STUDY TITLE

Independent Laboratory Validation of Ishihara Sangyo Kaisha (ISK) Residue Analytical Method for the Determination of IKI-220 and Metabolites (TFNA, TFNG, TFNA-AM, and TFNG-AM) in Surface Water and Drinking Water
(Document Number: 834131)

GUIDELINE REQUIREMENTS

US EPA Ecological Effects Test Guidelines, OCSPP 850.6100

- HPLC vials, clear glass: 1.8 mL
- Ultrasonic Cleaner: Branson 5510
- SPE Cartridges: ENV+ 500 mg, 6 mL, Biotage Isolute P/N 915-0050-C
- Rotary Evaporators: Labconco Rotary Evaporators
- pH Meter: VWR symphony SB70P Meter
- SPE Manifold: Burdick & Jackson (24 position)
- SPE Reservoirs: ~ 60 mL Varian Bond Elut
- AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD XR HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC HT Autosampler

  Note: Both APCI (Atmospheric-Pressure Chemical Ionization) and ESI (Electrospray Ionization) probes were used.

B. Reagents and Standards

The following chemicals were used:

Chemical	Distributer	Part No:
Acetonitrile	Fisher	A996-4
Dichloromethane	VWR	MK487910
Ethanol	VWR	EM-EX0278-6
Ethanol	Fisher	AC61509-0020
Formic Acid	Fisher	A118P-500
Hydrochloric Acid	VWR	EMD-HX0603-3
Methanol	VWR	MK304110
Water	Fisher	W5-4

All chemicals were of HPLC-grade quality or better.

Preparation of Reagent Solutions:

Acetonitrile/HPLC grade water (10:90, v/v): Prepared by adding 100 mL of acetonitrile to 900 mL of HPLC-grade water and mixing well. Other proportional preparations were also made.

Hydrochloric Acid Solution (about 0.1 mol/L): Prepared by adding 0.5 mL of concentrated hydrochloric acid to 500 mL of HPLC-grade water and mixing well. *Note: The concentration of this solution can also be stated as "0.1% (v/v)". The use of "about 0.1 mol/L" is consistent with the wording of the solution name in the analytical reference method.*

Hydrochloric Acid Solution (about 1 mol/L): Prepared by adding 5 mL of concentrated hydrochloric acid to 500 mL of HPLC-grade water and mixing well. *Note: The concentration of this solution can also be stated as "1% (v/v)". The use of "about 1 mol/L" is consistent with the wording of the solution name in the analytical reference method.*

Mobile Phase A: 100% Methanol was used as mobile phase A with no additional preparation necessary.

Mobile Phase B:
0.05% (v/v) Formic Acid in HPLC-grade water: Prepared by adding 1 mL of formic acid to 1999 mL of HPLC-grade water and mixing well.

Needle Wash (APCI Methods):
Methanol/Water (10:90, v/v): Prepared by mixing 50 mL of methanol with 450 mL of HPLC-grade water and mixing well.

Needle Wash (ESI Methods):
Acetonitrile/Water (50:50, v/v): Prepared by mixing 500 mL of acetonitrile with 500 mL of HPLC-grade water and mixing well.

1. Reference Substances

The IKI-220, TFNA, TFNA-AM, and TFNG (original lot) analytical reference standards were received in good condition on April 30, 2014 from Midwest Research Institute (MRI Global), Kansas City, MO. The TFNG-AM reference standard was received in good condition on April 23, 2015 from MRI Global. The second lot of TFNG reference standard was received on March 9, 2016. The certificate of analysis for the standards are in the archives at GPL. The following table contains detailed information for the analytical standards used in this study.

Analytical Standard	CAS #	Lot/Batch #	Purity (%)	Expiration Date
IKI-220 (Flonicamid)	158062-67-0	9809	99.6	12/12/2018
TFNA	158063-66-2	0006	100.0 100.0	01/03/2016 11/17/2020
TFNG	NA	20110328	99.37	02/29/2016
TFNG	NA	20110920	99.83	03/31/2017
TFNA-AM	158062-71-6	0006	99.7 99.9	01/03/2016 11/19/2020
TFNG-AM	158062-96-5	0006	99.5 99.6	01/04/2016 11/19/2020

Upon receipt, the neat reference standards were stored in a freezer set to maintain ≤ -10 °C.

2. Preparation of Standard Solutions

The IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM reference substances were used in the preparation of the fortification and calibration solutions. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data. For clarity, this section has been further divided into two sections going forward, one for the failed method validation trial attempts (surface water trial 1, surface water trial 2, drinking water trial 1) and one for the successful method validation attempts (surface water trial 3 and drinking water trial 2)

a. Failed Method Validation Attempts

(1) Stock Solutions

On December 9, 2015, the following stock solutions were prepared:

An aliquot of the IKI-220 reference standard (11.2 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.12 mg/mL IKI-220 (Solution A).

An aliquot of the TFNA reference standard (14.2 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.42 mg/mL TFNA (Solution B).

An aliquot of the TFNG reference standard (10.3 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.02 mg/mL TFNG (Solution C).

An aliquot of the TFNA-AM reference standard (10.8 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.08 mg/mL TFNA-AM (Solution D).

An aliquot of the TFNG-AM reference standard (10.6 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.05 mg/mL TFNG-

AM (Solution E).

All stock solutions were sonicated for approximately 5 minutes after preparation. The stock solutions were stored frozen (in a freezer set to maintain ≤ -10 °C) when not in use.

(2) Intermediate and Fortification Solutions

A 2-mL aliquot of solutions A through E were individually diluted with acetonitrile to a volume (approximately 20 mL, each individual volume was different) to make individual 100 µg/mL intermediate solutions. A 10-mL aliquot of each of these intermediate solutions was combined and diluted to a volume of 100 mL with acetonitrile resulting in a solution that contained 10.0 µg/mL IKI-220, TFNA, TFNG, TFNA-AM and TFNG-AM (Solution F). Note: Solution F and all subsequent dilutions also contained the compound TFNA-OH which was later amended out of the study and will not be reported. From solution F, a 5-mL aliquot was diluted to 50 mL with acetonitrile, resulting in a solution that contained 1.00 µg/mL IKI-220, TFNA, TFNG, TFNA-AM and TFNG-AM (Solution G). From solution G, a 5-mL aliquot was diluted to 50 mL with acetonitrile, resulting in a solution that contained 100 ng/mL IKI-220, TFNA, TFNG, TFNA-AM and TFNG-AM (Solution H).

Aliquots of solution F were used to fortify at 10x the LOQ for the trial 1 surface water method validation attempt. Aliquots of solution G were used to fortify at the LOQ for the trial 1 surface water method validation attempt and at 10x the LOQ for both the trial 2 surface water and trial 1 drinking water method validation attempts. Solutions F and G were diluted to prepare calibration standards. Subsequent dilutions were made from the calibration standards to prepare additional standards when necessary. All intermediate and fortification solutions were given an expiration date of three months from the date of preparation or when the neat reference standard expired, whichever was earlier. The intermediate and fortification solutions were stored refrigerated (in a refrigerator set to maintain 4 ± 5 °C) when not in use.

(3) Calibration Standards

All calibration standards were diluted into HPLC-grade water. The calibration standards were given an expiration date of three months from the date of preparation (or an expiration date not to exceed the expiration date of the neat reference standards). The calibration solutions were stored refrigerated when not in use. The calibration standards were prepared by diluting the solutions as listed in the table as follows:

Initial Solution ID	Volume of Solution (mL)	Final Volume (mL)	Final Solution ID	Approximate Standard Concentration (ng/mL)
F	5	100	I	500
F	2	100	J	200
G	10	100	K	100
G	5	100	L	50
G	2	100	M	20
G	1	100	N	10
G	1	200	O	5
G	0.5	200	P	2.5
M	1	10	Q	2
N	1	10	R	1
O	1	10	S	0.5
P	1	10	T	0.25

b. Successful Method Validation Attempts

(1) Stock Solutions

On October 12, 2016, the following stock solutions were prepared:

An aliquot of the IKI-220 reference standard (10.1 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.01 mg/mL IKI-220 (Solution AA).

An aliquot of the TFNA reference standard (11.3 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.13 mg/mL TFNA (Solution BB).

An aliquot of the TFNG reference standard (11.4 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.14 mg/mL TFNG (Solution CC).

An aliquot of the TFNA-AM reference standard (10.5 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.05 mg/mL TFNA-AM (Solution DD).

An aliquot of the TFNG-AM reference standard (11.0 mg) was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.10 mg/mL TFNG-AM (Solution EE).

All stock solutions were sonicated for approximately 5 minutes after preparation. The stock solutions were stored frozen (in a freezer set to maintain ≤ -10 °C) when not in use.

(2) Intermediate and Fortification Solutions

Aliquots (approximately 5 mL, each individual volume was different) of solutions AA, BB, CC, DD, and EE were diluted with acetonitrile to 50.1 mL, resulting in a solution containing approximately 100 µg/mL IKI-220, TFNA, TFNG, TFNA-AM and TFNG-AM (Solution FF). A 1-mL aliquot of solution FF was diluted to a volume of 101 mL with acetonitrile resulting in a solution that contained approximately 1.00 µg/mL IKI-220, TFNA, TFNG, TFNA-AM and TFNG-AM (Solution GG). Additionally, from solution FF, a 100 µL aliquot was diluted to 101 mL with acetonitrile, resulting in a solution that contained approximately 100 ng/mL IKI-220, TFNA, TFNG, TFNA-AM and TFNG-AM (Solution HH). An approximately 100 ng/mL IKI-220, TFNA, TFNG, TFNA-AM and TFNG-AM intermediate solution was also prepared from solution FF by diluting a 100 µL aliquot to 101 mL with HPLC-grade water (Solution II).

Aliquots of solution GG and HH were used to fortify at 10x the LOQ and LOQ, respectively, for both the trial 3 surface water and trial 2 drinking water method validation

attempts. Solution II was diluted to prepare calibration standards. All intermediate and fortification solutions were given an expiration date of three months from the date of preparation. The intermediate and fortification solutions were stored refrigerated (in a refrigerator set to maintain 4 ± 5 °C) when not in use.

(3) Calibration Standards

All calibration standards were diluted into HPLC-grade water. The calibration standards were given an expiration date of three months from the date of preparation. The calibration solutions were stored refrigerated when not in use. The calibration standards were prepared by diluting the solutions as listed in the table as follows:

Initial Solution ID	Volume of Solution (mL)	Final Volume (mL)	Final Solution ID	Approximate Standard Concentration (ng/mL)
II	10	100	JJ	10
II	5	100	KK	5
II	2	100	LL	2
II	1	100	MM	1
II	0.5	100	NN	0.5
II	0.5	200	OO	0.25

C. Safety and Health

Safety Data Sheets (SDS) and/or Material Safety Data Sheets (MSDS) for the reference substances can be found in the raw data and are available from the Sponsor. Reagent and solvent SDS are available on the distributor's websites. Proper personal protective equipment was worn during the execution of this method. Staff avoided breathing chemical vapor and avoided chemical contact with eyes and skin. There were no procedural steps that required special precautions to avoid safety or health hazards, however a fume hood was used during the steps requiring concentrated acids.

III. METHODS

A. Principle of Analytical Method

The analysis of surface and drinking water was performed according to the method described in the study report "Validation of the Residue Analytical Method for IKI-220 and Metabolites (TFNA, TFNG, TFNA-AM, TFNA-OH and TFNG-AM) in Surface Water and Drinking Water" dated with the study

completion date August 22, 2002 (Document Number 834131 from RCC Ltd Switzerland). After the first method validation trial attempt in surface water, the method was modified as necessary to result in a successful validation attempt for both surface and drinking water. Those modifications are discussed herein.

The limit of quantitation (LOQ) for IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM was 0.1 µg/L (ppb). The method defined limit of detection LOD was 0.1 µg/L (ppb), however, 0.05 µg/L (ppb) was the achieved LOD for all analytes. The method validation trial 1, 2, and 3 attempts for surface water were performed on December 15, 2015, January 19, 2016, and November 2, 2016, respectively. The method validation trial 1 and 2 attempts for drinking water were performed on January 22, 2016 and October 31, 2016, respectively. All samples for the validation trial attempts were extracted in one analytical set each. The sets consisted of one reagent blank sample (HPLC-grade water), two control samples, five LOQ laboratory fortification samples and five 10x LOQ laboratory fortification samples. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number, the sample set designation and a sample number (e.g., 589ILV01-1).

Aliquots (1000 mL or 100 mL) of control matrix water were fortified. Samples were acidified with a dilute hydrochloric acid solution. Samples were drawn through a SPE cartridge and the analytes were eluted with methanol. Sample extracts were concentrated using rotary evaporation. Samples were vialed and analyzed by LC-MS/MS.

B. Analytical Procedure

1. Control Matrices

a. Drinking Water

The drinking water control matrix samples were obtained from the municipal supply at GPL on January 21, 2016 and October 27, 2016. The sample was collected into a 4-L amber bottle and was kept refrigerated until use. The drinking water sampled on January 21, 2016 was used as the control source for the first method validation trial attempt, whereas the drinking water sampled on October 27, 2016 was used as the control source for the second validation attempt. A newly sampled drinking water sample was used for trial 2 due to the duration of time between trial 1 and 2. The characterization of the Fresno municipal supply water is presented in the 2014 and 2015 Water Quality Reports located in Appendix C.

b. Surface Water

The first surface water control matrix sample was obtained from the Fresno Irrigation District Canal "Herndon No. 39" at a point near the Gates Avenue Bridge on December 14, 2015. Sub-portions of this sample were taken and labeled as "Herndon-39-151214" (with additional suffixes to denote individual containers) and stored refrigerated when not in use. One of these sub-aliquots was shipped overnight (on blue ice) to Agvise Laboratories in Northwood, North Dakota for GLP characterization. The GLP sample characterization results are presented below:

Parameter	Result
pH	7.4
Calcium	6.8 ppm
Magnesium	1.4 ppm
Sodium	4.5 ppm
Hardness	23 mg equivalent $CaCO_3$/L
Conductivity	0.07 mmhos/cm
Sodium Adsorption Ratio (SAR)	0.41
Total Dissolved Solids	66 ppm
Turbidity	5.38 NTU

Sub-portions from the sample "Herndon-39-151214" (refrigerated after collection) were used for the trial 1 and 2 surface water method validation attempt.

The site where the first surface water control matrix sample was obtained did not have water at the time of the second sampling. As a result, the second surface water control matrix sample was obtained from a point on the San Joaquin River near Nees and Palm Avenues in Fresno, California on October 18, 2016. Sub-portions of this sample were taken and labeled as "SJR-161018" (with additional suffixes to denote individual containers) and stored refrigerated when not in use. One of these sub-aliquots was frozen and shipped overnight (on blue ice) to Agvise Laboratories in Northwood, North Dakota for GLP characterization. The GLP sample characterization results are presented below:

Parameter	Result
pH	7.1
Calcium	2.7 ppm
Magnesium	0.5 ppm
Sodium	2.8 ppm
Hardness	9 mg equivalent $CaCO_3$/L
Conductivity	0.04 mmhos/cm
Sodium Adsorption Ratio (SAR)	0.41
Total Dissolved Solids	94 ppm
Turbidity	1.24 NTU

Sub-portions from the sample "SJR-161018" (refrigerated after collection) were used for the trial 3 surface water method validation attempt.

2. Failed Method Validation Attempts

a. Surface Water Trial 1

(1) Preparation of Samples

Sub-samples (1000 mL) of the sample Herndon-39-151214 were measured into 1-Liter bottles.

(2) Fortifications

Independent laboratory validation samples were fortified at the LOQ (0.1 µg/L) or 10x the LOQ (1 µg/L). Fortifications were performed using Wiretrol disposable micropipettes to directly fortify the 1000-mL samples as follows:

Fortification Level	Amount and Concentration of Spiking Solution Used (IKI-220/TFNA/TFNG/TFNA-AM/ TFNA-OH/TFNG-AM)
LOQ (0.1 µg/L)	100 µL of 1 µg/mL
10x LOQ (1 µg/L)	100 µL of 10 µg/mL

(3) Extraction

After fortification, a 2-mL aliquot of hydrochloric acid solution (about 0.1 mol/L) was added to each sample. The ENV+ SPE cartridges were conditioned and equilibrated by passing 5 mL of dichloromethane, 5 mL of methanol, 5 mL of HPLC-grade water, and 5 mL of hydrochloric acid solution (about 0.1 mol/L) through the cartridges one after another. An aliquot (5 mL) of concentrated hydrochloric acid was passed through the cartridges. The eluate was discarded. A reservoir was added to each SPE cartridge and the entirety of each sample was loaded onto each SPE cartridge at a flow rate of less than 10 mL/minute. The eluate was discarded. After the entirety of the sample was loaded onto the cartridges, the cartridges were vacuumed to dryness for approximately 10 seconds. The cartridges were

then eluted with three 5-mL aliquots of methanol (i.e., 15 mL) which was collected in a 16-mL glass tube and then transferred (in parts so that the 16-mL did not overflow) to a 22-mL vial for refrigerated storage overnight. Following storage, each sample extract was poured into a 125-mL round bottom flask.

The sample extracts were evaporated to dryness using a rotary evaporator set at a temperature of 50-60 ºC. The residues were re-dissolved in 10 mL of ethanol. The sample extract was evaporated to dryness using a rotary evaporator set at a temperature of 50-60 ºC. The residues were re-dissolved in 2 mL of acetonitrile/water (10:90, v/v) using an ultrasonic bath (approximately 1 minute of sonication). The 10x LOQ samples were dilute 10-fold with acetonitrile/water (10:90, v/v).

Samples were vialed and submitted for analysis by LC-MS/MS. The samples were analyzed against a calibration curve that ranged from 5 ng/mL to 500 ng/mL.

Notes from trial 1:

During the load step, approximately 25 to 40 mL of sample 589ILV01-5 was inadvertently poured into the SPE cartridge for 589ILV01-6. Approximately 50-60 mL of 589ILV01-11 was lost during sample loading when the reservoir broke. For 589ILV01-5, 5 mL of the methanol eluent was lost.

b. Surface Water Trial 2 and Drinking Water Trial 1

(1) Preparation of Samples

Sub-samples (100 mL) of the sample Herndon-39-151214 and sample GPL-DW-160121, were measured into 250 mL glass bottles for surface water trial 2 and drinking water trial 1, respectively.

(2) Fortifications

Independent laboratory validation samples were fortified at the LOQ (0.1 µg/L) or 10x the LOQ (1 µg/L). Fortifications were performed using Wiretrol disposable micropipettes to directly fortify the 100-mL samples as follows:

Fortification Level	Amount and Concentration of Spiking Solution Used (IKI-220/TFNA/TFNG/TFNA-AM/ TFNA-OH/TFNG-AM)
LOQ (0.1 µg/L)	100 µL of 100 ng/mL
10x LOQ (1 µg/L)	100 µL of 1 µg/mL

(3) Extraction

After fortification, a 5-mL aliquot of hydrochloric acid solution (about 0.1 mol/L) was added to each surface water sample. For the drinking water samples, a 2-mL aliquot of hydrochloric acid solution (about 1 mol/L) was added to each sample. The ENV+ SPE cartridges were conditioned and equilibrated by passing 5 mL of dichloromethane, 5 mL of methanol, 5 mL of HPLC-grade water, and 5 mL of hydrochloric acid solution (about 0.1 mol/L) through the cartridges one after another. An aliquot (5 mL) of concentrated hydrochloric acid was passed through the cartridges. The eluate was discarded. The entirety of each sample was loaded onto each SPE cartridge at a flow rate of less than 10 mL/minute. The eluate was discarded. After the entirety of the sample was loaded onto the cartridges, the cartridges were vacuumed to dryness for approximately 10 seconds. The cartridges were then eluted with three 5-mL aliquots of methanol (i.e., 15 mL) which was collected in a 16-mL glass tube and then transferred (in parts so that the 16-mL did not overflow) into a 125-mL round bottom flask.

The sample extracts were evaporated to dryness using a rotary evaporator set at a temperature of 50-60 °C. The residues were re-dissolved in 10 mL of ethanol. The ethanol extract was evaporated to dryness using a rotary evaporator set at a temperature of 50-60 °C. The residues were re-dissolved in 2 mL of acetonitrile/water (10:90, v/v) using an ultrasonic bath (approximately 1 minute of sonication).

The final extracts were vialed and submitted for analysis by LC-MS/MS. The final extracts were analyzed against a calibration curve that ranged from 2.5 ng/mL to 200 ng/mL.

Re-analysis (for all analytes) of the trial 2 surface water extracts was conducted after the sample extracts were diluted 10-fold. The diluted final extracts were vialed and submitted for analysis by LC-MS/MS. The diluted final extracts were analyzed against a calibration curve that ranged from 0.25 ng/mL to 20 ng/mL.

3. Successful Method Validation Attempts

a. Preparation of Samples

Sub-samples (100 mL) of the sample SJR-161018 and sample GPL-DW-161027, were measured into 120-mL glass bottles for surface water trial 3 and drinking water trial 2, respectively.

b. Fortifications

Independent laboratory validation samples were fortified at the LOQ (0.1 µg/L) or 10x the LOQ (1 µg/L). Fortifications were performed using Wiretrol disposable micropipettes to directly fortify the 100-mL samples as follows:

Fortification Level	Amount and Concentration of Spiking Solution Used (IKI-220/TFNA/TFNG/TFNA-AM/ TFNG-AM)
LOQ (0.1 µg/L)	100 µL of 100 ng/mL
10x LOQ (1 µg/L)	100 µL of 1 µg/mL

c. Extraction

After fortification, a 2.5-mL aliquot of hydrochloric acid solution (about 0.1 mol/L) was added to each surface water sample. For the drinking water samples, a 2-mL aliquot of hydrochloric acid solution (about 1 mol/L) was added to each sample. The ENV+ SPE cartridges were conditioned and equilibrated by passing 5 mL of dichloromethane, 5 mL of methanol, 5 mL of HPLC-grade water, and 5 mL of hydrochloric acid solution (about 0.1 mol/L) through the cartridges one after another. An aliquot (5 mL) of concentrated hydrochloric acid was passed through the cartridges. The eluate was discarded. The entirety of each sample was loaded onto each SPE cartridge at a flow rate of less than 10 mL/minute. The eluate was discarded. After the entirety of the sample was loaded onto the cartridges, the cartridges were vacuumed to dryness for approximately 10 seconds. The cartridges were then

eluted with three 5-mL aliquots of methanol (i.e., 15 mL) which was collected in a 16-mL glass tube and then transferred (in parts so that the 16-mL did not overflow) into a 125-mL round bottom flask.

The sample extracts were evaporated to dryness using a rotary evaporator set at a temperature of 50-60 ºC. The residues were re-dissolved in 10 mL of ethanol. The ethanol extract was evaporated to dryness using a rotary evaporator set at a temperature of 50-60 ºC. The residues were re-dissolved in 20 mL of acetonitrile/water (10:90, v/v) using an ultrasonic bath (approximately 1 minute of sonication).

The final extracts were vialed and submitted for analysis by LC-MS/MS. The final extracts were analyzed against a calibration curve that ranged from 0.25 ng/mL to 10 ng/mL.

Re-analysis (for TFNA-AM) of the trial 2 drinking water extracts was conducted after the sample extracts were diluted 5-fold. The diluted final extracts were vialed and submitted for analysis by LC-MS/MS. The diluted final extracts were analyzed against a calibration curve that ranged from 0.05 ng/mL to 2 ng/mL.

Notes from surface water trial 3:

During the load step, approximately 5 mL of sample 589ILV05-6 was lost prior to loading onto the SPE cartridge.

C. Instrumentation

1. Failed Method Validation Attempts

Instrument:	AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD XR HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC HT Autosampler
HPLC Column:	Phenomenex Luna C8 100A 150 x 3.0 mm, 3 µm Part # 00F-4123-Y0
Guard Column:	Phenomenex Security Guard Cartridge C8 4 x 2 mm Part #AJ0-4289
Data System:	Analyst Chromatography Data System version 1.6, AB Sciex

Mobile Phases:

A) Methanol
B) 0.05% Formic Acid in Water

Flow Rate: 0.5 mL/minute
Run Time: 28 minutes
Injection Volume: 10 µL (surface water trial 1)
25 µL (surface water trial 2, drinking water trial 1)
Gradient Program:

Time (minutes)	%A	%B
0.0	10	90
2.0	10	90
4.0	22	78
12.0	70	30
15.0	90	10
20.0	90	10
20.1	10	90
28.0	10	90

Column Heater: 40 °C
Needle Wash: methanol/water (10:90, v/v)

Approximate Retention Times:

IKI-220: 8.2 minutes
TFNA: 9.7 minutes
TFNG: 8.0 minutes
TFNA-AM: 6.7 minutes
TFNG-AM: 6.3 minutes

Typical Mass Spectrometer Parameters (operated in LC-MS/MS mode):

AB Sciex API-4000 Acquisition Parameters (TurboIonSource, APCI interface, MRM mode, positive mode, Unit/Low Resolution)						
Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	DP	CE	CXP
IKI-220	229.8	203.1	100	75	25	10
TFNA	191.6	148.1	100	75	31	10
TFNG	248.8	203.0	100	70	28	12
TFNA-AM	190.6	148.2	100	80	30	10
TFNG-AM	247.7	174.0	100	60	27	10

Parameter	Setting
CUR:	25
GS1:	60
GS2:	NA
NC:	4
TEM:	550
CAD:	10
EP:	10
ihe:	ON

The instrument parameters were optimized for analyte sensitivity and resolution prior to the chromatographic run. The exact parameters were documented with the data set.

2. Successful Method Validation Attempts

The analytes IKI-220, TFNA, TFNA-AM, and TFNG-AM were analyzed using an APCI source, whereas TFNG was analyzed using an ESI source.

Instrument: AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD XR HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC HT Autosampler

HPLC Column: Phenomenex Luna C8 100A
150 x 3.0 mm, 3 µm
Part # 00F-4123-Y0

Guard Column: Phenomenex Security Guard Cartridge C8 4 x 2 mm
Part #AJ0-4289

Data System: Analyst Chromatography Data System version 1.6, AB Sciex

Mobile Phases:

A) Methanol
B) 0.05% Formic Acid in Water

Flow Rate: 0.5 mL/minute
Run Time: 28 minutes
Injection Volume: 25 µL

Gradient Program:

Time (minutes)	%A	%B
0.0	10	90
2.0	10	90
4.0	22	78
12.0	70	30
15.0	90	10
20.0	90	10
20.1	10	90
28.0	10	90

Column Heater: 40 °C
Needle Wash: methanol/water (10:90, v/v) for IKI-220, TFNA, TFNA-AM and TFNG-AM
acetonitrile/water (50:50, v/v) for TFNG

Approximate Retention Times:

IKI-220: 8.2 minutes

TFNA: 9.5 minutes

TFNA-AM: 6.7 minutes

TFNG-AM: 6.3 minutes

TFNG: 8.0 minutes

Typical Mass Spectrometer Parameters (operated in LC-MS/MS mode):

AB Sciex API-4000 Acquisition Parameters (TurboIonSource, APCI interface, MRM mode, positive mode, Unit/Low Resolution)					
Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	DP	CE
IKI-220	229.8	203.1	100	75	25
TFNA	191.6	148.1	100	75	31
TFNG	248.8	203.0	100	70	28
TFNA-AM	190.6	148.2	100	70	30
TFNG-AM	247.7	174.0	100	60	27

Parameter	Setting
CUR:	25
GS1:	60
GS2:	NA
NC:	4
TEM:	550
CAD:	10
EP:	10
CXP:	10
ihe:	ON

Typical Mass Spectrometer Parameters (operated in LC-MS/MS mode):

AB Sciex API-4000 Acquisition Parameters (TurboIonSource, ESI interface, MRM mode, positive mode, Unit/Low Resolution)					
Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	DP	CE
TFNG	249.1	203.0	300	70	28
TFNG	249.1	148.0	100	70	43

Parameter	Setting
CUR:	25
GS1:	40
GS2:	50
IS:	2500
TEM:	550
CAD:	12
EP:	10
CXP:	10
ihe:	ON

The instrument parameters were optimized for analyte sensitivity and resolution prior to the chromatographic run. The exact parameters were documented with the data set.

D. Potential Interferences

1. Matrix Interference

There were no interference peaks visible in the chromatograms at the retention times of the analytes of interest in the passing trials. However, signal enhancement was observed for IKI-220, TFNG, and TFNG-AM. This signal enhancement was resolved by further dilution of the matrix extracts and changing the ionization source (for TFNG). See the "Modification or Potential Problems" section for further discussion of the matrix enhancement problems.

2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. No interferences were observed.

3. Labware Interference

This method uses mostly disposable labware. No interferences from the labware that was used were observed. However, analyte residues were recovered in the reagent blank and control samples for the failed method attempts and the first two LOQ samples recovered unusually high compared to the other LOQ samples. This was attributed to a procedural bias. The rotary evaporator stations that were used for the 10x LOQ samples during the first evaporation were used to evaporate the reagent blank samples, control samples, and first two LOQ samples during the evaporation of the ethanol (second evaporation). Based on the recovery of residues in these samples, it was determined that cross-contamination was occurring at the rotary evaporator stations. For the successful method validation attempts, the rotary evaporator stations were thoroughly cleaned prior to the second evaporation to prevent cross-contamination.

It is also notable to mention, that since dichloromethane aggressively dissolves certain types of plastic materials and hydrochloric acid is highly corrosive, the materials used for SPE (i.e., reservoirs, valve, etc.) must not be degraded by dichloromethane or concentrated hydrochloric acid.

E. Confirmatory Techniques

The independent laboratory validation sets were run by LC-MS/MS with monitoring of a single set of transition ion pairs for IKI-220, TNFA, TFNA-AM, and TFNG-AM. For the successful method trial attempt, TFNG was analyzed by LC-MS/MS with monitoring of two transition ion pairs. As this method is highly selective, no additional confirmatory technique was used. However, due to observed signal enhancement, additional confirmation may be considered.

F. Time Required for Analysis

Six to ten hours were required for one person to prepare an analysis set from the time samples were prepared to LC-MS/MS analysis. Automated LC-MS/MS analysis was performed overnight. An additional 2 hours was spent on data calculation and tabulation the following day. An analytical set can be prepared, analyzed and tabulated over one to two calendar days. *Note: Reduction of the sample size from 1-Liter to 100 mL is suggested to shorten the time needed to prepare a sample set (e.g., from 10 hours to 6 hours).*

G. Modification or Potential Problems

1. Sample Size

There were several obstacles to final successful validation trials. First, a 1-Liter sample size is a large amount of sample to pass through a 6 mL SPE cartridge, even with the use of a reservoir. As a result, for all method validation trial attempts subsequent to the first surface water validation attempt, with permission from the Sponsor, the sample size was modified to 100 mL.

2. pH Adjustment

The recoveries of TFNA and TFNG for the first surface water method validation attempt were low. No TFNA was recovered and very little TFNG was recovered. Due to the similarity in their structures, it was predicted that the analytes were not recovered due to a pH adjustment problem. An experiment was conducted to confirm this prediction.

A 2-mL aliquot of hydrochloric acid solution (about 0.1 mol/L) was added to 1 liter of HPLC-grade water as prescribed in the first step in the method. The approximate pH of this water (4.3) was determined using a pH meter. Aliquots of a hydrochloric acid solution (about 0.1 mol/L) were added to a 100-mL aliquot of the surface water sample Herndon-39-151214 until the pH fell below 4.3. Approximately 5 mL of hydrochloric acid solution (about 0.1 mol/L) was required to reach a pH under 4.3.

Furthermore, the same experiment was conducted using a 100-mL aliquot of drinking water collected from the faucet at GPL (for the purposes of this experiment). Approximately 18 mL of hydrochloric acid solution (about 0.1 mol/L) was required to reach a pH of approximately 4.3. As a result, the experiment was repeated with a higher strength hydrochloric acid solution (about 1 mol/L). The drinking water collected from the tap required 2 mL of this solution to reach a pH of approximately 4. The pH adjustment steps for the subsequent method validation trial attempts were changed accordingly for each sample type. These experiments were

repeated when the second surface water and drinking water samples were collected prior to the successful method validation trial attempts.

Since it was determined to be critical that the pH is adjusted to a pH of approximately 4.3 or lower (the exact threshold was not determined over the course of this study), and since the buffering capacity of environmental samples can vary greatly, it is suggested that the method be modified to emphasize that all sample pH must be suitably adjusted rather than providing a blanket adjustment step for all samples.

3. Signal Enhancement

After pH adjustment, the next critical problem was signal enhancement. Signal enhancement was observed for IKI-220, TFNG, and TFNG-AM. After the data were reviewed for the surface water method validation trial 2 attempt, an experiment was conducted to confirm that signal enhancement was present for these three analytes. A 10x LOQ extract was diluted 10-fold and injected against a 2-point standard curve to determine whether the signal enhancement improved upon dilution. The signal enhancement was improved by dilution. All of the extracts from the surface water method validation trial 2 attempt were diluted and re-analyzed. The signal enhancement was suitably improved for IKI-220 and TFNG-AM using 10-fold dilution, however, the TFNG signal enhancement was not acceptably improved. Recoveries averaged 385% for TFNG.

The signal for TFNG using APCI was not strong enough for further dilution. As a result, the ionization source was changed to ESI for the purposes of mitigating the signal enhancement. New control matrix samples were obtained (the original samples had been stored refrigerated for almost a year by the time trials were reinitiated and suitably of the samples were suspect), pH adjusted, and three aliquots of each were extracted and analyzed using the method modification described above. These samples were analyzed using ESI for TFNG and there was no enhancement observed. As a result, for the successful method validation trial attempts, TFNG was analyzed using an ESI source rather than an APCI source.

The signal enhancement may have not been seen in the original validation study for multiple reasons. First, the control matrix sample may have not interfered with the analyte as seen herein. Secondly, the modern ionization sources are much more efficient at ionization. It is possible that the interference was not ionized using the older instrumentation. In order to decrease the possibility of signal enhancement, it may be suggested to use a more sensitive mass spectrometer, an ESI source, and aggressive final extract dilution.

There are likely other alternative approaches for resolving the TFNG signal enhancement problem. Another approach may include alternative chromatography.

H. Methods of Calculation

Analyst Chromatography Data System version 1.6, a product of AB Sciex, was used to acquire, integrate and calculate the concentrations of IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM as ng/mL using the power regression function with no weighting. For the successful method trial, TFNG was calculated as ng/mL using the linear regression function with 1/x weighting. The calibration was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak area response was designated as the dependent variable and plotted on the y-axis. From this regression curve, a slope, a correlation coefficient and other parameters of the standard curve were calculated. Calibration standards were injected every three to five sample injections as well as at the beginning and end of the injection sequence. Six or seven different standard concentrations were injected within each analytical set. The concentrations (ng/mL) of IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM detected in method validation sample extracts were interpolated from the standard calibration curve. The concentration as µg/L of residue found in samples was then calculated with Microsoft® Excel using the following equation:

$$\mu g/L = \frac{(ng/mL \text{ from curve}) \times (\text{Final Vol. in mL}) \times 1\ \mu g \times 1000\ mL}{(\text{Sample amount in mL}) \times 1000\ ng \times 1\ \text{Liter}}$$

Recovery of the analyte from fortified samples was calculated as follows:

$$\%\ \text{Recovery} = \frac{(\text{Measured Concentration, } \mu g/L) \times 100}{(\text{Theoretical Concentration, } \mu g/L \text{ added})}$$

An example calculation for drinking water for an IKI-220 laboratory fortification in set 589ILV04, sample 589ILV04-8 LOQ sample fortified at 0.100 µg/L, is as follows:

standard curve equation: $y = 4.05 \times 10^4 (x)^{1.02}$

where x = IKI-220 concentration in ng/mL and

y = peak response = 14644.1

IKI-220 concentration from the curve =0.369 ng/mL

$$\mu g/L = \frac{(0.369\ ng/mL\ \text{IKI-220}) \times (20\ mL) \times 1\mu g \times 1000\ mL}{(100\ mL) \times 1000\ ng \times 1\ \text{Liter}} = 0.0738\ \mu g/L$$

$$\% \text{ recovery} = \frac{0.0738\ \mu g/L}{0.100\ \mu g/L} \ X\ 100 \quad = 73.8\%$$

No detectable residues were measured in any control samples in any of the successful method trial attempts. Laboratory fortification samples were not corrected for reported control responses. Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.

I. Statistical Procedures

Laboratory statistical procedures included calculation of arithmetic mean, the corresponding standard deviation (where $n \geq 3$), coefficient of variation and 95% confidence interval for analyte recovery data. Power regression analysis was applied to LC-MS/MS calibration curves for the determination of slope, y-intercept and correlation coefficient values for IKI-220, TFNA, TFNA-AM, and TFNG-AM. For the successful method trial attempts for TFNG, linear regression analysis was used.

J. Chromatograms

Example chromatograms are presented in Appendix E of this report.

IV. RESULTS/DISCUSSION

A. Method Validation Results

The method was successfully validated at 0.1 and 1 µg/L for the determination of IKI-220, TNFA, TFNG, TFNA-AM, and TFNG-AM in surface and drinking water.

The method is precise and results meet all precision acceptance criteria.

D. Limit of Detection and Limit of Quantitation

The LOD for IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM as stated by the reference method in surface and drinking water is about 10 ng/mL (equivalent to 0.02 µg/L in sample matrix). This was defined by the reference method as the lowest calibration standard analyzed. However, for this study, the calibration range was decreased due to the dilutions that were necessary to resolve the signal enhancement difficulties. The lowest calibration standard analyzed was only at one half of the LOQ calibration standard. As a result, in the view of the reference method, the LOD for this study is defined as 0.05 µg/L.

The LOQ for IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM in surface and drinking water is 0.1 µg/L. This was defined by the reference method as the lowest fortification level at which acceptable recovery data was obtained.

The LOD and LOQ as stated above are estimated. In order to confirm the estimated values, the LOD and LOQ were calculated empirically by using the data generated for each analyte at 0.1 µg/L (the estimated LOQ). The surface and drinking water values were combined to create a set of 10 replicates. The calculations were performed using the statistical procedure described in the Handbook of Environmental Analysis, fourth edition, by Roy-Keith Smith, Genium Publishing, 1999.

LOD = $t_{0.99}$ x S LOQ = 3 x LOD

$t_{0.99}$ = the one-tailed statistic at the 99% confidence level for *n* replicates
S = the standard deviation of recovery results from *n* samples fortified at the estimated LOQ (0.1 ppm)

E. Selectivity and Specificity

In the successful method trial attempts, there was no apparent response in the unfortified samples in the region of the chromatograms at the retention times for IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM suggesting the method is selective in surface and drinking water. However, significant signal enhancement was seen when the samples were run un-diluted as per the method. Additional dilution resolved this problem. The addition of alternate transition ion pairs may increase the selectivity and specificity of the method.

F. Limitations

The method has been tested in surface water and drinking water. It can be assumed that the method may be applicable to other water matrixes not tested in these validations provided successful recovery tests are conducted at relevant fortification levels. Due to the enhancement problems found for a few of the analytes, each water source would need to be evaluated for this effect.

V. CONCLUSION

The reference method was successfully validated using Liquid Chromatography (LC) equipped with a tandem mass spectrometer (MS/MS) detector. The reference method was validated at 0.1 µg/L and 1 µg/L for the determination of IKI-220, TFNA, TFNG, TFNA-AM, and TFNG-AM in surface water during the third method trial and drinking water during the second method trial.

VI. PROTOCOL AMENDMENTS/DEVIATIONS

There were two protocol amendments in this study. Protocol amendment 1 amended the title of the study to delete TFNA-OH from the title. It replaced the original test/reference substance information with updated information from newly obtained certificates of analysis and new lot numbers/batches of material. Furthermore, all references to TFNA-OH were removed from the protocol since TFNA-OH is only an animal metabolite. Validation of the method for TFNA-OH was determined to be unnecessary. The second protocol amendment replaced the test/reference substance information for TFNA-AM and TFNG-AM to clarify the differences in purity used in the study for a single lot number of these reference substances. These protocol amendments have no negative impact on the study.

VII. CIRCUMSTANCES AFFECTING THE DATA

No circumstances were encountered that would affect the quality or integrity of the data generated in this study.

Appendix A

Study Protocol

STUDY PROTOCOL

Independent Laboratory Validation of Ishihara Sangyo Kaisha (ISK) Residue Analytical Method for the Determination of IKI-220 and Metabolites (TFNA, TFNG, TFNA-AM, TFNA-OH and TFNG-AM) in Surface Water and Drinking Water
(Document Number: 834131)

Guideline Requirements

USA EPA I Effects Test Guidelines, OCSPP 850.6100

I. STUDY OBJECTIVE

The objective of this study is to independently validate Ishihara Sangyo Kaisha's (ISK) Analytical Method (Document Number: 834131) for the determination of IKI-220 and metabolites (TFNA, TFNG, TFNA-AM, TFNA-OH and TFNG-AM) in water as an enforcement method in both surface and drinking water in accordance to Environmental Protection Agency (EPA) Ecological Effects Test Guidelines, OCSPP 850.6100. The study will be conducted in accordance with EPA's Good Laboratory Practice (GLP) Standards, 40 CFR Part 160.

II. JUSTIFICATION OF TEST SYSTEM

The test systems consist of a representative control (untreated) sample of surface water and drinking water. The representative control sample will be analyzed to determine matrix background of unfortified controls and procedural recoveries of fortified samples.

Analysis of this test system is required by independent laboratory analyst(s) according to EPA's Ecological Effects Test Guidelines, OCSPP 850.6100 to demonstrate method ruggedness prior to the Sponsor's submission of the method to the EPA.

III. MATERIALS AND METHODS

A. Test/Reference Substances

The following test/reference substances will be used for this study and will be the same.

Name:	IKI-220
CAS Number:	158062-67-0
Purity:	99.6%
Lot/Batch Number:	9809
Expiration Date:	December 12, 2018

Name:	TFNA
CAS Number:	158063-66-2
Purity:	100.0%
Lot/Batch Number:	0006
Expiration Date:	January 03, 2016

Name:	TFNG
CAS Number:	207502-65-6
Purity:	99.37%
Lot/Batch Number:	20110328
Expiration Date:	February, 2016

Name:	TFNA-AM
CAS Number:	158062-21-6
Purity:	99.7%
Lot/Batch Number:	0006
Expiration Date:	January 03, 2016

Name:	TFNA-OH
CAS Number:	Not Available
Purity:	100.0%
Lot/Batch Number:	0010
Expiration Date:	January 4, 2016

Name:	TFNG-AM
CAS Number:	158062-96-5
Purity:	99.5%
Lot/Batch Number:	0006
Expiration Date:	January 4, 2016

All six test/reference substances have been supplied by the Sponsor and were accompanied by a Certificate of Analysis (COA) for each substance, which included the purity and expiration date, and a Material Safety Data Sheets (MSDS) for IKI-220.

Characterization (such as identity, purity, synthesis documentation) and stability data of the reference substances will be the responsibility of the Sponsor. Results and supporting raw data of the characterization and stability data will be maintained by the Sponsor. Retention samples of the reference materials will also be retained by the Sponsor. All reference substances will be stored as specified by the Sponsor or certificate of analysis when not in use.

B. <u>Sample Identification and Storage Conditions</u>

The control (untreated) matrix samples will be obtained according to Golden Pacific Laboratories, LLC (GPL) Standard Operating Procedures (SOPs). Surface water will be obtained by GPL personnel from a suitable source in Fresno County, California. Drinking water will

be obtained from the municipal tap water source at GPL. The time, date, and location of each sampling event will be recorded.

New control samples undergo log-in according to GPL's SOPs. Control samples will be labeled (or re-labeled as necessary) with the following minimum information:

Study Number
Sample Number/Code
Sample Description
Initials of Responsible Analyst

Each sample will be identified with a unique sample number/code. This number/code will be used to track the use of the sample throughout the validation process. The samples will be kept isolated from reference substances during storage. The samples will be kept in a refrigerator set to maintain ± 4 °C (refrigerated) when not in use. Storage temperatures will be monitored and recorded per GPL's SOPs.

C. <u>Sample Characterization</u>

The following parameters will be determined for the characterization of the surface water control sample: pH, calcium, sodium, hardness, conductivity, sodium adsorption ratio, total dissolved solids, and turbidity.

The characterization of the control surface water sample will be conducted at Agvise Laboratories (Northwood, ND). A portion of the surface water control sample will be transferred to Agvise Laboratories via overnight shipment (e.g., overnight FedEx shipment) in a cooler with ice. The characterization will be conducted under GLP standards, 40 CFR Part 160. Agvise Laboratories will submit a report to GPL with the results of the characterization analyses.

Tap water will be the source of the drinking water sample. The drinking water sample will not be characterized. The water quality report for the Fresno municipal supply will be included in the raw data for information about the drinking water sample properties.

D. Analytical Procedure

The analysis for IKI-220, TFNA, TFNG, TFNA-AM, TFNA-OH, and TFNG-AM residues in the surface and drinking water samples shall be performed according to ISK residue analytical method contained in the study report entitled "VALIDATION OF THE RESIDUE ANALYTICAL METHOD FOR THE DETERMINATION FOR IKI-220 AND METABOLITES (TFNA, TFNG, TFNA-AM, TFNA-OH AND TFNG-AM) IN SURFACE WATER AND DRINKING WATER" (RCC Study Number 834131). The Limit of Quantitation (LOQ) for all six analytes will be 0.1 µg/L. The surface water and drinking water will be validated in two separate analytical sets. Three attempts will be allowed for the analysis of each type of water.

Calibration standards will be prepared to evaluate detector response, linearity, and preparation technique. Fortification solutions will also be analyzed against calibration standards to check preparation technique. Background responses from reagents, solvents, glassware, and all specified procedures within the method will be checked alongside with the analysis of the first set of validation samples (i.e., a reagent blank sample will be analyzed) to insure that interferences will not hinder the quantitation of the LOQ fortification recoveries.

1. Method Validation

Each set will include one reagent blank, duplicate controls (unfortified), five controls spiked at the LOQ and five controls spiked with at 10x LOQ. Total number of samples per analytical batch will be 13.

Spiking levels are as follows:

Sample Description	IKI-220/TFNA/TFNG/TFNA-AM/TFNA-OH/TFNG-AM Fortification Level (µg/L)
Reagent Blank	NA
Duplicate Untreated Controls	NA
Five LOQ Spikes	0.1
Five (10x LOQ) Spikes	1

Control (untreated) samples prepared as described in the analytical method are to be fortified with an appropriate amount of each of the six analytes. Recovery of each analyte will be determined.

The method will be considered successfully validated for a matrix if mean recoveries are in the range of 70-120%. Individual recoveries falling outside the 70-120% range will require approval from the Sponsor Representative. Mean recoveries outside 70-120% will require review of the methodology with the sponsor representative and reanalysis. The first method trial will be conducted following ISK's analytical method as written. Discussions prior to and following the first attempt between the Sponsor Representative and the performing laboratory group will be documented. If the first trial is not successful, a second (and third trial, if necessary) will be conducted following collaboration with the Sponsor Representative (i.e., to determine potential problem areas). All discussions will be documented and a summary of these discussions will be presented in the final report to the Sponsor.

Control of procedural bias is achieved by analysis of both reagent blanks and unfortified (control) samples along with the fortified samples. This enables ongoing assessment of method/sample background in the region of analyte response and thus ensures that analyte recovery is determined without offset (bias) from background.

2. Quantitation

A standard curve will be used for quantitation of all samples. The standard curve will be derived from standards injected within the analysis set. The standard curve will be generated from the LC-MS/MS run using the resulting peak area versus concentration. A minimum of six standards of different analyte concentrations will be prepared and used to generate the curve. The calibration curve will be constructed using the regression described in the analytical method. A correlation coefficient (r) will be calculated for each curve generated. The r value for each analytical set should be greater than 0.98.

A fixed injection volume will be used for quantitation purposes and will be the same for both samples and standards. Run sequences will begin and end with at least one, and preferably two,

standards. Standard injections are made throughout the run, generally with no more than five sample injections between each standard. Any injection that is rejected must be so indicated. The reason for rejecting the standard must be documented in the raw data.

3. Reporting of Results

Results will be reported in units of µg/L or ppb, and percent recovery. The mean percent recovery, standard deviation, relative standard deviation (RSD), and 95 or 99% Confidence Intervals for the true average recoveries will also be reported for each analyte at each spiking level.

The reagent blank and control samples should be chromatographically clean at the retention time of the analytes of interest. Recovery data for individually fortified samples should fall within the 70-120% range. Residues measured in matrix controls may be used to correct fortified sample recoveries. RSDs of replicate measurements of recoveries should not exceed the target level of less than or equal to 20% for each spiking level.

E. Statistical Procedures

Statistical procedures include the calculation of mean percent recovery, the corresponding standard deviation (where $n \geq 3$), RSD, and 95 or 99% Confidence Intervals. In addition, a linear regression analysis (as described within the analytical method) will be applied to the calibration curve to determine the slope, intercept values, and correlation coefficient.

IV. MINIMUM RAW DATA REQUIREMENTS

The following items will be included, but not limited to, in the raw data:

A. Copy of protocol with any amendments and/or deviations.
B. Personnel log including signatures, initials, and job titles.
C. Sample receipt and chain of custody documentation.
D. Sample inventory upon receipt of samples.
E. Copy of the analytical method.
F. Description of storage units and identity of units samples were stored.
G. Period of time samples were stored.
H. Temperature records/logs of storage units used for test/reference substance and sample storage.

I. Laboratory tracking data including, preparation, request for analysis, extraction records, etc.
J. Date of sample preparation.
K. Test/reference substance receipt, usage, and preparation.
L. Test/reference substance storage conditions.
M. Solution preparation records.
N. Calculation worksheets.
O. Chromatography data, standard curve plots and summary spreadsheets.
P. Chronological/Communications records.
Q. Observations and/or additional notes to the study file.
R. Error codes are to be coded, dated and initialed with an error code explanation to be supplied.
S. The following format requirements must be adhered to:
    a. Use black indelible ink.
    b. Sponsor (if applicable) and analytical phase study numbers are to be listed on each page of the raw data.
    c. Calculations are to be fully outlined and explained, with all units, conversion factors, constants and variables defined.

V. REPORTING OF RAW DATA

All study-specific raw data will be transferred to the Sponsor's custody along with all study summary reports. Such raw data is to be archived by the Sponsor to be kept as required by the GLP's, Section 160.195.

This data is intended to be used in the preparation of the final report for submission to regulatory agencies. The draft and final report will be prepared by GPL and submitted to the Sponsor Representative for review. The final report will be considered complete following the signature of the Study Director. The final report will be written according to the guidance and reporting format given in the OCSPP 850.6100 section (f) ecological effects test guideline.

VI. RECORDS TO BE MAINTAINED

A. All raw data and authorized copies of raw data will be archived at GPL. Once the final report is signed by the Study Director, all study specific raw data as well as the original final report will be transferred from GPL to the Sponsor. Such raw data is to be archived by the Sponsor to be kept as required by the GLP's, Section 160.195. The study specific data will include each of the following (if applicable), but will not be limited to: standard and sample chromatograms, sample preparation data sheets, sample requests, dilution records, sample tracking forms,

chain-of-custody records for standards and samples, sample shipping and receipt records, and all correspondence that is necessary to reconstruct the study. Facility records such as test/reference substance receipt, login and usage, standard preparation forms, temperature records for freezer, refrigerator, and ambient storage units used for sample and test/reference material storage will be maintained by GPL and verified copies of these records will be included in the raw data as necessary in order to reconstruct the study.

B. The following records will be maintained at GPL in the GLP archives or other location as directed by GPL's SOPs.

1) Original protocol and all deviations and amendments (or verified copies).
2) Maintenance logs and calibration records for equipment.
3) Training, signatures, initials, and records of experience of personnel involved in the study.
4) A copy of the master schedule and records of all QAU inspections.
5) A copy of the final report, along with any copies of amended final reports that may be issued.
6) A verified copy of raw data.
7) An electronic copy of the analytical method used for the study.

VII. QUALITY ASSURANCE

GPL's QAU will monitor the study and report whether facilities, equipment, personnel, practices, procedures, records, and controls are designed and function in conformance with US EPA GLP Standards, 40 CFR Part 160, and all GPL's SOPs.

The protocol will be reviewed prior to the initiation of the study. Three types of audits will be conducted by QAU to ensure the integrity of the study, e.g., in-progress, raw data, and final report. The in-progress inspection must be of an activity that is critical to the outcome of the study. Written documentation of audit findings will be provided to the Study Director, Testing Facility Management, and the Sponsor Representative. Appropriate corrective action, if needed, will be instituted promptly.

A QA statement will be signed and submitted by the QAU at the conclusion of the study and included with the final report. The format of the QA statement will adhere to the requirements of the GLP's, Section 160.35(b)(7). The QA statement will include audit dates, audit description (e.g. processing procedures, extraction procedures) and date that findings were reported to the Study Director and Testing Facility Management.

VIII. PROTOCOL AMENDMENTS

All amendments (planned changes) in or revisions of the approved protocol and the reasons for the changes will be documented, signed, and dated by the Study Director. The amendment will not go into effect until approved by the Study Director and signed by the Sponsor Representative. The reason for the amendment, the effective date, and any effect on the integrity of the study are to be reported as soon as possible after the need for change is recognized.

IX. PROTOCOL DEVIATIONS

All deviations (unplanned changes) from the approved protocol will be documented, signed, and dated by the Study Director and signed by the Sponsor Representative. Reference will also be made in the supporting raw data. The reason for the deviation, the date of occurrence, and any effect on the integrity of the study are to be reported to the Study Director as soon as possible after the deviation is recognized.

X. STANDARD OPERATING PROCEDURES (SOPs) REQUIREMENTS

SOPs will be in place for all phases and activities of this study and will be adhered to, unless superseded by the specific provisions of this protocol. All SOP deviations will be properly documented by study personnel. The Study Director is to be notified within one week of identification of the deviation. Effects of the deviation on the study will also be determined and documented.

XI. SPONSOR AUTHORIZATION DURING THE STUDY

Should a problem develop while the study is in progress, GPL will notify the Sponsor Representative as soon as possible. The problem and suggested test modifications will be discussed and analysis will proceed with the changes necessary. A request for written authorization will then be submitted by GPL to the Study Representative and handled in the same manner as discussed in Sections VIII and IX.

XII. DISPOSITION OF SAMPLES

Samples will not be retained for this study.

PROTOCOL AMENDMENT NO.: 1
GPL Study #150589

Study Title:
Independent Laboratory Validation of Ishihara Sangyo Kaisha (ISK) Residue Analytical Method for the Determination of IKI-220 and Metabolites (TFNA, TFNG, TFNA-AM, TFNA-OH and TFNG-AM) in Surface Water and Drinking Water (Document Number: 834131)

Effective Date: October 11, 2016

Description of Amendment:

1. **Title of the Study**

The title of the study is updated from:

Independent Laboratory Validation of Ishihara Sangyo Kaisha (ISK) Residue Analytical Method for the Determination of IKI-220 and Metabolites (TFNA, TFNG, TFNA-AM, TFNA-OH and TFNG-AM) in Surface Water and Drinking Water (Document Number: 834131)

to

Independent Laboratory Validation of Ishihara Sangyo Kaisha (ISK) Residue Analytical Method for the Determination of IKI-220 and Metabolites (TFNA, TFNG, TFNA-AM, and TFNG-AM) in Surface Water and Drinking Water (Document Number: 834131)

2. **Pages 3 and 4**

This amendment replaces the original test/reference substance information with the following information:

Name:	IKI-220
CAS Number:	158062-67-0
Purity:	99.6%
Lot/Batch Number:	9809
Expiration Date:	December 12, 2018

Name:	TFNA
CAS Number:	158063-66-2
Purity:	100.0%
Lot/Batch Number:	0006
Expiration Date:	November 17, 2020

Name:	TFNG
CAS Number:	207502-65-6
Purity:	99.37%
Lot/Batch Number:	20110328
Expiration Date:	February, 2016

Name:	TFNG
CAS Number:	207502-65-6
Purity:	99.83%
Lot/Batch Number:	20110920
Expiration Date:	March, 2017

Name:	TFNA-AM
CAS Number:	158062-21-6
Purity:	99.9%
Lot/Batch Number:	0006
Expiration Date:	November 19, 2020

Name:	TFNG-AM
CAS Number:	158062-96-5
Purity:	99.6%
Lot/Batch Number:	0006
Expiration Date:	November 19, 2020

3. Eliminate All References to TFNA-OH

All references to the analyte TFNA-OH are eliminated from the protocol.

Justification:

1. Title has been updated to eliminate an analyte that does not need to be validated at the request of the Sponsor.
2. New Certificates of Analysis were provided for TFNA, TFNA-AM, and TFNG-AM. A new lot of test/reference substance was provided for TFNG. The information for IKI-220 remains unchanged.
3. The analyte TFNA-OH has been removed from the study.

PROTOCOL AMENDMENT NO.: 2
GPL Study #150589

Study Title:
Independent Laboratory Validation of Ishihara Sangyo Kaisha (ISK) Residue Analytical Method for the Determination of IKI-220 and Metabolites (TFNA, TFNG, TFNA-AM, and TFNG-AM) in Surface Water and Drinking Water (Document Number: 834131)

Effective Date: November 29, 2016

Description of Amendment:

This amendment replaces test/reference substance information from Amendment 1 for TFNA-AM and TFNG-AM with the following information:

Name:	TFNA-AM
CAS Number:	158062-21-6
Purity:	99.7% ,99.9%
Lot/Batch Number:	0006
Expiration Date:	January 3, 2016, November 19, 2020

Name:	TFNG-AM
CAS Number:	158062-96-5
Purity:	99.5%, 99.6%
Lot/Batch Number:	0006
Expiration Date:	January 4, 2016, November 19, 2020

Justification:

New Certificates of Analysis were provided for TFNA-AM, and TFNG-AM. However, both the old and new purities and expiration dates were used to support data in the study.

Effect on Study:

There is no negative impact on the study.

Appendix B

Method Flow Charts

(Successful Method Trial Attempts)

Analysis of IKI-220 and Metabolites in Surface Water (SJR-161018) by LC-MS/MS

Measure 100 mL of sample into a 120-mL glass bottle or other appropriate container

↓

Fortify as necessary

↓

Add 2.5 mL of hydrochloric acid solution (about 0.1 mol/L) to the sample

↓

Prepare an ENV+ SPE cartridge by passing 5 mL of dichloromethane, 5 mL of methanol, 5 mL of HPLC-grade water, and 5 mL of hydrochloric acid solution (about 0.1 mol/L) through the cartridge one after another

↓

Add 5 mL of concentrated hydrochloric acid to the column (Reservoir is optional)

↓

Shake the sample container briefly, and allow the sample to flow through the SPE cartridge at a flow rate of < 10 mL/minute

↓

After the sample has completely flowed through the cartridge, vacuum the cartridge to dryness for approximately 10 seconds

↓

Elute the cartridge with 3 x 5 mL of methanol using no vacuum or only slight vacuum into a 16-mL glass tube, making sure to pour off the first 5 to 7 mL collected into a 125-mL round bottom flask so that the collection tube does not overflow

↓

Combine the eluted methanol into the 125-mL round bottom flask

↓

Evaporate the sample to dryness using a rotary evaporator set at a temperature of 50-60 °C

↓

Continue to page 2

Analysis of IKI-220 and Metabolites in Surface Water (SJR-161018) by LC-MS/MS

Continued from page 1

↓

Re-dissolve the residue in 10 mL of ethanol

↓

Evaporate the sample to dryness using a rotary evaporator (same conditions)

↓

Re-dissolve the residues in 20 mL of acetonitrile/water (10:90, v/v) using an ultrasonic bath (use ultrasonic bath for 1 minute or longer)

↓

Load samples onto LC-MS/MS

If further dilution is necessary, dilute with acetonitrile/water (10:90, v/v)

Analysis of IKI-220 and Metabolites in Drinking Water (GPL-DW-161027) by LC-MS/MS

Measure 100 mL of sample into a 120-mL glass bottle or other appropriate container

↓

Fortify as necessary

↓

Add 2 mL of hydrochloric acid solution (about **1** mol/L) to the sample

↓

Prepare an ENV+ SPE cartridge by passing 5 mL of dichloromethane, 5 mL of methanol, 5 mL of HPLC-grade water, and 5 mL of hydrochloric acid solution (about 0.1 mol/L) through the cartridge one after another

↓

Add 5 mL of concentrated hydrochloric acid to the column (Reservoir is optional)

↓

Shake the sample container briefly, and allow the sample to flow through the SPE cartridge at a flow rate of < 10 mL/minute

↓

After the sample has completely flowed through the cartridge, vacuum the cartridge to dryness for approximately 10 seconds

↓

Elute the cartridge with 3 x 5 mL of methanol using no vacuum or only slight vacuum into a 16-mL glass tube, making sure to pour off the first 5 to 7 mL collected into a 125-mL round bottom flask so that the collection tube does not overflow

↓

Combine the eluted methanol into the 125-mL round bottom flask

↓

Evaporate the sample to dryness using a rotary evaporator set at a temperature of 50-60 °C

↓

Continue to page 2

Analysis of IKI-220 and Metabolites in Drinking Water (GPL-DW-161027) by LC-MS/MS

Continued from page 1

Re-dissolve the residue in 10 mL of ethanol

Evaporate the sample to dryness using a rotary evaporator (same conditions)

Re-dissolve the residues in 20 mL of acetonitrile/water (10:90, v/v) using an ultrasonic bath

Load samples onto LC-MS/MS

If further dilution is necessary, dilute with acetonitrile/water (10:90, v/v)