

Analytical method for fluazaindolizine (DPX-Q8U80) and its metabolites IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862 in water

Reports: ECM: EPA MRID No.: 50932067. Morgan, E., and J.P. Klems. 2015. Analytical Method for the Determination of DPX-Q8U80 and Metabolites in Surface, Ground and Drinking Water Using LC/MS/MS. Report prepared by E. I. du Pont de Nemours and Company, Newark, Delaware; and sponsored and submitted by E. I. du Pont de Nemours and Company, Wilmington, Delaware; 136 pages. Project Identification No.: DuPont-42574. Final report issued September 10, 2015.

ILV: EPA MRID No.: 50932113. Shen, Y. 2016. Independent Laboratory Validation of DuPont-42574, "Analytical Method for the Determination of DPX-Q8U80 and Metabolites in Surface, Ground and Drinking Water Using LC/MS/MS". Report prepared by Alliance Pharma, Malvern, Pennsylvania; and sponsored and submitted by E. I. du Pont de Nemours and Company, Wilmington, Delaware; 253 pages. DuPont Project ID: DuPont-45660. Alliance Pharma Project No.: 160513. Final report issued October 3, 2016.

Document No.: MRIDs 50932067 & 50932113

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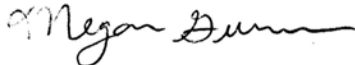
Statements: ECM: The study was not conducted in compliance with USEPA FIFRA (40 CFR Part 160) Good Laboratory Practices (GLP), which are compatible with OECD GLP, but it was conducted in a GLP compliant facility (p. 3 of MRID 50932067). Signed and dated Data Confidentiality, GLP, and Authenticity statements were provided (pp. 2-4). The Quality Assurance statement was not provided.

ILV: The study was conducted in compliance with USEPA FIFRA GLP standards, which are compatible with OECD GLP (p. 3 of MRID 50932113). Signed and dated Data Confidentiality, GLP, Quality Assurance, and Authenticity statements were provided (pp. 2-5).


Classification: **This analytical method is classified as supplemental.** The ILV was not conducted independently from the ECM since ECM personnel communicated directly with the ILV personnel. The specificity of the method for IN-QEK31 and IN-VM862 was not supported by ILV representative chromatograms. The LOD was not reported in the ILV.

PC Code: 129777


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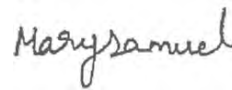
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This Data Evaluation Record may have been altered by the Environmental Fate and Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. The CDM/CSS-Dynamac JV role does not include establishing Agency policies.

Executive Summary

The analytical method, DuPont-42574, is designed for the quantitative determination of fluazaindolizine and its metabolites IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862 in water at the stated LOQ of 0.10 µg/L using HPLC/MS/MS. The LOQ is less than the lowest toxicological level of concern in water for all analytes. The ECM and ILV used characterized drinking, surface, and ground water matrices; the ILV drinking and surface water matrices were the same as those of the ECM. The ILV validated the method for all analytes in the first trial with insignificant analytical instrument and equipment modifications. Due to LC/MS issues, the first trial drinking water samples were re-injected twice. ILV was not conducted independently from the ECM since the ECM Study Author/Study Director, acting as the DuPont Study Monitor for the ILV, communicated directly with the ILV study author regarding technical issues. All ILV and ECM data regarding repeatability, accuracy, and precision were satisfactory for fluazaindolizine and its metabolites, except for the ECM confirmation ion analysis of IN-QEK31 at the LOQ. All ILV and ECM data regarding linearity was satisfactory for fluazaindolizine and its metabolites, except for the ILV confirmation ion analysis of IN-QEK31. Deviations of acceptability in the confirmation ion analysis did not affect the validity of the method since a confirmation method is not usually required when LC/MS or GC/MS is used as the primary method to generate study data. All ILV data regarding specificity was satisfactory for fluazaindolizine, IN-A5760, IN-F4106, IN-REG72, and IN-RYC33. The specificity of the method for IN-QEK31 and IN-VM862 was not supported by ILV representative chromatograms due to highly elevated baseline noise which encompassed the analyte interfering with identification and quantification. All ECM data regarding specificity was satisfactory for all analytes. IN-QEK31 was considered the least sensitive analyte in the ECM. The LOD was not reported in the ILV.

Table 1. Analytical Method Summary

Analyte(s) by Pesticide	MRID		EPA Review	Matrix	Method Date (dd/mm/yyyy)	Registrant	Analysis	Limit of Quantitation (LOQ)
	Environmental Chemistry Method	Independent Laboratory Validation						
Fluazaindolizine (DPX-Q8U80)	50932067 ¹	50932113 ²		Water	10/09/2015	E. I. du Pont de Nemours and Company	LC/MS/MS	0.10 µg/L
IN-A5760								
IN-F4106								
IN-REG72								
IN-RYC33								
IN-QEK31								
IN-VM862								

1 In the ECM, drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface (White Clay Creek) water (Trial ID: Newark DE; pH 8.1, conductivity 0.35 mmhos/cm, hardness 144 mg equiv. CaCO₃/L, total organic carbon 1.9 mg/L, total dissolved carbon 1.2 mg/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained locally, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 16; Appendix 5, pp. 131-136 of MRID 50932067).

2 In the ILV, drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface water (Trial ID: NA; pH 7.5, conductivity 0.24 mmhos/cm, hardness 68 mg equiv. CaCO₃/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained from the Sponsor, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 25; Appendices 2-4, pp. 223-233 of MRID 50932113). The drinking and ground water matrices were the same as those of the ECM.

I. Principle of the Method

Water (5 ± 0.1 mL) in 15-mL centrifuge tube was fortified with 0.100 mL of 5.0 or 50 ng/mL fortification solutions, if necessary (pp. 8, 14-17 of MRID 50932067). Two types of fortification solutions were prepared: one with IN-QEK31 and one with all other analytes. The water samples were centrifuged (10 minutes at 3500 rpm). An aliquot (900 µL) of the supernatant was diluted with 100 µL of methanol and analyzed using LC-MS/MS.

Samples are analyzed using an ABSciex QTRAP 6500 mass spectrometer coupled with an Agilent 1290 HPLC (pp. 12, 17-19; Appendix 3, p. 117 of MRID 50932067). The following LC conditions were used: Zorbax Eclipse Plus Phenyl-Hexyl RRHD column (2.1 mm x 50 mm, 2.1 µm; column temperature 50°C), gradient mobile phase of A) HPLC grade water and B) 0.01% formic acid in methanol [time, percent A:B; 0.0-0.1 min. 90:10, 3.5-5.5 min. 1.0:99, 5.6-8.0 min. 90:10], injection volume of 20.00 µL, MS/MS with TurboIonspray (ESI) source in positive (IN-RYC33, IN-QEK31, and IN-VM862) or negative (IN-A5760, IN-F4106, IN-REG72, and fluazaindolizine) polarity (source temperature 400°C). Two ion pair transitions were monitored for each analyte (quantitation and confirmation, respectively): *m/z* 466→157 and *m/z* 466→142 for fluazaindolizine, *m/z* 206→122 and *m/z* 206→142 for IN-A5760, *m/z* 220→156 and *m/z* 220→141 for IN-F4106, *m/z* 452→123 and *m/z* 452→244 for IN-REG72, *m/z* 264→157 and *m/z* 264→184 for IN-RYC33, *m/z* 265→219 and *m/z* 265→184 for IN-QEK31, and *m/z* 197→141 and *m/z* 197→114 for IN-VM862. Approximate retention times were not reported.

The ILV performed the ECM method for fluazaindolizine as written, except for insignificant analytical instrument and equipment modifications (pp. 26-27 of MRID 50932113). The LC/MS/MS instrument was an AB Sciex Triple Quad 5500 mass spectrometer coupled with a Shimadzu Nexera X2 UHPLC System. The LC conditions were the same as those of the ECM, except that Zorbax Eclipse Plus Phenyl-Hexyl RPHD column (4.6 mm x 50 mm, 1.8 μ m) was used. The ion pair transitions monitored for fluazaindolizine were the same as those of the ECM. Approximate retention times were 4.7, 2.5, 3.4, 4.5, 4.1, 3.9, and 4.1 minutes for fluazaindolizine, IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862, respectively.

In the ECM and ILV, the Limit of Quantification (LOQ) was 0.10 μ g/L for all analytes in water matrices (pp. 12, 24 of MRID 50932067; pp. 13, 21 of MRID 50932113). In the ECM, the Limit of Detection (LOD) was *ca.* 0.02 μ g/L for all analytes in water matrices; the LOD was not reported in the ILV.

II. Recovery Findings

ECM (MRID 50932067): Mean recoveries and relative standard deviations (RSDs) were within guidelines (mean 70-120%; RSD \leq 20%) for analysis of fluazaindolizine and its metabolites IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862 at fortification levels of 0.10 μ g/L (LOQ) and 1.0 μ g/L (10 \times LOQ) in three water matrices, except for the LOQ confirmation ion analysis of IN-QEK31 in ground water (RSD 22%; Tables 1-2, pp. 28-33). Two ion pair transitions were monitored, one quantitation and one confirmation; quantitation and confirmation recovery results were comparable, except that RSDs were higher in the confirmation ion analyses. The drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface (White Clay Creek) water (Trial ID: Newark DE; pH 8.1, conductivity 0.35 mmhos/cm, hardness 144 mg equiv. CaCO₃/L, total organic carbon 1.9 mg/L, total dissolved carbon 1.2 mg/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained locally, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 16; Appendix 5, pp. 131-136).

ILV (MRID 50932113): Mean recoveries and RSDs were within guidelines for analysis of fluazaindolizine and its metabolites IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862 at fortification levels of 0.10 μ g/L (LOQ) and 1.0 μ g/L (10 \times LOQ) in three water matrices (pp. 31-37). Two ion pair transitions were monitored, one quantitation and one confirmation; quantitation and confirmation recovery results were comparable. The drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface water (Trial ID: NA; pH 7.5, conductivity 0.24 mmhos/cm, hardness 68 mg equiv. CaCO₃/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained from the Sponsor, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 25; Appendices 2-4, pp. 223-233). The drinking and ground water matrices were the same as those of the ECM. The method was validated for all analytes in the first trial with insignificant analytical

instrument and equipment modifications (pp. 26-27, 30). Due to LC/MS issues, the first trial drinking water samples were re-injected twice.

Table 2. Initial Validation Method Recoveries for Fluazaindolizine and its metabolites IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862 in Water^{1,2,3}

Analyte	Fortification Level (µg/L)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
Drinking (Tap) Water						
Quantitation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	88-116	100	11	11
	1.0	5	92-100	96	3	3
IN-A5760	0.10 (LOQ)	5	70-89	79	7	9
	1.0	5	87-103	91	7	7
IN-F4106	0.10 (LOQ)	5	77-101	89	11	12
	1.0	5	80-85	82	2	2
IN-REG72	0.10 (LOQ)	5	70-83	76	6	8
	1.0	5	76-84	81	3	4
IN-RYC33	0.10 (LOQ)	5	85-99	91	5	6
	1.0	5	85-89	87	2	2
IN-QEK31	0.10 (LOQ)	5	85-108	100	9	8
	1.0	5	94-97	96	1	1
IN-VM862	0.10 (LOQ)	5	90-104	97	6	7
	1.0	5	86-91	89	2	2
Confirmation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	95-119	105	9	8
	1.0	5	86-93	91	3	3
IN-A5760	0.10 (LOQ)	5	71-82	75	4	5
	1.0	5	78-96	86	7	8
IN-F4106	0.10 (LOQ)	5	75-89	84	5	6
	1.0	5	77-83	80	2	3
IN-REG72	0.10 (LOQ)	5	69-96	88	11	13
	1.0	5	81-92	86	4	5
IN-RYC33	0.10 (LOQ)	5	81-88	84	3	4
	1.0	5	87-90	88	1	1
IN-QEK31	0.10 (LOQ)	5	79-136	87	10	12
	1.0	5	84-90	87	2	3
IN-VM862	0.10 (LOQ)	5	81-103	94	8	9
	1.0	5	90-99	95	4	4
Surface (River) Water						
Quantitation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	89-134	106	19	18
	1.0	5	82-89	86	3	3
IN-A5760	0.10 (LOQ)	5	95-115	101	8	8
	1.0	5	94-102	98	3	3
IN-F4106	0.10 (LOQ)	5	88-100	95	5	5
	1.0	5	80-85	83	2	2
IN-REG72	0.10 (LOQ)	5	84-94	88	5	6
	1.0	5	81-86	85	2	3
IN-RYC33	0.10 (LOQ)	5	86-97	91	5	5
	1.0	5	88-95	92	3	3
IN-QEK31	0.10 (LOQ)	5	83-111	98	11	11
	1.0	5	96-104	99	4	4

Analyte	Fortification Level (µg/L)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
IN-VM862	0.10 (LOQ)	5	95-108	103	5	5
	1.0	5	95-103	100	3	3
Confirmation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	85-128	100	17	16
	1.0	5	95-101	99	3	3
IN-A5760	0.10 (LOQ)	5	81-102	92	10	11
	1.0	5	85-93	88	3	4
IN-F4106	0.10 (LOQ)	5	83-104	94	9	10
	1.0	5	87-94	90	3	3
IN-REG72	0.10 (LOQ)	5	74-104	87	12	14
	1.0	5	86-94	90	3	4
IN-RYC33	0.10 (LOQ)	5	86-119	96	14	14
	1.0	5	91-94	92	1	1
IN-QEK31	0.10 (LOQ)	5	93-136	93	5	6
	1.0	5	89-100	94	4	4
IN-VM862	0.10 (LOQ)	5	75-113	94	14	15
	1.0	5	108-114	111	2	2
Ground Water						
Quantitation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	92-105	99	6	6
	1.0	5	87-94	90	3	3
IN-A5760	0.10 (LOQ)	5	81-91	88	5	5
	1.0	5	83-94	90	4	5
IN-F4106	0.10 (LOQ)	5	70-101	87	15	18
	1.0	5	84-88	86	2	2
IN-REG72	0.10 (LOQ)	5	72-98	82	10	12
	1.0	5	83-90	87	3	3
IN-RYC33	0.10 (LOQ)	5	83-101	89	7	8
	1.0	5	89-96	92	3	3
IN-QEK31	0.10 (LOQ)	5	93-108	101	6	6
	1.0	5	99-105	102	2	2
IN-VM862	0.10 (LOQ)	5	75-116	105	17	16
	1.0	5	109-117	112	3	3
Confirmation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	88-115	98	10	10
	1.0	5	95-98	96	1	1
IN-A5760	0.10 (LOQ)	5	80-99	89	8	9
	1.0	5	83-94	87	4	5
IN-F4106	0.10 (LOQ)	5	71-110	95	14	15
	1.0	5	86-95	89	3	4
IN-REG72	0.10 (LOQ)	5	71-90	79	8	10
	1.0	5	84-90	87	3	3
IN-RYC33	0.10 (LOQ)	5	77-100	87	8	10
	1.0	5	85-93	89	3	3
IN-QEK31	0.10 (LOQ)	5	80-136	99	22	22
	1.0	5	98-108	101	4	4

Analyte	Fortification Level (µg/L)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
IN-VM862	0.10 (LOQ)	5	91-111	98	8	9
	1.0	5	102-110	107	4	3

Data (uncorrected recovery results; pp. 20-21) were obtained from Tables 1-2, pp. 28-33 of MRID 50932067.

- The drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface (White Clay Creek) water (Trial ID: Newark DE; pH 8.1, conductivity 0.35 mmhos/cm, hardness 144 mg equiv. CaCO₃/L, total organic carbon 1.9 mg/L, total dissolved carbon 1.2 mg/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained locally, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 16; Appendix 5, pp. 131-136 of MRID 50932067).
- Two ion pair transitions were monitored for each analyte (quantitation and confirmation, respectively): *m/z* 466→157 and *m/z* 466→142 for fluazaindolizine, *m/z* 206→122 and *m/z* 206→142 for IN-A5760, *m/z* 220→156 and *m/z* 220→141 for IN-F4106, *m/z* 452→123 and *m/z* 452→244 for IN-REG72, *m/z* 264→157 and *m/z* 264→184 for IN-RYC33, *m/z* 265→219 and *m/z* 265→184 for IN-QEK31, and *m/z* 197→141 and *m/z* 197→114 for IN-VM862.

Table 3. Independent Validation Method Recoveries for Fluazaindolizine and its metabolites IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862 in Water^{1,2}

Analyte	Fortification Level (µg/L)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
Drinking (Tap) Water						
Quantitation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	69-76	72	3.0	4
	1.0	5	69-78	74	4.9	7
IN-REG72	0.10 (LOQ)	5	65-74	70	3.6	5
	1.0	5	67-76	72	4.0	6
IN-F4106	0.10 (LOQ)	5	96-110	103	5.1	5
	1.0	5	100-104	102	1.5	1
IN-A5760	0.10 (LOQ)	5	100-103	101	1.2	1
	1.0	5	99-103	101	1.7	2
IN-VM862	0.10 (LOQ)	5	85-111	102	10.8	11
	1.0	5	100-108	106	3.4	3
IN-QEK31	0.10 (LOQ)	5	89-107	98	8.5	9
	1.0	5	99-109	103	3.6	3
IN-RYC33	0.10 (LOQ)	5	86-111	95	10.4	11
	1.0	5	93-104	99	4.7	5
Confirmation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	66-77	72	4.7	6
	1.0	5	65-78	72	5.1	7
IN-REG72	0.10 (LOQ)	5	68-79	72	4.5	6
	1.0	5	66-77	71	4.5	6
IN-F4106	0.10 (LOQ)	5	93-107	100	5.5	5
	1.0	5	98-104	102	2.8	3
IN-A5760	0.10 (LOQ)	5	81-99	88	7.3	8
	1.0	5	101-107	103	2.9	3
IN-VM862	0.10 (LOQ)	5	90-122	103	12.8	12
	1.0	5	98-108	104	3.7	4
IN-QEK31	0.10 (LOQ)	5	86-111	102	10.0	10
	1.0	5	92-111	101	7.1	7
IN-RYC33	0.10 (LOQ)	5	86-98	94	5.1	5
	1.0	5	93-100	97	2.8	3
Surface (River) Water						
Quantitation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	94-110	101	5.7	6
	1.0	5	103-108	105	2.0	2
IN-REG72	0.10 (LOQ)	5	86-93	89	2.5	3
	1.0	5	91-93	92	1.0	1
IN-F4106	0.10 (LOQ)	5	90-101	95	4.3	5
	1.0	5	98-101	99	1.1	1
IN-A5760	0.10 (LOQ)	5	82-95	89	4.6	5
	1.0	5	95-101	99	2.4	2
IN-VM862	0.10 (LOQ)	5	106-118	114	4.8	4
	1.0	5	94-111	102	7.1	7
IN-QEK31	0.10 (LOQ)	5	100-109	103	3.5	3
	1.0	5	101-107	103	2.4	2

Analyte	Fortification Level (µg/L)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
IN-RYC33	0.10 (LOQ)	5	97-101	98	1.7	2
	1.0	5	95-102	98	2.6	3
Confirmation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	105-113	108	5.8	5
	1.0	5	106-110	108	1.7	2
IN-REG72	0.10 (LOQ)	5	80-90	85	5.3	6
	1.0	5	92-95	93	1.1	1
IN-F4106	0.10 (LOQ)	5	92-101	95	3.6	4
	1.0	5	98-101	99	1.2	1
IN-A5760	0.10 (LOQ)	5	77-106	93	11.8	13
	1.0	5	96-100	98	1.8	2
IN-VM862	0.10 (LOQ)	5	87-95	91	2.9	3
	1.0	5	92-108	99	6.9	7
IN-QEK31	0.10 (LOQ)	5	90-107	99	6.0	6
	1.0	5	100-106	103	2.8	3
IN-RYC33	0.10 (LOQ)	5	91-98	95	3.3	3
	1.0	5	98-102	101	1.4	1
Ground Water						
Quantitation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	95-111	101	6.2	6
	1.0	5	101-106	103	1.7	2
IN-REG72	0.10 (LOQ)	5	85-94	89	3.9	4
	1.0	5	96-100	98	2.0	2
IN-F4106	0.10 (LOQ)	5	90-98	95	3.5	4
	1.0	5	93-96	95	1.1	1
IN-A5760	0.10 (LOQ)	5	81-90	85	4.4	5
	1.0	5	89-92	90	1.2	1
IN-VM862	0.10 (LOQ)	5	86-108	97	10.1	10
	1.0	5	85-102	92	7.6	8
IN-QEK31	0.10 (LOQ)	5	81-111	96	12.6	13
	1.0	5	92-103	99	4.1	4
IN-RYC33	0.10 (LOQ)	5	91-101	96	3.8	4
	1.0	5	94-101	98	3.3	3
Confirmation Ion Transition						
Fluazaindolizine (DPX-Q8U80)	0.10 (LOQ)	5	92-104	97	4.7	5
	1.0	5	101-106	104	1.8	2
IN-REG72	0.10 (LOQ)	5	85-97	90	4.6	5
	1.0	5	96-99	97	1.2	1
IN-F4106	0.10 (LOQ)	5	88-96	92	2.8	3
	1.0	5	93-97	95	2.1	2
IN-A5760	0.10 (LOQ)	5	73-100	84	10.0	12
	1.0	5	88-91	90	1.1	1
IN-VM862	0.10 (LOQ)	5	83-96	87	6.1	7
	1.0	5	82-101	94	7.8	8
IN-QEK31	0.10 (LOQ)	5	84-103	96	7.1	7
	1.0	5	91-103	98	5.3	5

Analyte	Fortification Level (µg/L)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
IN-RYC33	0.10 (LOQ)	5	91-107	98	6.5	7
	1.0	5	94-101	98	3.1	3

Data (uncorrected recovery results; pp. 29-30) were obtained from pp. 31-37 of MRID 50932113.

- The drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface water (Trial ID: NA; pH 7.5, conductivity 0.24 mmhos/cm, hardness 68 mg equiv. CaCO₃/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained from the Sponsor, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 25; Appendices 2-4, pp. 223-233 of MRID 50932113). The drinking and ground water matrices were the same as those of the ECM.
- Two ion pair transitions were monitored for each analyte (quantitation and confirmation, respectively): *m/z* 466→157 and *m/z* 466→142 for fluazaindolizine, *m/z* 206→122 and *m/z* 206→142 for IN-A5760, *m/z* 220→156 and *m/z* 220→141 for IN-F4106, *m/z* 452→123 and *m/z* 452→244 for IN-REG72, *m/z* 264→157 and *m/z* 264→184 for IN-RYC33, *m/z* 265→219 and *m/z* 265→184 for IN-QEK31, and *m/z* 197→141 and *m/z* 197→114 for IN-VM862.

III. Method Characteristics

In the ECM and ILV, the LOQ was 0.10 µg/L for all analytes in water matrices (pp. 12, 24; Appendix 4, p. 130 of MRID 50932067; pp. 13, 21 of MRID 50932113). In the ECM, the LOQ was defined as the lowest fortification level evaluated at which acceptable average recoveries (70-120%, RSD <20%) were obtained, as well as the fortification level at which analyte peaks are consistently generated at a level of *ca.* 10-20xs the signal at the analytes in the untreated controls. In the ECM, the LOD was defined as the concentration of the least responsive analyte (IN-QEK31) at which analyte peaks are *ca.* 3x the chromatographic baseline noise observed near the retention time or *ca.* one-third the concentration of the LOQ. The ECM LOD values for fluazaindolizine and its metabolites were calculated as *ca.* 0.02 µg/L for all analytes in water matrices, based on the following equation:

$$\text{LOD}_{\text{calc}} = 3 \times \text{LOQ} \div (\text{signal-to-noise ratio})$$

The LOD was not reported in the ILV. No calculations or comparisons to background levels were reported to justify the LOQ for the method in the ECM or ILV.

Table 4. Method Characteristics Fluazaindolizine and its metabolites IN-A5760, IN-F4106, IN-REG72, IN-RYC33, IN-QEK31, and IN-VM862 in Water

Test Material		Fluazaindolizine	IN-A5760	IN-F4106	IN-REG72	IN-RYC33	IN-QEK31	IN-VM862
Limit of Quantitation (LOQ)	ECM	0.10 µg/L						
	ILV							
Limit of Detection (LOD)	ECM	<i>ca.</i> 0.02 µg/L (calculated)						
	ILV	Not reported						
Linearity (calibration curve <i>r</i> and concentration range)	ECM ¹	<i>r</i> = 0.9998 (Q) <i>r</i> = 0.9999 (C)	<i>r</i> = 1.0000 (Q & C)	<i>r</i> = 1.0000 (Q) <i>r</i> = 0.9999 (C)	<i>r</i> = 0.9998 (Q) <i>r</i> = 0.9999 (C)	<i>r</i> = 0.9999 (Q & C)	<i>r</i> = 1.0000 (Q) <i>r</i> = 0.9999 (C)	<i>r</i> = 0.9999 (Q) <i>r</i> = 0.9998 (C)
		0.050-5.0 ng/mL						
	ILV	<i>r</i> = 0.9992 (Q) <i>r</i> = 0.9986 (C)	<i>r</i> = 0.9995 (Q) <i>r</i> = 0.9996 (C)	<i>r</i> = 0.9984 (Q) <i>r</i> = 0.9976 (C)	<i>r</i> = 0.9998 (Q) <i>r</i> = 0.9967 (C)	<i>r</i> = 0.9971 (Q) <i>r</i> = 0.9959 (C)	<i>r</i> = 0.9970 (Q) <i>r</i> = 0.9917 (C) ²	<i>r</i> = 0.9985 (Q) <i>r</i> = 0.9967 (C)
		0.050-5.0 ng/mL						
Repeatable	ECM ³	Yes at LOQ and 10×LOQ in characterized drinking, surface, and ground water matrices.					Yes at LOQ and 10×LOQ in characterized drinking and surface water matrices. Yes at LOQ (Q) and 10×LOQ in characterized ground water matrix; No at LOQ (C; RSD 22%).	Yes at LOQ and 10×LOQ in characterized drinking, surface, and ground water matrices.
	ILV ^{4,5}	Yes at LOQ and 10×LOQ in characterized drinking, surface, and ground water matrices.						
Reproducible		Yes at LOQ and 10×LOQ.					Yes at LOQ and 10×LOQ (based on Q results).	Yes at LOQ and 10×LOQ.

Test Material		Fluazaindolizine	IN-A5760	IN-F4106	IN-REG72	IN-RYC33	IN-QEK31	IN-VM862
Specific	ECM	Yes, matrix interferences were ≤4% of the LOQ (based on peak area and quantified residues). Some minor baseline noise was noted at the LOQ.	Yes, matrix interferences were ≤6% (Q) of the LOQ (based on peak area and quantified residues); however, matrix interferences were ≤26% (C) ² of the LOQ (based on peak area and quantified residues). Some minor baseline noise interference was noted at the LOQ.	Yes, matrix interferences were ≤6% of the LOQ (based on peak area and quantified residues). Notable baseline noise was observed around the analyte peak at the LOQ and interfered with peak attenuation and integration. ⁶	Yes, matrix interferences were ≤6% of the LOQ (based on peak area and quantified residues). Some minor baseline noise was noted at the LOQ.		Yes, matrix interferences were ≤2% of the LOQ (based on peak area and quantified residues). Notable baseline noise and minor contaminants were observed around the analyte peak at the LOQ and interfered with peak attenuation and integration. ⁷	Yes, matrix interferences were ≤6% of the LOQ (based on peak area and quantified residues). Notable baseline noise was observed around the analyte peak at the LOQ and interfered with peak attenuation and integration in surface and drinking water matrices. ⁸
			Only representative chromatograms from the quantitation ion analysis were provided. No representative chromatograms from the confirmation ion analysis were provided. ²					
	ILV	Yes, matrix interferences were <2% of the LOQ (based on peak area). ⁶	Yes, matrix interferences were <5% of the LOQ (based on peak area)	Yes, matrix interferences were <2% of the LOQ (based on peak area).	Yes, matrix interferences were <5% of the LOQ (based on peak area).	Yes, matrix interferences were <2% of the LOQ (based on peak area)	No , matrix interferences were <7% of the LOQ (based on peak area); however, LOQ peak was relatively small compared to baseline noise. ⁹ Baseline was highly elevated around analyte RT.	No , matrix interferences were <10% of the LOQ (based on peak area) in drinking and ground water matrices, but <i>ca.</i> 23% of the LOQ (based on peak area) in surface water. Also, LOQ peak was very small compared to baseline noise

Test Material		Fluazaindolizine	IN-A5760	IN-F4106	IN-REG72	IN-RYC33	IN-QEK31	IN-VM862
								and difficult to distinguish in all three water matrices. ¹⁰ Baseline was highly elevated around analyte RT
Some minor baseline noise interference was noted at the LOQ which was more prominent in the representative chromatograms from the confirmation ion analysis.								

Data were obtained from pp. 12, 24; Appendix 4, p. 130 (LOQ/LOD); Tables 1-2, pp. 28-33 (recovery results); Figure 2, pp. 48-54 (calibration curves); Figures 3-4, pp. 55-110 (chromatograms) of MRID 50932067; pp. 13, 21 (LOQ/LOD); pp. 31-37 (recovery results); Figure 8, pp. 124-130 (calibration curves); Figures 9-30, pp. 131-214 (chromatograms) of MRID 50932113; DER Attachment 2. Q = quantitation ion transition; C = confirmation ion transition.

- 1 ECM correlation coefficients (r) were reviewer-calculated from r² values provided in the study report (Figure 2, pp. 48-54 of MRID 50932067; DER Attachment 2).
- 2 Deviations of acceptability in the confirmation ion analysis did not affect the validity of the method since a confirmation method is not usually required when LC/MS or GC/MS is used as the primary method to generate study data.
- 3 In the ECM, drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface (White Clay Creek) water (Trial ID: Newark DE; pH 8.1, conductivity 0.35 mmhos/cm, hardness 144 mg equiv. CaCO₃/L, total organic carbon 1.9 mg/L, total dissolved carbon 1.2 mg/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained locally, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 16; Appendix 5, pp. 131-136 of MRID 50932067).
- 4 In the ILV, drinking (tap) water (Trial ID: SHRC Newark DE; pH 7.9, conductivity 0.45 mmhos/cm, hardness 134 mg equiv. CaCO₃/L, total organic carbon 2.1 mg/L, total dissolved carbon 1.4 mg/L), surface water (Trial ID: NA; pH 7.5, conductivity 0.24 mmhos/cm, hardness 68 mg equiv. CaCO₃/L), and ground (well) water (Trial ID: Kemblesville PA); pH 7.6, conductivity 0.20 mmhos/cm, hardness 68 mg equiv. CaCO₃/L, total organic carbon 0.6 mg/L, total dissolved carbon 0.1 mg/L) were obtained from the Sponsor, characterized by Agvise Laboratories, Northwood, North Dakota, and used in the study (p. 25; Appendices 2-4, pp. 223-233 of MRID 50932113). The drinking and ground water matrices were the same as those of the ECM.
- 5 The ILV validated the method for all analytes in the first trial with insignificant analytical instrument and equipment modifications (pp. 26-27, 30 of MRID 50932113). Due to LC/MS issues, the first trial drinking water samples were re-injected twice.
- 6 Based on Figure 4, pp. 87-92, of MRID 50932067.
- 7 Based on Figure 4, pp. 93-98, of MRID 50932067.
- 8 Based on Figure 4, pp. 105-110, of MRID 50932067.
- 9 Based on Figure 14, p. 146; Figure 21, p. 167; and Figure 28, p. 188 of MRID 50932113 (quantitation ion chromatograms).
- 10 Based on Figure 13, p. 143; Figure 20, p. 164; and Figure 27, p. 185 of MRID 50932113 (quantitation ion chromatograms). Linearity is satisfactory when r ≥ 0.995.

IV. Method Deficiencies and Reviewer's Comments

1. The ILV was not conducted independently from the ECM since ECM personnel Joseph Klems (Study Author/Study Director) communicated directly with the ILV personnel (study author, Yixiao Shen) since Joseph Klems acted as the DuPont Study Monitor for the ILV (pp. 1, 4 of MRID 50932067; pp. 1, 6; Appendix 5, pp. 235-253 of MRID 50932113). Communication between the ECM and ILV personnel involved technical advice regarding the IN-RYC33 RT shift, data transfer and approval between water matrices, and LC/MS/MS issues requiring re-injection of samples. OCSPP guidelines state that the analysts, study director, equipment, instruments, and supplies of the two laboratories must have been distinct and operated separately and without collusion, and the analysts and study director of the ILV must have been unfamiliar with the method both in its development and subsequent use in field studies.
2. The specificity of the method for IN-QEK31 and IN-VM862 was not supported by ILV representative chromatograms due to highly elevated baseline noise which encompassed the analyte interfering with identification and quantification (Figures 13-14, pp. 143, 146; Figures 20-21, pp. 164, 167; and Figures 27-28, pp. 185, 188 of MRID 50932113). Representative chromatograms of both analytes showed similar issues; however, the LOQ analyte peak for IN-VM862 was only slightly distinguishable from the baseline noise in all three water matrices and matrix interferences for IN-VM862 were *ca.* 23% of the LOQ (based on peak area) in surface water (>LOD).
3. ECM performance data was not acceptable for the LOQ confirmation ion analysis of IN-QEK31 in ground water (RSD 22%; Tables 1-2, pp. 28-33 of MRID 50932067). OSCPP guidelines state that mean recoveries should be 70-120% with RSD \leq 20%. The reviewer noted that deviations of acceptability in the confirmation ion analysis did not affect the validity of the method since a confirmation method is not usually required when LC/MS or GC/MS is used as the primary method to generate study data.
4. ILV linearity was not satisfactory for the confirmation ion analysis of IN-QEK31 ($r = 0.9917$). Linearity is satisfactory when $r \geq 0.995$. The reviewer noted that deviations of acceptability in the confirmation ion analysis did not affect the validity of the method since a confirmation method is not usually required when LC/MS or GC/MS is used as the primary method to generate study data.
5. The ILV drinking and surface water matrices were the same as those of the ECM.
6. In the ECM, the specificity of the method was not well-supported by the representative chromatograms IN-F4106, IN-QEK31, and IN-VM862 since notable baseline noise was observed around the analyte peak at the LOQ and interfered with peak attenuation and integration (Figure 4, pp. 87-92, 93-98, 105-110 of MRID 50932067). Additionally, no representative chromatograms from the confirmation ion analysis were provided.
7. The determinations of LOD and LOQ in the ECM and ILV were not based on scientifically acceptable procedures as defined in 40 CFR Part 136 (pp. 12, 24; Appendix 4, p. 130 of MRID 50932067; pp. 13, 21 of MRID 50932113). In the ECM, the LOQ was defined as the lowest fortification level evaluated at which acceptable average recoveries (70-120%, RSD <20%) were obtained, as well as the fortification level at which analyte peaks are consistently generated at a level of *ca.* 10-20xs the signal at the analytes in the untreated controls. In the ECM, the

LOD was defined as the concentration of the least responsive analyte (IN-QEK31) at which analyte peaks are *ca.* 3x the chromatographic baseline noise observed near the retention time or *ca.* one-third the concentration of the LOQ. The ECM LOD values for fluazaindolizine and its metabolites were calculated as *ca.* 0.02 µg/L for all analytes in water matrices, based on the following equation: $LOD_{calc} = 3 \times LOQ \div (\text{signal-to-noise ratio})$. The LOD was not reported in the ILV. No calculations or comparisons to background levels were reported to justify the LOQ for the method in the ECM or ILV. Detection limits should not be based on arbitrary values.

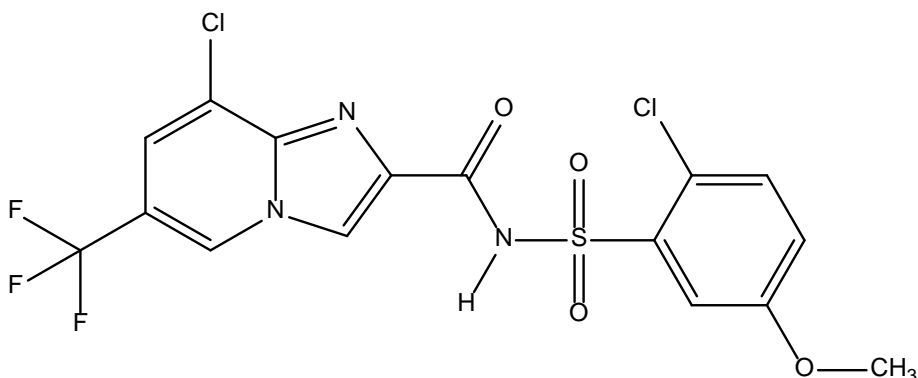
8. The reviewer noted that the ILV contained the significant typographical error of reporting “IN-RCY33”, instead of “IN-RYC33”, throughout the entire study report, i.e. pp. 20, 25 of MRID 50932113. The Certificate of Analysis provided in the ILV contained the correct name for the analyte (Appendix 1, p. 222).
9. In the ECM, the time requirement for 4 sets of 6 samples was an eight-hour day for preparation with LC/MS/MS analysis run overnight (p. 24 of MRID 50932067). In the ILV, one set of 12 samples required *ca.* 2 working days, including LC/MS/MS instrument run-time (p. 38 of MRID 50932113).

V. References

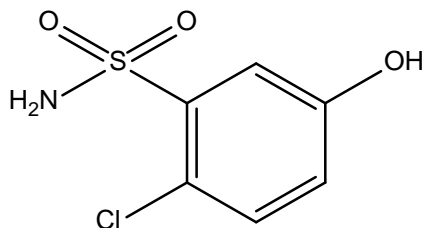
- U.S. Environmental Protection Agency. 2012. Ecological Effects Test Guidelines, OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation. Office of Chemical Safety and Pollution Prevention, Washington, DC. EPA 712-C-001.
- 40 CFR Part 136. Appendix B. Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11, pp. 317-319.

Attachment 1: Chemical Names and Structures**Fluazaindolizine (DPX-Q8U80)**

IUPAC Name: 8-Chloro-N-[(2-chloro-5-methoxyphenyl)sulfonyl]-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide
CAS Name: 8-Chloro-N-[(2-chloro-5-methoxyphenyl)sulfonyl]-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide
CAS Number: 1254304-22-7
SMILES String: C1C=CC(C(F)(F)F)=CN2C1=NC(C(N(S(C3=CC(OC)=CC=C3Cl))=O)=O)[H]=O)=C2

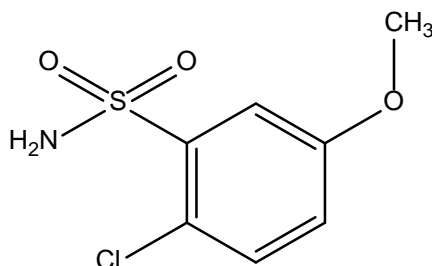
**IN-A5760**

IUPAC Name: 2-Chloro-5-hydroxybenzenesulfonamide
CAS Name: Not reported
CAS Number: 86093-06-3
SMILES String: OC1=CC(S(N)=O)=C(Cl)C=C1

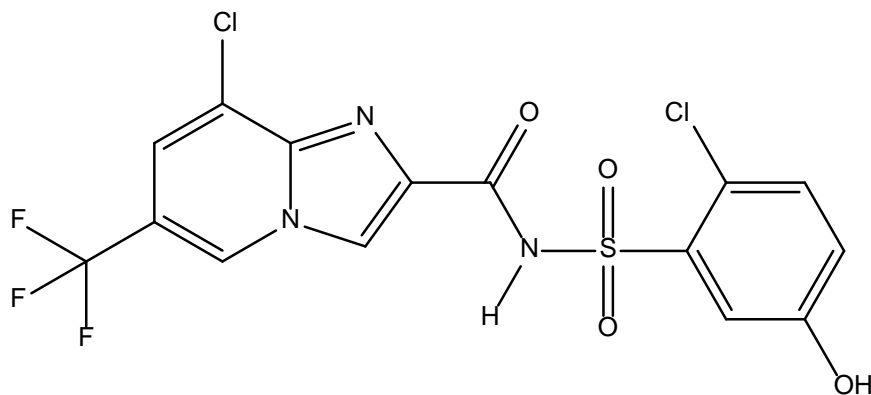


IN-F4106

IUPAC Name: 2-Chloro-5-methoxybenzenesulfonamide
CAS Name: Not reported
CAS Number: 502187-53-3
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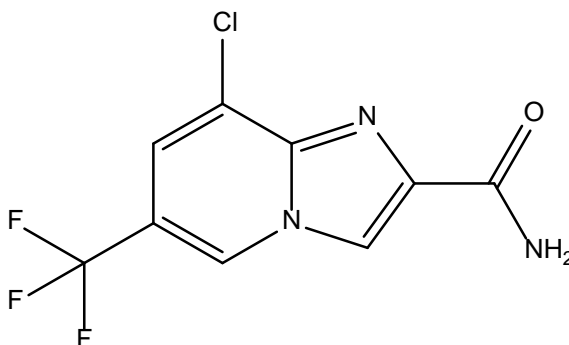
**IN-REG72**

IUPAC Name: 8-Chloro-N-((2-chloro-5-hydroxyphenyl)sulfonyl)-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide
CAS Name: Not reported
CAS Number: Not reported
SMILES String: ClC1=CC(C(F)(F)F)=CN2C1=NC(C(N(S(C3=CC(O)=CC=C3Cl))(=O)=O)[H])=O)=C2

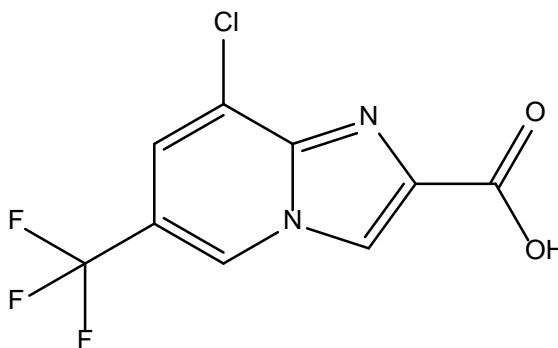


IN-RYC33

IUPAC Name: 8-Chloro-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide
CAS Name: Not reported
CAS Number: 1228376-01-9
SMILES String: C1C=CC(C(F)(F)F)=CN2C1=NC(C(N)=O)=C2

**IN-QEK31**

IUPAC Name: 8-Chloro-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxylic acid
CAS Name: Not reported
CAS Number: 353258-35-2
SMILES String: C1C=CC(C(F)(F)F)=CN2C1=NC(C(=O)O)=C2



IN-VM862

IUPAC Name: 3-Chloro-5-(trifluoromethyl)pyridin-2-amine
CAS Name: Not reported
CAS Number: 79456-26-1
SMILES String: ClC1=CC(C(F)(F)F)=CN=C1N

