → 381	100	81	21	18	~2.01
	427 → 152			41	18	
M-13	426 → 381	30	81	27	18	~1.61
	426 → 152	50		43	8	
M-36	443 → 218	50	76	51	12	~1.69
	443 → 353			23	18	
M-53	445 → 371	50	106	29	18	~1.70
	445 → 355	100		25	12	

SUMMARY

A method, based on the analytical method in soil and sediment (Reference1 and 2), was validated for the determination of DCC-3825 and its metabolites (M-01, M-12, M-13, M-36 and M-53) in two sediment types.

Samples were extracted with an acetonitrile/water/formic acid mixture, and clean-up with an OASIS HLB solid phase extraction (SPE) cartridge. Quantitation was performed using liquid chromatography with tandem mass spectrometric detection (LC/MS/MS).

Method validation

Since the correlation coefficient for the calibration curve was over 0.999 for DCC-3825 and its metabolites, linearity was demonstrated. Untreated samples of each sediment were analyzed using the analytical method and there was no apparent response (i.e. <30% of the LOQ) in the region in the chromatograms corresponding to the retention time of DCC-3825 and its metabolites. Therefore, specificity of the method was demonstrated. The recovery test was performed with fortification levels at 0.01 and 0.1 mg/kg for DCC-3825 and its metabolites. An LC/MS/MS scan with a different transition was used for confirmation. As a result, acceptable accuracy and precision were obtained (mean recovery in the range of 70-110 % and RSD<20%) for quantitation and confirmation monitored. The accuracy and precision data are summarized in the following tables.

1 OBJECTIVE

The objective of this study is to validate an analytical method for the determination of DCC-3825 and its metabolites (M-01, M-12, M-13, M-36 and M-53) in sediment.

Guideline and Guidance

EPA (Environmental Protection Agency) US: Residue Analytical Method, OPPTS 860.1340, August 1996.

Sanco/3029/99 rev.4 (11/07/00): Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414.

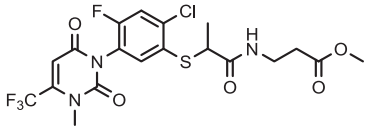
2 CONDUCT OF STUDY

The study was conducted at Ishihara Sangyo Kaisha, Ltd., Central Research Institute, Safety Science Research Laboratory, Environmental Sciences Group, 3-1, 2-Chome, Nishi-shibukawa Kusatsu-shi, Shiga-ken, 525-0025 Japan.

3 MATERIALS AND METHODS

3.1 Analytical standards

3.1.1 DCC-3825

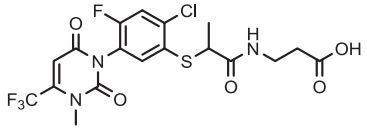
Identity:	DCC-3825
Common name:	Tiafenacil
Chemical name:	methyl 3-[(2*RS*)-2-{2-chloro-4-fluoro-5-[1,2,3,6-tetrahydro-3-methyl-2,6-dioxo-4-(trifluoromethyl)pyrimidin-1(6*H*)-yl] phenylthio}propionamido]propionate
Structure:	
Molecular weight:	511.87
Lot No.:	KILOLAB-140109
Purity:	98.7%

3.1.2 M-01

Identity: M-01

Chemical name: 3-(2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6*H*)-yl)phenyl)thio)propanamido)propanoic acid

Structure:



Molecular weight: 497.85

Lot No.: K20066-01

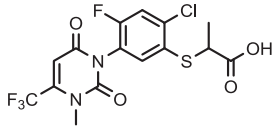
Purity: 96.1 %

3.1.3 M-12

Identity: M-12

Chemical name: 2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6*H*)-yl)phenyl)thio)propanoic acid

Structure:



Molecular weight: 426.77

Lot No.: KM02478-01

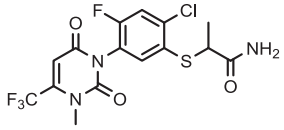
Purity: 97.4 %

3.1.4 M-13

Identity: M-13

Chemical name: 2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6*H*)-yl)phenyl)thio)propanamide

Structure:

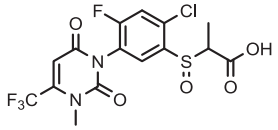


Molecular weight: 425.79

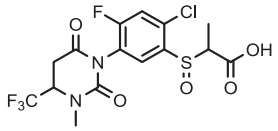
Lot No.: K20067-01

Purity: 98.6 %

3.1.5 M-36

Identity:	M-36
Chemical name:	2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)-2,3-dihydropyrimidin-1(6*H*)-yl) phenyl)sulfinyl)propanoic acid
Structure:	
Molecular weight:	442.77
Lot No.:	K20268-01
Purity:	94.1 %

3.1.6 M-53

Identity:	M-53
Chemical name:	2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoromethyl)tetrahydropyrimidin-1(2*H*)-yl) phenyl)sulfinyl)propanoic acid
Structure:	
Molecular weight:	444.79
Lot No.:	K20389-01
Purity:	94.7 %

3.2 Sediment

Two type sediments were used in the study. These sediments were supplied from Envigo CRS Ltd in June 2016. The sediment characterization data are shown in Table 1.

3.3 Reagents

All reagents were of analytical, HPLC or LC/MS/MS grade.

3.4 Standard solutions

3.4.1 Stock solutions

Individual stock solutions (100 µg/mL) of DCC-3825 and its metabolites were prepared by dissolving an accurately weighed amount of each material in a suitable volume of methanol.

3.4.2 Fortification solutions

The stock solutions were further diluted with methanol to obtain fortification solutions with a concentration of 1.0 and 10.0 µg/mL. These fortification solutions were prepared by mixing DCC-3825 and its metabolites.

3.4.3 Calibration solutions

Calibration solutions, over the concentration range 0.1 to 10.0 ng/mL, were prepared by serial dilution of the mixed fortification solutions in methanol:water (70:30, v/v).

3.5 Fortification

To demonstrate the validity of the method used, untreated sediments were fortified with the following levels for DCC-3825 and its metabolites.

0.01 mg/kg	0.2 mL of the fortification solution (1.0 µg/mL) was added to 20 g (dry mass) sediment.
0.1 mg/kg	0.2 mL of the fortification solution (10.0 µg/mL) was added to 20 g (dry mass) sediment.

3.6 Analytical method

The analytical method in sediment was based on the methods used for soil and sediment in the following studies - The Transformation of [14C]-DCC-3825 in Four Soils Under Aerobic Conditions and The Transformation of [14C]-DCC-3825 in Two Aquatic Sediment Systems under Aerobic Conditions (Reference1 and 2).

3.6.1 Extraction

20 g of the untreated sediment sample was weighed into a 250 mL HDPE screw-top bottle. 90 mL of acetonitrile:water (80:20, v/v) and 0.9 mL of formic acid were added to the sediment sample. The sample was shaken for 30 minutes using a reciprocal shaker. The mixture was centrifuged at 3000 rpm for 5 minutes and supernatant was decanted. The sediment residue was re-extracted with 90 mL of acetonitrile:water (80:20, v/v) and 0.9 mL of formic acid were added for 30 minutes. The mixture was centrifuged and decanted likewise. The extracts were combined in glass flask and diluted to volume (200 mL) with acetonitrile:water (80:20, v/v).

3.6.2 Sample clean up on SPE

A SPE cartridge (OASIS HLB VAC RC, 60 mg) was placed onto a SPE vacuum manifold and conditioned using methanol (5 mL) followed by water:acetic acid (100:1, v/v) (5 mL). 5.0 mL of the extract and 30 mL of water:acetic acid (100:1, v/v) were mixed and transferred into the SPE cartridge. The aqueous sample solution was sucked through the column followed by 5 mL of water. All eluates were discarded. DCC-3825 and its metabolites were eluted with 9.5 mL of methanol:water (70:30, v/v). The eluate was collected and then filled up to 10 mL with methanol:water (70:30, v/v).

3.6.3 Quantitation

Quantitation of the concentration of the DCC-3825 and its metabolites was performed by LC/MS/MS using the external standard method. The calibration standards at six concentrations (0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/mL) were used for construction of a calibration curve. The calibration curve was constructed by plotting the peak areas against the injected amount of standard. From the calibration curve, the concentration of DCC-3825 and its metabolites in sediment sample was determined.

3.7 LC/MS/MS conditions

Part A; HPLC

Instrument:	ACQUITY UPLC System (Waters)			
Column:	Kinetex Biphenyl 2.1×150 mm, 2.6 μm			
Column temp.:	40°C			
Mobile phase:	0.1% formic acid in water:0.1% formic acid in methanol (25:75, v/v)			
Flow rate:	0.4 mL/min			
Injection volume:	4 μL			
Retention time:	DCC-3825;	1.74 min	M-13;	1.33 min
	M-01;	1.34 min	M-36;	1.43 min
	M-12;	1.63 min	M-53;	1.11 min

Part B; MS/MS

Instrument:	API5000 (AB SCIEX)
Ionization mode:	ESI
Scan mode:	MRM
Mass resolution:	Q1; unit, Q3; unit
Heater gas temp.:	600°C
Ion voltage:	5000 V
Gas flow settings:	Gas1; 60, Gas2; 80, CUR; 10, CAD; 11

Quantitation transition monitored

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
DCC-3825	Pos. $[M+H]^+$	512.2	381.0	37	141	10	28
M-01	Pos. $[M+H]^+$	498.1	381.0	35	101	10	10
M-12	Pos. $[M+H]^+$	427.2	380.7	23	81	10	26
M-13	Pos. $[M+H]^+$	426.1	380.9	27	131	10	12
M-36	Pos. $[M+H]^+$	443.1	218.1	51	121	10	12
M-53	Pos. $[M+H]^+$	445.1	371.0	33	81	10	26

Confirmation transition monitored

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
DCC-3825	Pos. $[M+H]^+$	512.2	152.2	57	141	10	8
M-01	Pos. $[M+H]^+$	498.1	59.1	79	101	10	10
M-12	Pos. $[M+H]^+$	427.2	152.0	45	81	10	20
M-13	Pos. $[M+H]^+$	426.1	152.0	47	131	10	18
M-36	Pos. $[M+H]^+$	443.1	353.0	25	121	10	26
M-53	Pos. $[M+H]^+$	445.1	355.0	23	81	10	34

3.8 Calculation

The concentration of DCC-3825 and its metabolites in sediment was calculated according to equation 1.

$$C = \frac{X \times V_F \times D \times CF}{V_I \times V_S} \quad (1)$$

Where

C = Concentration of DCC-3825 and its metabolites in sediment sample [mg/kg]

X = Injected amount of DCC-3825 and its metabolites [pg]

V_I = Injection volume [4 µL]

V_F = Final volume [10 mL]

D = Dilution factor [if applicable]

V_S = Aliquot of sediment sample [0.5 g]

CF = Conversion factor ($\times 10^3$: from mL to µL, $\times 10^{-6}$: from pg to µg)

The recovery of DCC-3825 and its metabolites was calculated according to equation 2.

$$R = \frac{C \times 100}{F} \quad (2)$$

Where

R = Recovery of DCC-3825 and its metabolites [%]

C = Concentration of DCC-3825 and its metabolites in sediment sample [mg/kg]

F = Fortification level [mg/kg]

ISK

Amendment **Document No.** MFT03817E

AMENDMENT

Validation of an analytical method for the determination of DCC-3825 and its metabolites (M-01, M-12, M-13, M-36, M-53) in Sediment

Date Report issued: February 8, 2017 Date of amendment: September 4, 2017

We declare that this amendment is accurate and correct.

Kumiko Ogawa 2017. 9. 4

Kumiko Ogawa
Study Director
Environmental & Analytical Sciences Group
Safety Science Research Laboratory

Date

Daisuke Kishimoto 2017. 9. 4

Daisuke Kishimoto, Ph.D.
Group Leader
Environmental & Analytical Sciences Group
Safety Science Research Laboratory

Date

Details of amendment	Reason for amendment
Page 17, Confirmation transition monitored The product ion of M-01 (m/z) is corrected from 359.1 to <u>59.1</u>.	To amend as incorrect value.

<u>Underlined part</u> is revision.

Confirmation transition monitored

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
DCC-3825	Pos. $[M+H]^+$	512.2	152.2	57	141	10	8
M-01	Pos. $[M+H]^+$	498.1	59.1	79	101	10	10
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M-13	Pos. $[M+H]^+$	426.1	152.0	47	131	10	18
M-36	Pos. $[M+H]^+$	443.1	353.0	25	121	10	26
M-53	Pos. $[M+H]^+$	445.1	355.0	23	81	10	34