ABSTRACT

The purpose of this study was to demonstrate that the ISK Analytical Method MFT03717E: "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 surface water and drinking water" 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 surface water and drinking water samples by using acetic acid. An SPE cartridge (Oasis HLB VAC RC 60 mg) was conditioned using methanol followed by water:acetic acid (100:1, v/v). The acidified water sample was transferred into the SPE cartridge and the aqueous sample solution was vacuumed through the column followed by water. All 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, untreated surface water and drinking water samples were 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 surface water and drinking water was 0.1 ppb as stated in the method and the LOD was set to 30% of the LOQ or 0.03 ppb.

Selectivity
The method determines residues of DCC-3825 and its metabolites in surface water and drinking water 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 1.0 – 10.0 ng/mL range (r = 0.9954 or greater.

1. INTRODUCTION

1.1 Purpose of the Study

Analytical Method MFT03717E 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 surface water and drinking water using LC-MS/MS. The purpose of this study was to perform an Independent Laboratory Validation of the method MFT03717E. 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 0.1 ppb, and 10xLOQ, which is 1.0 ppb, in surface water and drinking water. 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 50 mL homogeneous surface water and drinking water samples by mixing each with 50 µL of acetic acid. An SPE cartridge (Oasis HLB VAC RC 60 mg) was conditioned using 5 mL methanol followed by 5 mL water:acetic acid (100:1, v/v). The acidified water sample was transferred into the SPE cartridge and the aqueous sample solution was vacuumed through the column followed by water. All eluates were discarded. The residues of DCC-3825 and its metabolites were eluted using 9.5 mL methanol:water (70:30, v/v) in a clean polypropylene test tube and were vialed for LC-MS/MS analysis.

Detailed validation results for DCC-3825 and its metabolites in surface water are presented in **Tables 1 through 12**. Detailed validation results for DCC-3825 and its metabolites in drinking water are presented in **Tables 12 through 24**.

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

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

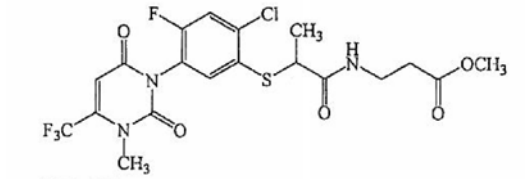
The test systems used in this study were surface water and drinking water.

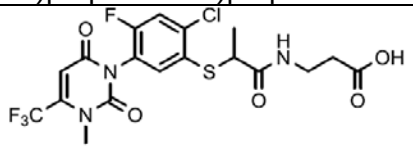
The surface water control sample was collected on November 4, 2014 from Pond Creek Lake and the drinking water was finished tap water. Both samples remained refrigerated at an average temperature of 5.0 ºC until they were used for this study. The samples were inspected, and logged into the Laboratory Information Management System (LIMS) in Order Identification Number 170815002. A unique laboratory code was assigned to the sample by the LIMS (e.g. 170815002-001). The sample was stored in refrigerator E-57, which maintained an average temperature of 5 °C during the course of the study. The surface water and drinking water characterization report from Agvise Laboratories is presented in **Appendix A**.

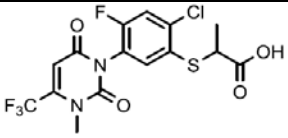
2.2 Test and Reference Substances

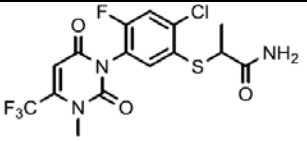
Analytical standards (reference substances) of DCC-3825 and its metabolites (M-01, M-12, M-13, M-36, and M-53) were received at ADPEN Laboratories, Inc. on May 26, 2017 frozen and in good condition. The reference substances were stored frozen (≤ -5°C) until use. All calibration and fortification standards were prepared using the reference substances and kept refrigerated in E- 109, which operated at an average temperature of 5 ºC for the duration of this study. An example of standard preparation and dilution data is shown in **Table 25**.

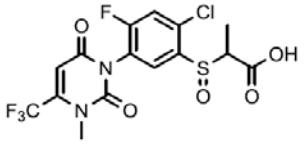
ISK Biosciences has retained a reserve of these reference chemicals, and has documentation specifying the location of the synthesis and characterization information. The certificates of analysis for all reference substances are presented in **Appendix A**. A summary of the reference substances is presented below.

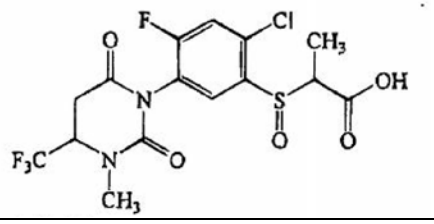
Identity:	DCC-3825
Common Name:	Tiafenacil
Lot Number:	KILOLAB-140109
Storage Condition:	Frozen
Molecular Weight:	511.87 g/mol
Purity:	98.9% (w/w)
Expiration Date:	December 16, 2018
Chemical Name:	methyl 3-[(2RS)-2-{2-chloro-4-fluoro-5-[1,2,3,6-tetrahydro-3-methyl-2,6-dioxo-4-(trifluoromethyl)pyrimidin-1(6H)-yl] 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 MFT03717E 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 surface water and drinking water” using LC-MS/MS, which is attached in **Appendix B**. Instrument parameters used for analysis are described in **Table 26**. No major modifications were made to the analytical method.

The independent laboratory validation of the method was successfully completed without any clarification of the method or consultation with the sponsor. A detailed recovery data for surface water for including the peak area, average recovery, standard deviation and relative standard deviations are presented in **Tables 1 through 12**. A detailed recovery data for drinking water including the peak area, average recovery, standard deviation and relative standard deviations are presented in **Tables 13 through 24**. The detailed spreadsheet reports representing the analytical data are presented in **Appendix C**.

In summary, the ILV was completed successfully during the first trial. All mean recoveries were within the acceptable range (70-120%).

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 ppb 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) Amount of sample injected = (sample volume × injection size) / final sample volume

c) Residue found (ppb) = ng found/mL of sample injected

As an example, calculations to obtain recovery results are shown below for a control surface water sample fortified with DCC-3825. The residues of all metabolites were calculated in the same manner in both matrices.

Lab Code:	17081504-Recovery1-1
Sample ID:	Control + 0.1 ppb
Set Name:	WO-17081504
Peak Area:	2100602
Matrix:	Surface Water

a) Calibration curve: y = mx + b

$$y = (1.0016e+008)\,x + 1.0767e+005$$

Solving for x: $x = \frac{y - b}{m}$

$$= (2100602 - 1.0767e+005)/\,(1.0016e+008)$$
$$= 0.019897484 \text{ ng}$$

b) Amount of sample injected = (50 mL × 0.04 mL)/10 mL
= 0.2 mL

$$\text{Residue Found (ppb)} = \frac{0.019897484 \text{ ng}}{0.2 \text{ mL}}$$

$$= 0.09948742 \text{ ng/mL}$$

c) $\text{Recovery (\%)} = \frac{0.09948742 \text{ ppb}}{0.10 \text{ ppb}} \times 100\% = 99.5\%$

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 Surface water and drinking water

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 MFT03717E: "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 Surface water and drinking water"

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 0.03 ppb

LOQ 0.1 ppb (lowest fortification level)

Levels of Fortification 0.1 ppb and 1.0 ppb

Time Required A set of 13 samples requires approximately 18 hours of work (the calculation of the results included).

7. DISCUSSION

Recovery Findings

Method MFT03717E proved to be suitable to determine residues of DCC-3825 and its metabolites in surface water and drinking water to an LOQ of 0.1 ppb. The mean recovery values of the validation experiments were within 70-120%, which fulfills the guideline requirements for mean recovery values.

Linearity
Good linearity ($r \geq 0.9954$) was observed in the range of 0.1 to 10.0 ng/mL for DCC-3825 and its metabolites.

Specificity
Method MFT03717E determines residues of DCC-3825 and its metabolites in surface water and drinking water. No interfering peaks were found at the retention time for DCC-3825 and its metabolites in surface water and drinking water.

Limit of Quantification and Limit of Detection
The LOQ for DCC-3825 and its metabolites in surface water and drinking water was 0.1 ppb 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 MFT03717E 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 surface water and drinking water.

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 MFT03717E can be performed successfully for quantitation of DCC-3825 and its metabolites in surface water and drinking water. The method is thorough and contains sufficient guidance to aid the analyst through the procedure for the first time. However, the matrix matched calibration standards were necessary to achieve the recoveries within the acceptable range of 70 – 120%., especially for the metabolites.

9. PROTOCOL, AMENDMENTS, AND DEVIATIONS

The study protocol was followed as written with two amendments during this study.

Amendment 1 removed the guideline requirements from the protocol which were not pertinent to the conduct of this study.
Amendment 2 changed the information regarding the Study Monitor.

10. COMMUNICATION

There were no communications necessary between the Study Director and the Study Monitor regarding the analytical method or its procedures. The sponsors did not visit the testing facility during the course of the study.

Table 26 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:	1.0% formic acid in water (Optima Grade)		
Mobile Phase B:	1.0% 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	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	~2.02
	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	~1.72
	443 → 353			23	18	
M-53	445 → 371	50	106	29	18	~1.32
	445 → 355	100		25	12	

SUMMARY

The objective of the study is to establish an analytical method for the determination of DCC-3825 and its Metabolites (M-01, M-12, M-13, M-36, M-53) in surface water and drinking water.

Samples were acidified using acetic acid, 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).

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 water 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 0.1 and 1.0 ng/mL for DCC-3825 and its metabolites. A 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, M-53) in surface water and drinking water.

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.

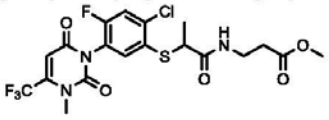
2 CONDUCT OF STUDY

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.

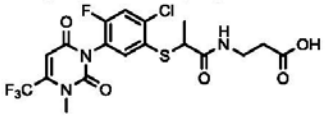
3 MATERIALS AND METHODS

3.1 Analytical standards

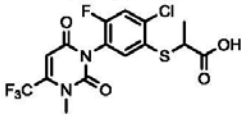
3.1.1 DCC-3825

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% (HPLC)

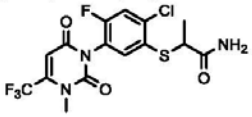
3.1.2 M-01

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% (HPLC)

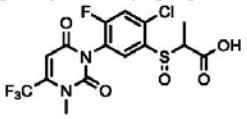
3.1.3 M-12

Identity:	M-12
Chemical name:	2-((2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(fluoromethyl)-2,3-dihydropyrimidin-1(6*H*)-yl)phenyl)thio)propanoic acid
Structure:	
Molecular weight:	426.77
Lot No.:	KM02478-01
Purity:	97.4% (HPLC)

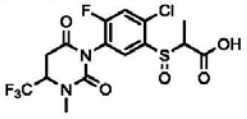
3.1.4 M-13

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% (HPLC)

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% (HPLC)

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% (HPLC)

3.2 Water

Surface water and drinking water were used in the study. Surface water was supplied from Envigo CRS Ltd in June 2016. Tap water supplied by Kusatsu City was used for drinking water. Tap water was aerated to remove the chlorine in water prior to starting the study. The water characterization data is 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:water (70:30, v/v) to obtain fortification solutions with a concentration of 10 and 100 ng/mL.

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 water was fortified with the following levels for DCC-3825 and its metabolites.

0.1 ng/mL	0.5 mL of the fortification solution (10 ng/mL) was added to 50 mL of water.
1.0 ng/mL	0.5 mL of the fortification solution (100 ng/mL) was added to 50 mL of water.

3.6 Analytical method

3.6.1 Acidification

50 mL of the untreated water sample was transferred to a 100 mL-volume Erlenmeyer flask and 500 µL of acetic acid were added to the water sample.

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). The acidified sample was 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 concentration 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 sample was determined.

3.7 LC/MS/MS conditions

Part A-1; HPLC for DCC-3875, M-12, M-13, M-36 and M-53

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-36;	1.43 min
	M-12;	1.63 min	M-53;	1.11 min
	M-13;	1.33 min		

Part A-2; HPLC for M-01

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 (35:65, v/v)	
Flow rate:	0.4 mL/min	
Injection volume:	4 µL	
Retention time:	M-01;	2.21 min

Part B; MS/MS

Instrument:	API 5000™ (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	359.1	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 surface water and drinking water was calculated according to equation 1.

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

Where

C = Concentration of DCC-3825 and its metabolites in water sample [ng/mL]
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 = Sample volume [50 mL]

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

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

Where

R = Recovery of DCC-3825 and its metabolites [%]
C = Concentration of DCC-3825 and its metabolites in water sample [ng/mL]
F = Fortification level [ng/mL]