INDEPENDENT LABORATORY VALIDATION REPORT

STUDY TITLE

Independent Laboratory Validation of "Development and Validation of an Analytical Method for the Determination of NF-180 and its Metabolite QP-1-7 in Surface and in Ground Water"

DATA REQUIREMENTS
Residue Chemistry Test Guidelines

U.S. EPA OCSPP 850.6100
U.S. EPA OPPTS 860.1340
SANCO/825/00 rev. 8.1 (2010)

2.0 INTRODUCTION

The purpose of this study was to demonstrate the method's (1) accuracy, precision, ruggedness, linearity, specificity, matrix effects, and limits of detection and quantitation as well as demonstrate its suitability for the determination of NF-180 in one type of water (surface water) following the method (1) as written.

This study was designed to satisfy guideline requirements described in U.S. EPA OCSPP 850.6100 (2), SANCO/825/00 rev. 8.1 (2010) (3), and OPPTS 860.1340 (4). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (5).

The method was suitable for determination of NF-180 in surface water samples. Aliquots of water samples were mixed with acetonitrile. Then, magnesium sulphate, sodium chloride and buffering citrate salts were added, and the mixture was shaken intensively followed by centrifugation for phase separation. An aliquot of 500 µL of the acetonitrile layer was mixed with 500 µL of water to obtain a 1.0 mL extract in acetonitrile/water (1/1, v/v). The final extracts were analyzed using LC-MS/MS. A method summary is provided in Section 3.6 and the full method is presented in Appendix II.

The method was used as written with the exception of use of the guard column during instrumentation analysis. Communication with the Sponsor consisted of 1) questions regarding acquisition of water matrices; 2) clarification of test substance identification and corresponding Certificates of Analysis; 3) protocol changes/updates during the draft process and receipts of signed final versions; and 4) discussion of protocol and method deviations that occurred (see Section 8.0) between the Study Monitor and Study Director.

3.0 EXPERIMENTAL DETAILS

3.1 Test Substance

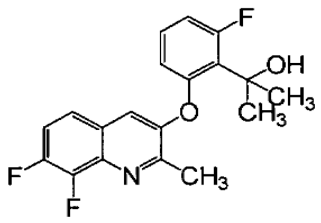
The test substances for this study was NF-180.

3.2 Analytical Reference Substance

The test substance mentioned in the previous section also served as the analytical reference substance. Information regarding the reference substance is summarized below.

Common Name:	NF-180
Chemical Name:	2-[2-(7,8-difluoro-2-methylquinolin-3-yloxy)-6-fluorophenyl]propan-2-ol

Structural Formula:



CAS No.:	1314008-27-9
Molecular Formula:	$C_{19}H_{16}F_3NO_2$
Molecular Weight:	347.33 g/mol
Receipt Date:	06 July 2018
Source:	Nisso Chemical Analysis Services Co., Ltd.
Lot Number:	TRED-001
Purity:	99.1%
Expiration Date:	06 July 2021
Storage:	Frozen (approximately -20 °C)

The test substance was supplied by the Sponsor. Information pertaining to the characterization and stability of the test substance is archived by the Sponsor.

3.3 Test System

The test system evaluated in this study was surface water. This matrix was one for which the method was designed.

The control surface water sample for Trial 1 was collected on the site of the test facility on 26 July 2017. The control surface water sample for Trial 2 was received from the Sponsor on 12 July 2019.

3.4 Equipment

Equipment used throughout the course of the study (including balances, pipettors and various glassware) was the same or of similar technical quality as that specified in the analytical method.

Equipment Description	Product ID	Supplier
Analytical Balances	Mettler Toledo ML3002E/103 Mettler Toledo XPE205	Mettler Instrument Corp
Balance Weights	Troemner 7228-2W	Troemner
Centrifuge	Sorvall Legend XTR	Thermo Fisher Scientific
HPLC	Applied Biosystems/Sciex API 6500 Q-trap LC-MS/MS System with Shimadzu UHPLC System including Controller, Degasser, Pump, Column Heater, and Autosampler	Applied Biosystems/Sciex and Shimadzu

Equipment Description	Product ID	Supplier
Column	Waters Acquity BEH C18 100 mm x 2.1 mm, 1.7 µm	Waters
Pipettors	Positive displacement Model M1000, 100-1000 µL capacity Positive displacement Model M250, 50-250 µL capacity Positive displacement Model M100, 10-100 µL capacity	Gilson, Inc.
	Repeater Xstream, 1 µL-50 mL capacity	Eppendorf North America

3.5 Solvents, Chemicals, and Reagents

Solvents, chemicals and reagents used were of equivalent grade as those specified in the analytical method.

Solvent, Chemical or Reagent (Purity)	Supplier
Water (Optima, Optima-LC/MS, HPLC Grade)	Thermo Fisher Scientific
Methanol (Optima, A.C.S.)	
Acetonitrile (Optima)	
Acetic Acid (Optima LC/MS, 99.7%)	
Water (Milli-pore)	Eurofins Columbia, MO
Supelco dSPE Citrate Extraction Tube	Sigma-Aldrich
Acetonitrile (ACS Reagent)	Burdick & Jackson

3.6 Method Summary

The analytical method independently validated in this study was EAG Laboratories GmbH ID: P 4906 G, entitled "Development and Validation of an Analytical Method for the Determination of NF-180 and its Metabolite QP-1-7 in Surface and in Ground Water" with Method Deviation, dated 23 September 2019. See Appendix II for the complete text of the method and method deviation. The following is a summary of the method:

Ten grams (10.00 g) of water were weighed into a BD Falcon 50-mL polypropylene centrifuge tube with a screw cap and fortified according to the study design. The samples were extracted with 10 mL of acetonitrile, and each tube was capped and shaken vigorously by hand for ~1 minute. The contents of dispersive SPE (dSPE) Citrate Extraction Tube were added to each sample which was then capped and vigorously shaken by hand before centrifuging at ~4000 rpm for ~5 minutes. A 500-µL aliquot of the acetonitrile layer from each sample was transferred to a vial and 500 µL of milli-pore water were added. Matrix and solvent post-spikes were prepared, vialed in 2-mL amber glass autosampler tubes, and submitted for LC-MS/MS analysis along with calibration standards and samples.

3.7 Sample Receipt and Storage

The samples were logged in according to Eurofins SOPs. Samples were stored under refrigerator conditions at approximately 2-8 ºC.

3.8 Instrumentation

All samples were analyzed by HPLC (High Pressure Liquid Chromatography) tandem mass spectrometric (MS/MS) detection. The conditions listed below are those employed for the control suitability evaluation and the successful validation trials.

- **Operating conditions**

Instrument: Applied Biosystems/Sciex API 6500 Q-trap LC-MS/MS System with Shimadzu UHPLC System including Controller, Degasser, Pump, Column Heater, and Autosampler with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.6.2)

Analytical column: Waters Acquity BEH C18 100 mm x 2.1 mm, 1.7 µm

Guard column: In-line Frit

Mobile phase: High purity water, HPLC grade acetonitrile, acetic acid
Component A: 0.1% acetic acid (aqueous)
Component B: 0.1% acetic acid in acetonitrile

Gradient:

Time (min)	% A	% B	Flow Rate (mL/min)
5.00	10	90	0.300
6.00	10	90	0.300
6.10	80	20	0.300
8.00	Stop		

Divert valve: Valco Valve

Interface: Heated Nebulizer

Ionization mode: Positive (+)

Acquisition mode: MRM

Resolution: Q1-Unit, Q3-Unit (Note: Unit is equivalent to medium)

Source temperature: 500 °C

Curtain gas: Nitrogen @ setting of "20"

Collision gas: Nitrogen @ setting of "Medium"

Gas 1: 50.00

Gas 2: 50.00

Exit potential: 10.00

Cell exit potential: 10.00

Transitions monitored:

	Ion, *m/z* Q1	Q3	Time, *ms*	CE, *v*	
NF-180	348	330	75	30	(quantitation)
NF-180a	348	180	75	37	(confirmation)

Injection volume: 10 µL

Column temperature: 40°C

Retention times: NF-180: ~4.75 minutes

- **Calibration/Sample Analysis**

  A six-point standard curve was prepared by injecting constant volumes of calibration standards at specified concentrations. Constant volume injections were used for sample extracts as well. A curve check standard was injected at least every six sample injections.

3.9 Sample Identification

All samples were assigned a unique sample identification number beginning with the five-digit Eurofins study number (87623) followed by an abbreviation indicating the matrix being analyzed and consecutive number designations that were assigned as samples were prepared. For example, the first surface water sample (a reagent blank) was assigned the unique identification of 87623-SW002, the second sample (a control) 87623-SW003, and so on.

3.10 Calculations

Calculations were performed using Analyst® 1.6.2 software to create a standard curve based on linear regression. Linear regression was monitored to support the response linearity of the mass spectrometer detector. The regression functions were used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak response) to demonstrate that a linear relationship exists between analyte concentration and peak response. 1/x weighting was used.

The equation used for the least squares fit was:

$$y = mx + b \quad \text{or} \quad x = \frac{y - b}{m}$$

where:

x	=	ng/mL found for peak of interest
y	=	peak response (area)
b	=	y-intercept
m	=	slope of the standard curve

The calculations for ppb found and percent recovery (for fortified samples) were:

1. The amount of NF-180 (in ppb) found in the sample was calculated according to the following equation:

$$ppb\ Found = \frac{ng/mL\ found\ \times\ Final\ Volume\ (mL) \times Extraction\ Volume\ (mL)\ \times DF}{Sample\ Weight\ (g) \times Aliquot\ Volume\ \ (mL)}$$

where:

ng/mL found	=	ng/mL of analyte found as determined by Analyst®
Final Volume (mL)	=	volume of final sample extract (1 mL)

Extraction Volume (mL)	=	volume of extraction solvent (10 mL)
DF	=	dilution factor
Aliquot Volume (mL)	=	aliquot of extracted sample (0.5 mL)
Sample Weight (g)	=	amount of sample taken through the extraction procedure (10.00 g)

2. Percent recovery of fortified samples (procedural fortifications) was determined using the following equation:

$$\%\ Recovery = \frac{ppb\ found\ in\ fortified\ sample}{ppb\ added} \times 100$$

3. Limit of Detection (LOD) was calculated by multiplying the standard deviation of the ppb found for the LOQ fortifications of each method validation by the Student's t test value for n-1 Degrees of Freedom at the 99% confidence level for each matrix.

4. Limit of Quantitation (LOQ) was calculated by multiplying the LOD by three.

- **Example calculations**

NF-180 ppb residue found in untreated control surface water and in fortified control surface water was calculated as follows:

1. Eurofins ID# 87623-SW016, NF-180, Set MV2, Control Surface Water:

$$ppb\ found = \frac{0.00000\ ng/mL\ \times 1\ mL \times 10\ mL\ \times 1}{10.00\ g \times 0.5\ mL}$$

$$ppb\ found = 0.00000$$

$$ppb\ reported = None\ Detected$$

2. Eurofins ID# 87623-SW020, NF-180, Set MV2, Control Surface Water + 0.05 ppb (LOQ):

$$ppb\ found = \frac{0.02195\ ng/mL\ \times 1\ mL \times 10\ mL\ \times 1}{10.00\ g \times 0.5\ mL}$$

$$ppb\ found = 0.04390$$

$$ppb\ reported = 0.0439$$

$$\%\ Recovery = \frac{0.04390\ ppb}{0.0500\ ppb} \times 100$$

$$\%\ Recovery = 88\%$$

3.11 Statistics

Statistical methods used were limited to calculations of the mean, standard deviation and relative standard deviation. Microsoft Excel® 2016 software was employed to develop all statistical data.

APPENDIX II

Method and Method Deviation

REPORT TITLE

Development and Validation of an Analytical Method for the Determination of NF-180 and its Metabolite QP-1-7 in Surface and in Ground Water

STUDY IDENTIFICATION

EAG Laboratories ID: P 4906 G
Sponsor Study Code: JAS-574: Nisso-NF-180

GUIDELINES COVERED

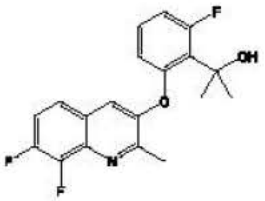
EC Guidance Document on Residue Analytical Methods:
SANCO/825/00 rev. 8.1, 16-Nov-2010
OECD Guidance Document on Pesticide Residue Analytical Methods:
ENV/JM/MONO (2007) 17, 13-Aug-2007
US EPA: OCSPP 860.1340 and OCSPP 850.6100.

1. INTRODUCTION

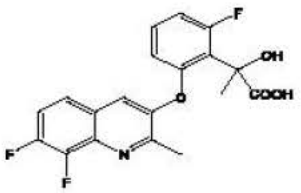
Background and Objective:

To develop and to validate analytical methods for the determination of NF-180 and its metabolite QP-1-7 in surface and ground water with a target limit of quantitation (LOQ) of 0.05 µg/L for each analyte and each water type.

NF-180



QP-1-7



2. GENERAL EXPERIMENTAL

2.1 Test System

Ground water was taken from Buxheim and surface water was obtained from the river Danube, near Leipheim (sampled at Landeswasserversorgung Langenau), Germany. The waters were stored refrigerated until analysis. The analysis data sheets of the 2 water types are attached in Appendix 2. Characteristics of water matrices are listed in the table below:

Parameter	Ground Water	Surface Water
Sampling Date	06-Aug-2018	21-Jun-2018
pH	7.40	8.20
Conductivity at 25 °C in [µS/cm]	827	544
Filterable solids in [mg/L]	0.6	3.6
TOC (total organic carbon) in [mg/L]	1.2	2.1
DOC (dissolved organic carbon) in [mg/L]	1.1	2.0
Magnesium in [mg/L]	17.3	12.7
Calcium in [mg/L]	104	83
Total hardness in [mmol/L]	3.31	2.59
Total german hardness in [°d]	18.5	14.5

2.2 Test/Reference Items

The test/reference items were supplied by the Sponsor, with relevant information concerning identity, molecular mass, purity, and expiration date (see Appendix 1 for Certificate of Analysis)

2.2.1 NF-180

Chemical Name:	2-[2-(7,8-difluoro-2-methylquinolin-3-yloxy)-6-fluorophenyl]propan-2-ol	
Empirical formula:	$C_{19}H_{16}F_3NO_2$	Molar mass: 347.33 g/mol
Lot/Batch No.:	11426-T.Kinoshita	Purity: 98.3 %
Certification:	19-May-16	Expiry Date: 15-June-20
Storage conditions	Keep in a freezer	Date of receipt: 06-May-16

2.2.2 QP-1-7

Chemical Name:	2-[2-(7,8-difluoro-2-methylquinolin-3-yloxy)-6-fluorophenyl]-2-hydroxypropanoic acid	
Empirical formula:	$C_{19}H_{14}F_3NO_4$	Molar mass: 377.31 g/mol
Lot/Batch No.:	31-16149-D.SATO	Purity: 98.1 %
Certification:	09-May-18	Expiry Date: 26-Jun-20
Storage conditions	Keep in a freezer	Date of receipt: 06-Jul-18

2.3 Material and Methods

2.3.1 Equipment and Instrumentation

Balances: Mettler Toledo XS205DU.

Ultrasonic bath:USC 300 TH, VWR international bvba, Belgium.

Centrifuge: Hettich Rotanta 460.

Typical glassware and laboratory equipment.

All the glassware was cleaned in a laboratory dishwasher and air-dried before use.

LC-MS/MS Instrumentation:

Agilent 1290 Series LC system (vacuum solvent degasser, binary LC pump), MayLab MistraSwitch column oven and a PAL System HTS-xt Autosampler.

HPLC column: Waters ACQUITY UPLC BEH C_{18} column (length: 100 mm, i.d.: 2.1 mm, particle size: 1.7 µm)

Pre-column: Phenomenex C18 (UPLC).

Applied Biosystems MDS Sciex API 6500+ triple quadrupole LC-MS/MS system with TurboIonspray (ESI) source.

2.3.2 Solvents, Chemicals and Reagents

Acetonitrile, HPLC grade (Promochem).

LC-MS acetonitrile, ≥ 99.9 % (Honeywell).

Millipore water Supply at test facility

LC-MS water, (LC-MS grade, Honeywell).

Glacial acetic acid, (LC-MS grade Sigma-Aldrich).

Citrate extraction tube (Supelco, 55227-U)

Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Safety information could be obtained from the supplier or literature (e.g. H- and P-phrases). Disposal of waste materials, reagents, reactants, and solvents must be in compliance with applicable governmental requirements.

Acetonitrile and acetic acid are flammable and should be used in well-ventilated areas away from ignition sources. Acetic acid is corrosive.

It is imperative that proper eye and personal protection equipment is worn when handling these reagents.

Safety for reference items: toxicity not specified, usual pre-cautions with toxic compounds.

2.3.3 Preparation of Solutions

Acetonitrile/water (1/1, v/v)

Measure 500 mL Millipore water and add 500 mL of acetonitrile. Mix well.

Water containing 0.1 % acetic acid (mobile phase A)

Measure 1000 mL of water (LC-MS grade). Add 1 mL of concentrated acetic acid (LC-MS grade). Mix well.

Acetonitrile containing 0.1 % acetic acid (mobile phase B)

Measure 1000 mL of acetonitrile (LC-MS grade). Add 1 mL of concentrated acetic acid (LC-MS grade). Mix well.

2.3.4 Preparation of Stock and Fortification Solutions

Separate stock solutions for NF-180 and QP-1-7 were prepared in acetonitrile. During the study each stock solution was prepared two times (2 sets). One set of stock solutions was used for the validation purpose and the second set was used for stability investigations. The concentrations of the NF-180 respectively QP-1-7 were always 0.20 mg/mL.

These concentrations of the stock solutions could be obtained as follows:

Substance name	Weight* [mg]	Dissolve in [mL]	Obtain [mg/mL]
NF-180 (purity 98.3 %)	10.17	50	0.20
QP-1-7 (purity 98.1 %)	10.19	50	0.20

*: Purity taken into account.

Fortification solutions containing both analytes were prepared by accurate dilution into acetonitrile to obtain the following concentrations:

Use solution with	Pipette [mL]	Dilute to [mL]	Concentration of each analyte [µg/mL]
0.20 mg/mL (NF-180 stock solution)	0.050	10	1.0
0.20 mg/mL (QP-1-7 stock solution)	0.050		
1.0 µg/mL (fortification solution)	1.0	10	0.10
0.10 µg/mL (fortification solution)	1.0	10	0.010

All stock and fortification solutions were stored refrigerated and protected from light when not in use.

2.3.5 Preparation of Calibration Solutions in Solvent

Calibration solutions containing NF-180 and QP-1-7 as mix were prepared by accurate dilution into acetonitrile/water (1/1 v/v) to obtain the following concentrations:

Solution Used [ng/µL]	Pipette [µL]	Dilute to [mL]	Concentration of each analyte [ng/mL]
200 (NF-180 stock solution)	500	10	10000
200 (QP-1-7 stock solution)	500		
10 (calibration solution)	100	10	100
0.10 (calibration solution)	1000	10	10
0.10 (calibration solution)	100	10	1.0
0.010 (calibration solution)	250	10	0.25
0.010 (calibration solution)	100	10	0.10
0.0010 (calibration solution)	250	10	0.025
0.0010 (calibration solution)	100	10	0.010
0.0010 (calibration solution)	50	10	0.0050

All calibration solutions were stored refrigerated and protected from light when not in use.

2.3.6 Preparation of Calibration Solutions in Matrix

Preparation of Matrix-Matched Standards

One matrix-matched standard solution containing NF-180 and QP-1-7 at 0.025 ng/mL was prepared for both water types. 0.450 mL of final blank extract was mixed with 0.050 mL of the 0.25 ng/mL calibration solution (containing NF-180 and QP-1-7). These solutions were used to establish the matrix-effect on LC-MS/MS response.

2.3.7 LC-MS/MS Determination

The final extracts were analyzed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) using a Sciex API 6500+ mass spectrometer:

LC System	Agilent 1290 Series LC system (vacuum solvent degasser, binary LC pump), MayLab MistraSwitch column oven and a PAL System HTS-xt Autosampler.
LC Column	Pre-column: Phenomenex C_{18} (UPLC). Column: Waters ACQUITY UPLC BEH C18 column (length: 100 mm, i.d.: 2.1 mm, particle size: 1.7 µm). Column temperature: 40 °C.
LC Injection Volume	10 µL.
LC Method	Solvent A: Water + 0.1 % acetic acid Solvent B: Acetonitrile + 0.1 % acetic acid Mobile Phase Composition: Time (min) / Flow rate (mL/min) / % A / % B 0.00 / 0.30 / 80 / 20 5.00 / 0.30 / 10 / 90 6.00 / 0.30 / 10 / 90 6.10 / 0.30 / 80 / 20 8.00 / 0.30 / 80 / 20
Retention time	≈ 4.9 min for NF-180. ≈ 3.8 min for QP-1-7.
MS/MS System	Applied Biosystems MDS Sciex API 6500+ triple quadrupole LC-MS/MS system with TurboIonspray (ESI) source.
Ion Source Conditions ESI Positive Polarity	Source temperature: 400°C Gas supply (GS 1): 60 (arbitrary units) Gas supply (GS 2) 60 (arbitrary units) Curtain gas: 35 (arbitrary units) CAD gas: 10 Entrance potential: 10 V Declustering potential (DP): 41 V IonSpray voltage: 5500 V Resolution: Q1: Unit, Q3: Unit
MS/MS Conditions for NF-180 and QP-1-7	<u>*NF-180:*</u> *MS/MS transition for quantification:* *348 m/z > 330 m/z* Collision energy (CE): 31 V Cell exit potential (CXP): 22 V Dwell time: 200 ms *MS/MS transition for confirmation* *348 m/z > 180 m/z* Collision energy (CE): 39 V Cell exit potential (CXP): 12 V Dwell time: 200 ms <u>*QP-1-7:*</u> *MS/MS transition for quantification:* *378 m/z > 332 m/z* Collision energy (CE): 31 V Cell exit potential (CXP): 22 V Dwell time: 200 ms *MS/MS transition for confirmation.* *378 m/z > 314 m/z* Collision energy (CE): 43 V Cell exit potential (CXP): 20 V Dwell time: 200 ms

Mass spectra are shown in Appendix 3. Details about the LC-MS/MS method are shown in Appendix 7.

The $[M+H]^+$ ion of NF-180 at 348 m/z was used as parent ion for MS/MS detection. The MS/MS transition to the daughter ion at 330 m/z was used for quantification of the analyte. The transition 348 m/z-> 180 m/z was used as confirmation transition.

The $[M+H]^+$ ion of QP-1-7 at 378 m/z was used as parent ion for MS/MS detection. The MS/MS transition to the daughter ion at 332 m/z was used for quantification of the analyte. The transition 348 m/z-> 314 m/z was used as confirmation transition.

Calibration functions as exemplified in Figure 1 and Figure 2 were established with standards in solvent ranging from 0.0050 to 1.0 ng/mL for both analytes (≥ 5 levels) by injecting 10 µL of standard solutions in solvent. The LC-MS/MS calibration functions were calculated and plotted by regression analysis, using the LC-MS/MS software. The correlation coefficients (r) were always > 0.99. Matrix interference on LC-MS/MS response must be always examined for each analyte / matrix and if significant, compensated by the use of matrix-matched standard calibration solutions. Matrix effect observed in the course of the present study were not significant (≤ 20 %).

Figure 3 and Figure 4 show representative LC-MS/MS daughter ion chromatograms of calibration solutions. Figure 5 to Figure 18 show representative LC-MS/MS daughter ion chromatograms of fortified extracts, blank controls and reagent blank.

2.3.8 Calculation

The following equation was used to calculate the residues R of the analytes in µg/L:

$R = C_{End} \times [(V_{Ex} \times V_{End}) / (V_1 \times V_{sample})$

$= C_{End} \times$ Multiplier M

R: Residue in µg/L.

C_{End}: Final concentration of analyte in extract in ng/mL.
(where multiple injections were evaluated: mean).

V_{Ex}: Extraction volume: 10 mL of acetonitrile.

V_1: Aliquot of V_{Ex} used for preparation of final extract: 0.50 mL

V_{End}: Final volume of extract: 1.0 mL.

V_{sample}: Volume of water used for extraction: 10 mL (equivalent with 10 g).

Residues R of the analytes are calculated in µg/L. The values reported in the tables are calculated with full precision, but displayed rounded. Minor/insignificant discrepancies may be observed when recalculated.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$Rec. = (R / R_{fortified}) \times 100\ \%$

The calculation of the residue R is demonstrated with a surface water specimen, which was fortified at the LOQ level (0.05 µg/L) with NF-180 and QP-1-7 and assigned with the specimen ID P 4906-56. The final extract was examined by LC-MS/MS (P4906API6#076, see Table 1 and Figure 6 top).

The daughter ion chromatogram of the final extract was evaluated by the instrument software using external calibration functions.

NF-180 elutes at 4.9 min and gives upon integration a peak area of 1.13 x 10^5 counts (quantified using the primary transition 348 m/z > 330 m/z), resulting in a concentration C_{End} of 0.0216 ng/mL.

The residue was calculated with:

R = C_{End} x [(V_{Ex} x V_{End}) / (V_1 x V_{sample})]
= C_{End} x Multiplier M
= 0.0216 ng/mL x [(10 mL x 1.0 mL) / (0.50 mL x 10 mL)]
= 0.0216 ng/mL x 2 = 0.0432 ng/mL = 0.0432 µg/L)

Recoveries (Rec.) were calculated for the fortified specimen as follows:

Rec. = (R / $R_{fortified}$) x 100 % = (0.0432 µg/L / 0.050 µg/L) x 100 %
= 86 %

3. METHOD

3.1 Extraction Procedure for Surface and Ground Water

1. Weigh 10 g (equivalent with 10 mL) of surface or ground in a 50 mL centrifuge tube.
2. Accurately fortify recovery control specimens with fortification solutions. For LOQ recoveries pipette 50 µL of the 0.010 µg/mL fortification solution containing NF-180 and QP-1-7. For the higher-level recoveries, add 50 µL of the 0.10 µg/mL fortification solution containing NF-180 and QP-1-7.
3. Add 10 mL of acetonitrile and shake vigorously for 1 minute.
4. Add contents of Citrate Extraction Tube* (Supelco, 55227-U). Shake vigorously for 1 minute and centrifuge for 5 minutes at 4000 rpm.
5. Dilute the extract (acetonitrile layer) by dilution factor (DF 2) i.e. by mix 500 µL of acetonitrile layer with 500 µL milli-pore water to obtain a 1.0 mL extract in acetonitrile/water (1/1, v/v)

*: Citrate extraction tubes (12 ml) containing 4 g $MgSC_4$, 1 g NaCl, 0.5 g NaCitrate dibasic sesquihydrate and 1 g NaCitrate tribasic dehydrate equivalent to QuEChERS[1] (EN 15662:2008 or EN 15662:2009-02) were used for clean-up of extracts.

3.2 Effects of Matrix on Analyte Response

Matrix effects were tested by comparing LC-MS/MS response of a standard in neat solvent with a standard in matrix at 0.025 ng/mL concentration (see Table 3).

No significant matrix (< 20%) effect was observed for NF-180 or QP-1-7 in both surface and ground water matrices. Therefore, calibration standards prepared in solvent (acetonitrile/water (1/1, v/v)) were used to quantify the final extracts.

3.3 Stability in Working Solutions and Final Extracts

Stability of stock solutions in acetonitrile was proven by comparing the areas of 10 ng/mL solutions prepared by diluting the aged and the freshly prepared stock solution.

[1] EN 15662:2009-02: Foods of plant origin –Determination of pesticide residues using GC-MS and/or LC/MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE – QuEChERS-method.

The stock solutions of NF-180 and QP-1-7 prepared in acetonitrile were found to be stable for at least 28 days when stored refrigerated.

Stability of the calibration solutions containing NF-180 and QP-1-7 were proven by comparing the area of original, aged calibration solutions with a dilution made from a freshly prepared stock solution. The calibration solutions prepared in acetonitrile/water (1/1, v/v) were stable for at least 27 days when stored refrigerated. Details concerning the stability of working solutions are shown in Table 4.

Selected final extracts for both water matrices were re-injected after storage for 7 or 9 days under refrigerated conditions to demonstrate stability. These selected final extracts were quantified using newly prepared calibration solutions. The results obtained showed small differences (always less than 20 %) in comparison to the initial analysis. Thus it can be concluded that NF-180 and QP-1-7 are stable in final extracts for at least 7 days under refrigerated storage conditions.

3.4 Time Required for Analysis

The time required for extraction of 13 samples is about 2 hours. LC-MS/MS analyses can be performed unattended overnight and requires about 8 min per run. Thus, a set of 13 specimens injected interspersed with calibration solution needs about 3-4 hours. Evaluation of the LC-MS/MS results need about 3 hours.
Thus, a complete set of 13 samples can be completed in approximately one and a half working days.

3.5 Extraction Efficiency

In the current study, extraction efficiency was not conducted experimentally because the radiolabeled samples with incurred residues were not available. The determination of extraction efficiency was not a subject/objective of the present study

4. Results And Discussion

The objective of this study was to develop and validate analytical methods for the determination of NF-180 and its metabolite QP-1-7 in surface water and ground water at a target limit of quantitation (LOQ) of 0.05 µg/L and at 10-fold LOQ (0.50 µg/L) for each analyte and each water type.

4.1 LC-MS/MS Selectivity and Sensitivity

The highly specific LC-MS/MS method used at least two mass transitions for each of the two analytes:

- *For NF-180*: The transition 348 m/z⟶ 330 m/z was used for quantitation and transition 348 m/z ⟶ 180 m/z was used for confirmation for all matrices.

- *For QP-1-7:* The transition 378 m/z-⟶ 332 m/z was used for quantitation and transition 378 m/z ⟶ 314 m/z was used for confirmation for all matrices.

LC-MS/MS using the commonly available Sciex API6500 + instrument allows the detection of NF-180 and its metabolite QP-1-7 at concentrations as low as 0.0050 ng/mL with 10-µL injections, therefore providing sufficient sensitivity to determine and to confirm residues of both analytes in the extracts.

4.2 Specificity and Limit of Detection

Final specimen extracts were analyzed by the highly specific LC-MS/MS technique, monitoring at least two mass transitions (MRMs) per analyte.

The limits of detection (LOD) are estimated (based on the signal obtained for the lowest calibration) to be 0.0050 ng/mL corresponding to 0.01 µg/L (20 % of the LOQ). No interfering signals > LOD (0.01 µg/L) were detected at the relevant retention times in the blank control specimens.

4.3 Linearity

Linear calibration functions (standard calibrations in solvent) with 1/x weighting were calculated and plotted by regression analysis, using the Analyst software (for calibration curves exemplified see Figure 1 and Figure 2. Calibration functions ranging from 0.0050 to 1.0 ng/mL for both analytes (≥ 5 levels) were established by injecting 10 µL of the calibration solutions in solvent. The correlation coefficients (r) for both analytes were always > 0.99.

4.4 Validation Results for Analytical Method

The method was validated for both water types at the 0.050 µg/L LOQ level and at 10-fold LOQ (0.50 µg/L) level for both NF-180 and QP-1-7.

Individual and average recoveries as well as relative standard deviations obtained were all within the acceptance criteria (70 % to 120 % with relative standard deviations (RSD) ≤ 10 %) for method validation,

5. CONCLUSIONS

The method was validated for the determination of resicues of NF-180 and its metabolite QP-1-7 in surface water and in ground water by LC-MS/MS, demonstrating the LOQ of 0.05 µg/L and the limit of detection (LOD) of 0.01 µg/L for both analytes.

The method is applicable as an enforcement method as it fulfils the validation requirements as defined in the following guidelines:

- EC Guidance Document on Pesticide Residue Analytical Methods: SANCO/825/00/rev. 8.1, 16-Nov-2010.
- OECD Guidance Document on Pesticide Residue Analytical Methods: ENV/JM/MONO(2007)17, 13/08/2007
- US EPA OCSPP 860.1340 Residue Analytical Method.
- US EPA OCSPP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation

APPENDIX 6 AMENDMENT TO STUDY PLAN

Amendment No. 1 to Study Plan

1.0 Title: Development and Validation of an Analytical Method for the Determination of NF-180 and its Metabolite QP-1-7 in Surface and in Ground Water

2.0 Study Identification: EAG Laboratories ID P 4906 G

3.0 Reason for Amendment and Impact:
Upon request of the Sponsor, ground water will be added as additional water type to this study. The target limit of quantitation (LOQ) will be 0.05 µg/L for each analyte.

No negative impact on the outcome of the study is expected.

4.0 Changes in the sections of the study plan are written in *italics* and deleted sections are **~~marked deleted~~**.

1.0 **Title (old):** Development and Validation of an Analytical Method for the Determination of NF-180 and its Metabolite QP-1-7 in Surface Water
1.0 **Title (new):** Development and Validation of an Analytical Method for the Determination of NF-180 and its Metabolite QP-1-7 in Surface *and in Ground* Water

3.0 Objectives: To develop and to validate analytical methods for the determination of NF-180 and its metabolite QP-1-7 in surface water and *in ground water* with a target limit of quantitation (LOQ) of 0.05 µg/L for each analyte and *each water type*.

[...]

6.0 Proposed Schedule: Experimental: Jul/*Aug*-18 Study Completion: ~~Sep-18~~ Oct-18

8.1 Test Systems:
Surface (pond or river) *and ground water* obtained locally. Sampling site of the water type will be reported. Water will be characterized for physical and chemical properties, including e.g. pH, conductivity, total hardness, DOC (dissolved organic carbon) and silt content (filtered particles). Some characterization parameters will be performed (non-GLP, DIN/EN methods) in environmental laboratory Institut Alpha (Ulm).

8.2 Method Validation:
Water will be mixed with acetonitrile, filtered, diluted (when necessary) and injected into LC-MS/MS.

If LC-MS/MS is not sensitive enough ~~a pre-concentration step e.g. via liquid/liquid extraction (LLE) or solid phase extraction will be used~~ *a clean-up step will be necessary*.

[...]

APPENDIX IV

Preparation of Standard Solutions

Preparation of Standard Solutions

The preparation of standard solutions used for this study is described below. Stock solutions (corrected for purity) and fortification solutions were prepared in acetonitrile and intermediate and calibration standard solutions were prepared in acetonitrile:water (50:50, v/v). All solutions were stored as recommended in the method when not in use (protected from light and refrigerated at approximately 2-8°C).

Stock Solutions

Solution Identification	Analyte	Purity (%)	Amt. Weighed (mg)	Dilution Vol. (mL)	Final Concentration (mg/mL)
CDG 17552[a]	NF-180	99.1	10.17	50	0.202
CDG 18106	NF-180	99.1	10.09	50	0.200

[a] The incorrect purity (98.3%) was inadvertently used when preparing this stock standard solution. This resulted in the final concentration of the stock standard solution and of any fortification, intermediate, and calibration standard solutions being slightly above those stated in the method.

Fortification Solutions

New Solution Identification	Parent Solution Identification	Parent Concentration	Aliq. Vol (µL)	Dilution Vol. (mL)	Final Concentration (µg/mL)
17552-F1[a]	CDG 17552	0.202 mg/mL	50	10	1.01
17552-F2[a]	17552-F1	1.01 µg/mL	1000	10	0.101
17552-F3[a]	17552-F2	0.101 µg/mL	1000	10	0.0101
18106-F1	CDG 18106	0.200 mg/mL	50	10	1.00
18106-F2	18106-F1	1.00 µg/mL	1000	10	0.100
18106-F3	18106-F2	0.100 µg/mL	1000	10	0.0100

[a] The incorrect purity (98.3%) was inadvertently used when preparing this stock standard solution. This resulted in the final concentration of the stock standard solution and of any fortification, intermediate, and calibration standard solutions being slightly above those stated in the method.

Intermediate and Calibration Standard Solutions

New Solution Identification	Parent Solution Identification	Parent Concentration	Aliq. Vol (µL)	Dilution Vol. (mL)	Final Concentration (ng/mL)
17552-C1[a]	CDG 17552	0.202 mg/mL	500	10	10100
17552-C2[a]	17552-C1	10100 ng/mL	100	10	101
17552-C3[a]	17552-C2	101 ng/mL	1000	10	10.1
17552-C4[a]	17552-C2	101 ng/mL	100	10	1.01
17552-C5[a]	17552-C3	10.1 ng/mL	250	10	0.253
17552-C6[a]	17552-C3	10.1 ng/mL	100	10	0.101
17552-C7[a]	17552-C4	1.01 ng/mL	250	10	0.0253
17552-C8[a]	17552-C4	1.01 ng/mL	100	10	0.0101
17552-C9[a]	17552-C4	1.01 ng/mL	50	10	0.00505

[a] The incorrect purity (98.3%) was inadvertently used when preparing this stock standard solution. This resulted in the final concentration of the stock standard solution and of any fortification, intermediate, and calibration standard solutions being slightly above those stated in the method.

New Solution Identification	Parent Solution Identification	Parent Concentration	Aliq. Vol (µL)	Dilution Vol. (mL)	Final Concentration (ng/mL)
18106-C1	CDG 18106	0.200 mg/mL	500	10	10000
18106-C2	18106-C1	10000 ng/mL	100	10	100
18106-C3	18106-C2	100 ng/mL	1000	10	10.0
18106-C4	18106-C2	100 ng/mL	100	10	1.00
18106-C5	18106-C3	10.0 ng/mL	250	10	0.250
18106-C6	18106-C3	10.0 ng/mL	100	10	0.100
18106-C7	18106-C4	1.00 ng/mL	250	10	0.0250
18106-C8	18106-C4	1.00 ng/mL	100	10	0.0100
18106-C9	18106-C4	1.00 ng/mL	50	10	0.00500