FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: JMD-HER 387 OD

Product name(s): Jockey 387 OD

Chemical active substances:
2,4-D, 250 g/L (as 2,4-D 2EHE, 377 g/L)
Iodosulfuron-methyl-sodium, 10 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Pestila Spółka z ograniczoną odpowiedzialnością

Submission date: December 2022, March 2024

Finalisation date: December 2023; March 2024; August 2024

Version history

When	What
12.2023	zRMS assesment
03.2024	Final version of RR after commenting period
03.2024	List of data submitted or referred to by the applicant and relied on, but already evaluated at EU peer review updated by Applicant
08.2024	zRMS addition

Table of Contents

5 Analytical methods

5.1 Conclusion and summary of assessment

Analytical method HPLC-DAD for determination of active substances: iodosulfuron, 2,4-D and UV spectrophotometer for determination of relevant impurity free phenols and GC-MS method for determination of relevant impurities dioxins and furans in plant protection product JMD-HER 387 OD have been validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev. 5, therefore it is acceptable. There are no data gaps.

Sufficiently sensitive and selective analytical methods are available for the active substance(s) and relevant impurities in the plant protection product.
Noticed data gaps are:

- none

Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.
Noticed data gaps are:

- ~~List of data submitted or referred to by the applicant and relied on, but already evaluated should be completed before registration~~. The list was completed in *iodosulfuron 2-4 D_fRR Part A JMD-HER 387 OD_Pestila_ PL_03.2024 v2* updated in 08.2024.

Commodity/crop	Supported/ Not supported
Spring wheat	Supported
Spring triticale	Supported
Winter wheat	Supported
Winter triticale	Supported
Rye	Supported

5.2 Methods used for the generation of pre-authorization data (KCP 5.1)

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

5.2.1.1 Determination of active substance and/or variant in the plant protection product (KCP 5.1.1)

An overview on the acceptable methods and possible data gaps for analysis of 2,4-D and iodosulfuron-methyl-sodium in plant protection product is provided as follows:

Comments of zRMS:	Analytical method HPLC-DAD for determination of active substances iodosulfuron and 2,4-D in plant protection product JMD-HER 387 OD has been validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev. 5, therefore it is acceptable.

Reference: 5.1.1/01

	5.1.1/02
Report	JMD-HER 387 OD. Determination of active substances content in preparation in an COEX bottle. Stage 1: Determination of active substances content in initial preparation and Stage 3: Determination of active substances content in preparation stored at temperature 40±2°C for 8 weeks., Ciach J., 2021, report no 001/DPL/2021
Guideline(s):	Yes, SANCO/3030/99 rev.5 (22/03/19)
Deviations:	Yes. During the storage of the test item in the warehouse for some days occurred the deviations from the instruction IO/L-01, 5th edition, defining the required temperature and humidity range in this room. These deviations were no more than +1.1°C (26.1°C), over the required range temperature (15÷25°C) and +4% RH, over the required range humidity (15÷80% RH). This deviation had no impact on the test results.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Determination of iodosulfuron-methyl-sodium and 2,4-D in JMD-HER 387 OD was performed with high performance liquid chromatography technique (HPLC) with DAD detection wavelength 270 nm and external standard.

Equipment and chromatographic conditions for iodosulfuron-methyl-sodium analysis

- Test system: HPLC - Agilent Technologies 1220 Infinity II (AKP/15)
- Detector: DAD: wavelength 250 nm
- Column: Kinetex C18, 150 mm x 4.6 mm x 5 µm (045/HPLC)
- Eluent: A) ACN/H_2O/5%H_3PO_4 (900/100//5) ml – 43%
  B) ACN/H_2O/5%H_3PO_4 (900/100//5) ml – 57%
- Solvent: 5 ml THF (directly into a volumetric flask) + ACN/H_2O/5%H_3PO_4 (900/100//5) ml (to the volume)
- Column temperature: 30°C
- Flow rate: 1.2 ml/min
- Sample injected volume: 3 µl
- Syringe filters: 0.22 µm
- Time of analysis: 18 min
- Retention time of iodosulfuron: ~4,7 min

The preparation of standard solution for iodosulfuron-methyl-sodium analysis

Weight 0,0500±0,0100 g of the iodosulfuron-methyl-sodium standard with accuracy of 0.0002 g into the flask of 100 ml. Next, add 5 ml THF into the flask and mix until the standard is dissolved. Next the flas was filled up to ¾ of the volume with the solvent Eluent A The solution was left in an ultrasonic bath for 15 minutes. After this time, it was cooled to ambient temperature (18÷25ºC) in a water bath for 30 minutes. The flask was made up to the volume of the solvent and was mixed. 20 ml of the prepared standard was transferred to a 100ml flask, made up to volume with solvent and mixed, and filtered through a 0,22 µm PTFE syringe filter prior to injection.

The preparation of samples for iodosulfuron-methyl-sodium analysis

Weigh 1,00±0,20 g of iodosulfuron standard with accuracy of 0.0002 g into the flask of 100 ml. Next, add 5 ml THF into the flask and mix until the standard is dissolved. The solution was left in an ultrasonic bath for 15 minutes. After this time, it was cooled to ambient temperature (18÷25ºC) in a water bath for 30

minutes. The flask was later made up to the volume of the eluent A, the content was mixed and filtered through a 0,22 µm PTFE syringe pilter prior to injection.

Equipment and chromatographic conditions for 2,4-D analysis

- Test system: HPLC - Agilent Technologies 1220 Infinity II (AKP/15)
- Detector: DAD: wavelength 270 nm
- Column: Kinetex C18, 150 mm x 4.6 mm x 5 µm (045/HPLC)
- Eluent: A) ACN/H_2O/5%H_3PO_4 (900/100//5) ml – 43%
  B) ACN/H_2O/5%H_3PO_4 (900/100//5) ml – 57%
- Solvent: 5 ml THF (only for test item solution, directly into a volumetric flask) + ACN/H_2O/5%H_3PO_4 (900/100//5) ml (to the volume)
- Saponification solution: 2-propanol/H_2O/KOH (700/300) ml/15 g
- Neutralization solution: 5% orthophosphoric acid solution
- Column temperature: 30°C
- Flow rate: 1.2 ml/min
- Sample injected volume: 3 µl
- Syringe filters: 0.22 µm
- Time of analysis: 18 min
- Retention time of 2,4-D: ~3,3 min

The preparation of the standard solution for 2,4-D analysis

Weigh 0.0800±0.0160 g of 2,4-D standard with accuracy of 0.0002 g into the flask of 100 ml. Next, make up to ¾ of the volume of the solvent. Leave in an ultrasonic bath for 10 minutes and after this time cool the solution to room temperature (18÷25°C) in a water bath for 30 min. Then make up the flask to the volume of the solvent, mix and filter through a 0.22 µm PTFE syringe prior to injection.

The preparation of samples for 2,4-D analysis

Weigh 0,30±0.06 g of preparation with accuracy of 0.0002 g into a flask of 100 ml. Next, add 5 ml THF into a flask and mix until the preparation is dispersed. Add 25 ml of saponification solution, mix and leave the flask in an ultrasonic bath for 60 minutes. After this time add 20 ml of neutralization solution, mix and cool the solution to room temperature (18÷25°C) in a water bath for 30 min. Then make up the flask to the volume of the solvent, mix and filter through a 0.22 µm PTFE syringe filter prior to injection.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances iodosulfuron and 2,4-D in plant protection product JMD-HER 387 OD

	Iodosulfuron-methyl-sodium	**2,4-D**
Author(s), year	Ciach J., 2021	
Principle of method	~~SANCO/3030/99 rev.5, 22 March 2019~~ HPLC - DAD	
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of the analytical method was assessed using seven iodosulfuron-methyl-sodium standard solutions (n=7) in the concentration range from 6.0 mg/L (0.6 g/kg) to 239.5 mg/L (22.6 g/kg). y=3.802x+3.2221 Correlation coefficient: $R^2 = 1$ Required: $R^2 \geq 0.99$	The linearity of the analytical method was assessed using seven 2,4-D standard solutions (n=7) in the concentration range from 99.6 mg/L (29.4 g/kg) to 1593.2 mg/L (469.7 g/kg). y=0.5233x+2.4802 Correlation coefficient: $R^2 = 0.9999$ Required: $R^2 \geq 0.99$
Precision – Repeatability Mean	Mean concentration: 0.918%	Mean concentration: 23.49%

	Iodosulfuron-methyl-sodium	**2,4-D**
n = ~~6~~ 7 (%RSD)	H_r = 0.27 Required: $H_r \leq 1$ RSD = 0.74 % Required: RSD ≤ 2.71%	H_r = 0.66 Reqiured: $H_r \leq 1$ RSD = 1.10% Required: RSD ≤ 1.67%
Accuracy **n = ~~6~~ 2 for each level** **(% Total Recovery)**	Level I: 9.1 g/kg – 101.65% Level II: 8.1 g/kg – 101.23% Mean 101.44% (range: 101.10% - 102.20%) Required: 90% ÷ 110%	Level I: 234.8 g/kg – 97.98% Level II: 187.9 g/kg – 98.58% Mean 98.28% (range: 97.26% - 99.89%) Required: 97% ÷ 103%
Interference/ Specificity	There are no any interferences coming from impurities for the peak of the target analyte – iodosulfuron-methyl-sodium.	There are no any interferences coming from impurities for the peak of the target analyte – 2,4-D.
Comment	No comments.	No comments.

Conclusion

The HPLC method, used to quantify iodosulfuron-methyl-sodium in JMD-HER 387 OD was fully validated. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

The HPLC method, used to quantify 2,4-D in JMD-HER 387 OD was fully validated. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

5.2.1.2 Description of analytical methods for the determination of relevant impurities (KCP 5.1.1)

An overview on the acceptable methods and possible data gaps for analysis of relevant impurities in plant protection product is provided as follows:

Comments of zRMS:	Analytical method UV spectrophotometer for determination of relevant impurity: free phenols in plant protection product JMD-HER 387 OD has been validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev. 5, therefore it is acceptable.

Reference:	5.1.1/03
Report	Determination of physicochemical properties, Wołoszynowska M., 2021, report no BA-05/21
Guideline(s):	Yes, SANCO/3030/99 rev. 5 (22/03/19)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods for free phenols content

~~Determination of residues of free phenols content in 2,4-D~~ Determination of free phenols in plant protection product JMD-HER 387 OD was performed with UV spectrophotometer at wavelength 520 nm.

Equipment and chromatographic conditions for free phenols analysis

- Spectrophotometer UV -1700 PharmaSpec, Shimadzu
- Analytical balance Mettler AT261 DR, accuracy 0.01 mg
- Glass graduated pipettes
- Automatic pipette
- Pipette 5ml, 10 ml
- Beaker 25 ml
- Graduated measuring cylinders with stoppers
- Glass graduated flasks 100 ml
- Typical laboratory equipment
- 4-aminoantipyrine 98 %
- Potassium hexacyanoferrate (III), K3[Fe(CN)6]
- Ammonia
- Acetone a.g.
- Ethanol a.g. 96 %
- 2,4-D 2-EHE
- 2,4-dichlorophenol

The preparation of standard solution for free phenols analysis

Solution A About 10 mg of 2,4-dichlorophenol standard (10,11 mg for initial, and 10,09 mg for after accelerated storage) was dissolved in 1ml of acetone and replenished up to 100ml with water
Solution B (for blank sample preparation) was prepared by weighing: 2,4-D 2-EHE standard – 54,01 mg for initial preparation and 54,11 mg for preparation after accelerate storage, iodosulfuron-methyl-sodium standard – 1,41mg for initial preparation and 1,41mg for preparation after accelerated storage and JMD-HER 387 OD placebo – 95,18 mg for initial preparation and 99,28mg for preparation after accelerated storage into a 100ml volumetric flask. Then 5ml of ethanol and 9ml of ammonia solution were added and flask was filled with water up to the mark.
Specimen preparation About 150 mg of JMD-HER 387 OD preparation was weighed into a 100ml volumetric flask. Then 5ml of ethanol and 9ml of ammonia solution were added and water was added up to the mark. Afterward 10ml of the sample was transferred to a graduated measuring cylinder. Then 5ml of each solution: ammonia, 4-aminoantipyrine and potassium ferricyanide were added and mixed. After 5 minutes' absorbance was measured.
Validation - Results and discussions

Table 5.2-2a: Methods suitable for the determination of the relevant impurities (free phenols) in plant protection product (PPP) JMD-HER 387 OD

	Relevant impurity free phenols max. content in PPP
Author(s), year	Wołoszynowska M., 2021
Principle of method	~~SANCO/3030/99 rev.5, 22 March 2019~~ UV spectrophotometer at wavelength 520 nm.
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	The absorbance of prepared solutions of 2,4-dichlorophenol were measured. The calibration curve was plotted in range 0.072 – 0.24 g/kg. n=5 y=2.3992x+0.0022 Correlation coefficient: $R^2 = 0.9933$ Required: $R^2 \geq 0.99$
Precision – Repeatability Mean n = 6 (%RSD)	RSD= 4.38% $RSD_r \leq 6.63\%$ $Hr = 0.66 \leq 1$
Accuracy n = ~~12~~ 6 for each level	Level I: LOQ – 99.57% Level II: 0.135 g/kg – 100.77%

	Relevant impurity free phenols max. content in PPP
(% Total Recovery)	Mean 100.17% (Range: 97.97% - 102.97%) Required: (70 - 130%)
Interference/ Specificity	Fulfilled
LOQ	~~0,0072~~ 0.072 g/kg
Comment	No comments.

Comments of zRMS:	Analytical method GC-MS for determination of relevant impurities: – dioxins and furans in plant protection product JMD-HER 387 OD have been validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev. 5, therefore it is acceptable.

Reference:	5.1.1/04
Report	GC method for determination of dioxins and furans in JMD-HER 387 OD, Pstuś J., 2022, report no RVM/2022/48
Guideline(s):	Yes, SANCO/3030/99 rev.5, 22/03/19.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods for dioxins and furans content

Determination of residues of dioxins and furans content in 2,4-D was performed with GC system equipped with solvent vent injector and MS single-quad detector.

Equipment and chromatographic conditions for dioxins and fruans analysis

- GC system equipped with solvent vent injector (MMI) and MS single-quad detector, Agilent Technologies;
- GC column: DB-DIOXIN, 60 m x 0.25 mm, film thickness 0.25 µm, Agilent Technologies, Part No.: 122-2462 or equivalent;
- Liner, Agilent Technologies 5190-2296;
- Class A volumetric glassware and pipettes;
- Rotary evaporator;
- Vacuum pump system;
- VLM Metal Block Thermostat;
- Silica column, Intarchim, part no.: PF-50SIHP-JP-F0120;
- Supelco Dioxin Prep System (6.35mm MULTI-LAYER SILICA GEL DIOXIN COLUMN, part no. 28397-U; lot no. 123684, 6.35/10MM DUAL LAYER CARBON Reversible Tube (Micro-Column), part no. 28399-U),
- Laboratory vacuum dryer, Jeio Tech (SAN-LAB), type OV-11;
- Magnetic stirrer, IKA RCT Classic, thermometer IKA ETS-D5;
- Eppendorf Multipette, internal no.: P3.LA.44;
- Vortex mixer, IKA VORTEX3, internal no.: C19.VT.04,
- Methanol gradient grade for liquid chromatography; Supelco; batch no. I1059907945; purity 99.93%;
- Dichloromethane HPLC Plus for HPLC, GC and residue analysis, ≥ 99.9%, contains 50-150 ppm amylene as stabilizer; Sigma Aldrich; batch no MKCN9794; purity 99.95%; expiry date: 14.01.2025;

- n-Hexane for HPLC ≥ 95%; Sigma - Aldrich; batch no. no STBH5346; purity ≥95%;
- Toluene for gas chromatography MS; Supelco; batch no. I1104549030; purity ≥ 99.8%;
- n-Nonane; 99%; Alfa Aesar; batch no. 10223919; purity 99.4%;
- Nitrogen 5.0; Linde Gas.

The preparation of test solution for dioxins and furans analysis

To the round bottom flask transfer 10 mL of JMD-HER 387 OD add 100 µL of Labelled Compound Stock Solution EDF-8999 (containing 15 labelled analogues of congeners). Use a rotary evaporator to eliminate the solvents from the sample at temperature 80°C and 2 mbar vacuum for 20 minutes. The next steps of sample preparation are described in points 2-10 (see paragraph below).

The preparation of samples for dioxins and furans analysis

The sample preparation was based on method 1613.
The non-spiked sample solution was prepared according the procedure below:

1. To the round bottom flask transfer 10 mL of JMD-HER 387 OD add 100 µL of Labelled Compound Stock Solution EDF-8999 (containing 15 labelled analogues of congeners). Use a rotary evaporator to eliminate the solvents from the sample at temperature 80°C and 2 mbar vacuum for 20 minutes.
2. After initial removal of solvents by rotary evaporator, perform a vacuum distillation of the sample at 80°C and c.a. 0.05 mbar for 30 minutes.
3. Dissolve the dried sample in 10 mL of mix dichloromethane : methanol (in ratio 1:1) an transfer quantitatively sample with another 10 mL mix dichloromethane : methanol (in ratio 1:1) to the preparative silica column (PF-50SIHP-JP-F0120, Intarchim). Dry the column with use of vacuum dryer at 40°C and under 10 mbar pressure for 30 minutes.
4. Wash out all analytes of interest with use of hexane for 25 minutes at flow rate of 60 mL (1500 mL of hexane). Collect all of eluate and reduce the volume up to 5 – 10 mL with use of rotary evaporator (200 mbar and 40°C).
5. Prepare the Supelco Dioxin Prep System for purifying and concentration of eluate: Precondition the multi-layer column: mount the column, vacuum adapter and round bottom flask with use of standard flow of 200 mL of hexane with use of slight vacuum to obtain the constant flow of eluent. Leave a wet column filling. The column is ready to use. Precondition of the dual layer carbon column: mount the column, vacuum adapter and round bottom flask with use of standard flow of 40 mL of toluene and then 100 mL of hexane with use of slight vacuum to obtain the constant flow of eluent. Leave a wet column filling. The column is ready to use.
6. Mount the Supelco Dioxin prep System (see the Figure 1) with two columns and introduce the eluate from the point 4.2.5 to the filing. Wash the round bottom flask with 5 mL of hexane and add this solution to the column. Turn on the vacuum pump and allow the eluate to be almost fully absorbed. Then add the 250 mL of hexane to the top flask and perform the purification. After finishing the chromatography remove the multi-layer column and flush the dual layer carbon column with additional portion of 50 mL of hexane.
7. Reverse the dual layer carbon column and remove the analytes of interest under vacuum to the clean round bottom flask with use of 100 mL of toluene.
8. Add 2 mL of n-nonane to collected toluene eluate and reduce to about 2mL with use of rotary evaporator (50 mbar and 50°C). Transfer quantitively the condensate to the glass conical vial. Rinse the flask with two 0.5 mL portions of n-nonane and transfer washes to the vial.
9. Add 100 µL of n-nonane to the sample and reduce the volume of the solution to about 90 µL with use of nitrogen stream. Measure the final volume of the sample by Hamilton syringe and adjust it up to 90 µL with use of n-nonane if necessary.
10. Transfer the whole solution to injection vial. Add 10 µL of Internal Standard Spiking Solution EDF-5999 (containing 1,2,3,4-TCDD - $^{13}C_{12}$ and 1,2,3,7,8,9-HxCDD - $^{13}C_{12}$ labelled internal standards). Mix gently with use of vortex. The sample is ready for injection to GC-MS system.

Validation - Results and discussions

Table 5.2-3b: Methods suitable for the determination of the relevant impurities (dioxins and furans) in plant protection product (PPP) FLD-HER 306 SE

	~~**Relevant impurity – dioxins max. content in PPP**~~	~~**Relevant impurity – furans max. content in PPP**~~
	Relevant impurity – dioxins and furans max. content in PPP	
Author(s), year	Pstuś J.	
Principle of method	~~SANCO/3030/99 rev.5, 22 March 2019~~ GC-MS	
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	Linearity range 0.5 – 198.8 ng/ml for tetra congners ($4.8x10^{-6}$-$1.9x10^{-3}$ mg/kg range). 2.5-995 ng/ml for penta-hepta congners ($2.4x10^{-5}$-$9.5x10^{-3}$ mg/kg range). 4.9-1978 mg/ml for octa congners ($4.7x10^{-5}$-$1.9x10^{-2}$ mg/lg range). Correlation Coefficient R > 0.99	
Precision – Repeatability Mean n = ~~17~~ 5 for each congener (%RSD)	~~H_r = 0.238 (range 0.10–0.42)~~ ~~Required: $H_r \leq 1$~~ ~~RSD = 8.13 % (range 4.3–14.2)~~ ~~RSDr = 34.8% (range 30.0–43.9)~~ ~~Required: % RSD < % RSDr~~	~~H_r = 0.186 (range 0.08–0.30)~~ ~~Reqiured: $H_r \leq 1$~~ ~~RSD = 6.47% (range 2.2–10.2)~~ ~~RSDr = 34.57% (range 29.4–43.6)~~ ~~Required: % RSD < % RSDr~~
	$9x10^{-5}$ mg/kg for tetra congners Range %RSD = 4.3-8.3. %RSDr = 43.5 Range Hr = 0.10-0.19 $4.0x10^{-4}$ – $5.4x10^{-4}$ mg/kg for penta-hepta congners Range %RSD = 4.1-14.2 Range %RSDr = 33.3-34.8 Range Hr = 0.12-0.42 $1.2x10^{-5}$ mg/kg for octa congners Range %RSD = 2.2-6.6 Range %RSDr = 30 Range Hr = 0.08-0.22 Required: $H_r \leq 1$	
Accuracy n = ~~17~~ 3 for each level of each congener (% Marginal Recovery)	~~103.21% (range: 83% – 118%)~~ ~~Required: (70 – 130%)~~	~~102.55% (range: 86% – 129%)~~ ~~Required: (70–130%)~~
	for tetra congners LOQ level – range 112-114% $9.0x10^{-5}$ mg/kg – range 91-92% for penta-hepta congners LOQ level – range 93-115% $4.3x10^{-4}$ mg/kg – 83-112% for octa congners LOQ level – range 118-125% $1.1x10^{-3}$ mg/kg – 115-129% Required: (70 – 130%)	
Interference/ Specificity	fulfilled	fulfilled
LOQ	~~2.21 µg (range 0.5–5.0)~~	~~2.77 µg (range 0.5–4.9)~~
	$6.0x10^{-6}$ mg/kg for tetra congners (0.5 ng/ml). $2.5x10^{-5}$ - $3.0x10^{-5}$ mg/kg for penta-hepta congners (2.5 ng/ml) $6.2x10^{-5}$ – $6.7x10^{-5}$ mg/kg for octa congners (5.0 ng/ml)	

	~~Relevant impurity – dioxins max. content in PPP~~	~~Relevant impurity – furans max. content in PPP~~
	Relevant impurity – dioxins and furans max. content in PPP	
Comment	No comments.	No comments.

Conclusion

Determination of residues of sum of dioxins and furans was was fully validated. The methods for determination are specific. The validation parameters for linearity, instrument precision, limit of quantification, repeatability and accuracy are within the acceptance range. There are not any interferences between relevant impurities and other ingredients of the samples. The methods had good precision, accuracy and the linearity and fulfil requirements of SANCO/3030/99 rev.5.

5.2.1.3 Description of analytical methods for the determination of formulants (KCP 5.1.1)

The product JMD-HER 387 OD does not contain any co-formulant which is of toxicological, ecotoxicological or environmental significance. Therefore, analytical methods for formulants are not required.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

For 2,4-D salt aqueous solutions CIPAC Method 1.4/SL/M2/- (CIPAC Handbook 1C, page 2066) is suitable.
For iodosulfuron-methyl-sodium no CIPAC method is available for the active substance in the preparation.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

2,4-D
An overview on the acceptable methods and possible data gaps for analysis of residues of 2,4-D for the generation of pre-authorization data is given in the following table. the detailed evaluation of additional studies, it is referred to Appendix 2.

Table 5.2-4: Validated methods for the generation of pre-authorization data

Component of residue definition: 2,4-D (sum of 2,4-D and its esters expressed as 2,4-D)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
50% sucrose solution (Honey bee-oral, chronic and Bumbble bee-oral, acute) (Ecotoxicology)	Primary	0.33188 mg/kg	HPLC with UV-DAD	Włodarczyk M., 2021 / Study code: 0005/0099/FA

Component of residue definition: 2,4-D (sum of 2,4-D and its esters expressed as 2,4-D)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Aqueous solution (Bumbble bee-contact, acute) (Ecotoxicology)	Primary	0.40587 mg/L	HPLC with UV-DAD	Włodarczyk M., 2021 / Study code: 0005/0102/FA
Artificial soil (*Eisenia andrei)* (Ecotoxicology)	Primary	0.01 mg/kg (2,4-D 2-EHE) 0.1 mg/kg (2,4-D acid)	GC with ECD HPLC with DAD	Arendarczyk, A., 2021 / Study code: G-03-21
Artificial soil (*Folsomia candida)* (Ecotoxicology)	Primary	0.05 mg/kg (2,4-D 2-EHE) 0.2 mg/kg (2,4-D acid)	GC with ECD HPLC with DAD	Gierbuszewska, A., 2021 / Study code: G-04-21
Water (*Raphidocelis subcapitata)* (Ecotoxicology)	Primary	0.001 mg/L (2,4-D 2-EHE) 0.001 mg/L (2,4-D acid)	GC with ECD HPLC with DAD	Czarnecka, M., 2021 / Study code: W-03-21
Water (Vegetative Vigour; Seedling Emergence and Seedling Growth) (Ecotoxicology)	Primary	0.01 mg/L (2,4-D 2-EHE) 0.1 mg/L (2,4-D acid)	GC with ECD HPLC with DAD	Arendarczyk, A., 2021 / Study code: G-07-21 and Study code: G-08-21
Elendt M7 medium (*Chironomus* sp.*)* (Ecotoxicology)	Primary	0.01 mg/L (2,4-D 2-EHE) 0.1 mg/L (2,4-D acid)	GC with ECD HPLC with DAD	Czarnecka, M., 2021 / Study code: W-01-21
Water (*Lemna gibba)* (Ecotoxicology)	Primary	0.001 mg/L (2,4-D 2-EHE)	GC with ECD	Czarnecka, M., 2021 / Study code: W-04-21
Water (*Myriophyllum spicatum)* (Ecotoxicology)	Primary	0.05 mg/kg (2,4-D 2-EHE)	GC with ECD	Turek-Lipka, T., 2021 / Study code: W-05-21
Sediment (*Myriophyllum spicatum)* (Ecotoxicology)		0.001 mg/L (2,4-D 2-EHE)		

An overview on the acceptable methods and possible data gaps for analysis of residues of Iodosulfuron-methyl-sodium for the generation of pre-authorization data is given in the following table. For the detailed evaluation of new studies, it is referred to Appendix 2.

Table 5.2-5: Validated methods for the generation of pre-authorization data

Component of residue definition: iodosulfuron-methyl-sodium				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
50% sucrose solution (Honey bee-oral, chronic and Bumbble bee-oral, acute) (Ecotoxicology)	Primary	0.02083 mg/kg	HPLC with UV-DAD	Włodarczyk M., 2021 / Study code: 0005/0099/FA
Aqueous solution (Bumbble bee-contact, acute) (Ecotoxicology)	Primary	0.01795 mg/L	HPLC with UV-DAD	Włodarczyk M., 2021 / Study code: 0005/0102/FA
Artificial soil (*Eisenia andrei)* (Ecotoxicology)	Primary	0.2 mg/kg	HPLC with DAD	Arendarczyk, A., 2021 / Study code: G-03-21
Artificial soil (*Folsomia candida)* (Ecotoxicology)	Primary	0.2 mg/kg	HPLC with DAD	Gierbuszewska, A., 2021 / Study code: G-04-21
Water (*Raphidocelis subcapitata)* (Ecotoxicology)	Primary	0.001 mg/L	HPLC with DAD	Czarnecka, M., 2021 / Study code: W-03-21
Water (Vegetative Vigour; Seedling Emergence and Seedling Growth) (Ecotoxicology)	Primary	0.02 mg/L	HPLC with DAD	Arendarczyk, A., 2021 / Study code: G-07-21 and Study code: G-08-21
Elendt M7 medium (*Chironomus* sp.*)* (Ecotoxicology)	Primary	0.02 mg/L	HPLC with DAD	Czarnecka, M., 2021 / Study code: W-01-21
20X AAP medium (*Lemna gibba)* (Ecotoxicology)	Primary	0.01 µg/L	LC-MS/MS	Czarnecka, M., 2021 / Study code: W-04-21
Aqueous phase – Smart and Barko medium (*Myriophyllum spicatum)* (Ecotoxicology)	Primary	0.01 µg/L	LC-MS/MS	Turek-Lipka, T., 2021 / Study code: W-05-21

5.3 Methods for post-authorization control and monitoring purposes (KCP 5.2)

5.3.1 Analysis of the plant protection product (KCP 5.2)

The methods already submitted in accordance with the requirements set out in point 5.2.1 can be applied.

5.3.2 Description of analytical methods for the determination of residues of 2,4-D (KCP 5.2)

5.3.2.1 Overview of residue definitions and levels for which compliance is required

Compared to the residue definition proposed in the Draft Assessment Report (incl. its addenda) the current legal residue definition is identical.

Table 5.3-1: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Sum of 2,4-D, its salts, esters and conjugates expressed as 2,4-D	0.05 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Plant, high acid content		0.05 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Plant, high protein/high starch content (dry commodities)		0.05 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Plant, high oil content		0.05 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Plant, difficult matrices (hops, spices, tea)		0.1 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Muscle	Sum of 2,4-D, its salts, its esters and its conjugates expressed as 2,4-D	0.05 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Milk		0.01 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Eggs		0.01 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Fat		0.05 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)
Liver, kidney		0.05 mg/kg	MRL Reg. (EU) 2019/1791 (MRL Reg. (EU) 2022/1363 will apply from 25/02/2023)

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Soil (Ecotoxicology)	2,4-D	0.05 mg/kg	common limit
Drinking water (Human toxicology)	2,4-D	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	2,4-D	11 µg/L EC_{50} *Myriophyllum spicatum*	EFSA Journal 2014;12(9):3812
Air	2,4-D	6 µg/m^3 (AOEL sys = 0.02 mg/kg bw/day)	Calculated according to SANTE/2020/12830, Rev.1 24. February 2021
Tissue (meat or liver)	Not relevant	~~Not required~~	Not classified as T / T+
Body fluids		~~Not required~~	Not classified as T / T+

5.3.2.2 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of 2,4-D in plant matrices is given in the following tables. The methods include a hydrolysis step and are therefore considered adequate for the analysis of 2,4-D according to the residue definition. New studies were not provided.

Table 5.3-2: Validated methods for food and feed of plant origin (required for all matrix types, "difficult" matrix only when indicated by intended GAP)

Component of residue definition: Sum of 2,4-D, its salts, esters and conjugates expressed as 2,4-D				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
High water content	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013a / Report No. 130886 / EU agreed (RAR; Greece, 2014)
	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell, JT.; Li, Q, 2013a (Study number: 110357 / EU agreed (RAR; Greece, 2014)
	ILV	~~Not available, not required~~ 0.01 mg/kg	- HPLC-MS/MS	EFSA Journal 2014;12(9):3812
	Confirmatory (if required)	Not required	-	-
High acid content	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013a / Report No. 130886 / EU agreed (RAR; Greece, 2014)
	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell, JT.; Li, Q, 2013a (Study number: 110357 / EU agreed (RAR; Greece, 2014)
	ILV	Not available, not required	-	-

Component of residue definition: Sum of 2,4-D, its salts, esters and conjugates expressed as 2,4-D				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
	Confirmatory (if required)	Not required	-	-
High oil content	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013a / Report No. 130886 / EU agreed (RAR; Greece, 2014)
				Gesell, JT.; Li, Q, 2013a (Study number: 110357 / EU agreed (RAR; Greece, 2014)
	ILV	0.01 mg/kg	HPLC-MS/MS	Langridge, G, 2012 / Report No. CEMS-5229 / EU agreed (RAR; Greece, 2014)
				Bendler, S. E., 2013b / Report number: 130888 / EU agreed (RAR; Greece, 2014)
	Confirmatory (if required)	Not required	-	-
High protein/high starch content (dry)	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013a / Report No. 130886 / EU agreed (RAR; Greece, 2014)
				Gesell, JT.; Li, Q, 2013a (Study number: 110357 / EU agreed (RAR; Greece, 2014)
	ILV	0.01 mg/kg	HPLC-MS/MS	Langridge, G, 2012 / Report No. CEMS-5229 / EU agreed (RAR; Greece, 2014)
				Bendler, S. E., 2013b / Report number: 130888 / EU agreed (RAR; Greece, 2014)
	Confirmatory (if required)	Not required	-	-

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Renewal Assessment Report, Final Addendum, vol. 3, B.5.2.1
Not required, because:	-

5.3.2.3 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of 2,4-D in animal matrices is given in the following tables. New studies were not provided.

Table 5.3-4: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: Sum of 2,4-D, its salts, esters and conjugates expressed as 2,4-D				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Milk	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013b / Report No. 130887 / EU agreed (RAR; Greece, 2014)
				Gesell, J. T.; Li, Q., 2013b / Report number: 110468 / EU agreed (RAR; Greece, 2014)
	ILV	0.01 mg/kg	HPLC-MS/MS	Bendler S.E., 2013a / Report No. 205G584 / EU agreed (RAR; Greece, 2014)
				Garcia-Alix, M., 2012a / Report number: CEMS-5230, DAS Protocol No. 110763 / EU agreed (RAR; Greece, 2014)
	Confirmatory (if required)	Not required	-	-
Eggs	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013b / Report No. 130887 / EU agreed (RAR; Greece, 2014)
				Gesell, J. T.; Li, Q., 2013b / Report number: 110468 / EU agreed (RAR; Greece, 2014)
	ILV	0.01 mg/kg	HPLC-MS/MS	Bendler S.E., 2013a / Report No. 205G584 / EU agreed (RAR; Greece, 2014)
				Garcia-Alix, M., 2012a / Report number: CEMS-5230, DAS Protocol No. 110763 / EU agreed (RAR; Greece, 2014)
	Confirmatory (if required)	Not required	-	-
Muscle	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013b / Report No. 130887 / EU agreed (RAR; Greece, 2014)
				Gesell, J. T.; Li, Q., 2013b / Report number: 110468 / EU agreed (RAR; Greece, 2014)
	ILV	0.01 mg/kg	HPLC-MS/MS	Bendler S.E., 2013a / Report No. 205G584 / EU agreed (RAR; Greece, 2014)

Component of residue definition: Sum of 2,4-D, its salts, esters and conjugates expressed as 2,4-D				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
				Garcia-Alix, M., 2012a / Report number: CEMS-5230, DAS Protocol No. 110763 / EU agreed (RAR; Greece, 2014)
	Confirmatory (if required)	Not required	-	-
Fat	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013b / Report No. 130887 / EU agreed (RAR; Greece, 2014)
				Gesell, J. T.; Li, Q., 2013b / Report number: 110468 / EU agreed (RAR; Greece, 2014)
	ILV	0.01 mg/kg	HPLC-MS/MS	Bendler S.E., 2013a / Report No. 205G584 / EU agreed (RAR; Greece, 2014)
				Garcia-Alix, M., 2012a / Report number: CEMS-5230, DAS Protocol No. 110763 / EU agreed (RAR; Greece, 2014)
	Confirmatory (if required)	Not required	-	-
Kidney, liver	Primary	0.01 mg/kg	HPLC-MS/MS	Gesell J.T., Li Q., 2013b / Report No. 130887 / EU agreed (RAR; Greece, 2014)
				Gesell, J. T.; Li, Q., 2013b / Report number: 110468 / EU agreed (RAR; Greece, 2014)
	ILV	0.01 mg/kg	HPLC-MS/MS	Bendler S.E., 2013a / Report No. 205G584 / EU agreed (RAR; Greece, 2014)
				Garcia-Alix, M., 2012a / Report number: CEMS-5230, DAS Protocol No. 110763 / EU agreed (RAR; Greece, 2014)
	Confirmatory (if required)	Not required	-	-

Table 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	Renewal Assessment Report, Final Addendum, vol. 3, B.5.2.2
Not required, because:	-

5.3.2.4 Description of methods for the analysis of soil (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of 2,4-D in soil is given in the following tables. New studies were not provided.

Table 5.3-6: Validated methods for soil (if appropriate)

Component of residue definition: 2,4-D			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.05 mg/kg	HPLC-MS/MS and GC-MS (for 2,4-DCA)	Gesell J.T., 2012a / Report No. 110503 / EU agreed (RAR; Greece, 2014)
Confirmatory	Not required	-	-

5.3.2.5 Description of methods for the analysis of water (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of 2,4-D in surface and drinking water is given in the following tables. New studies were not provided.

Table 5.3-7: Validated methods for water (if appropriate)

Component of residue definition: 2,4-D				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	0.1 µg/L	HPLC-MS/MS and GCMS (for 2,4-DCA)	Gesell J.T., 2012b / Report No. 110504 / EU agreed (RAR; Greece, 2014)
	ILV	0.1 µg/L	HPLC-MS/MS and GCMS (for 2,4-DCA)	Garcia-Alix M., 2012b / Report No. CEMS-5324 / EU agreed (RAR; Greece, 2014)
	Confirmatory	Not required	-	-
Surface water	Primary	0.1 µg/L	HPLC-MS/MS and GCMS (for 2,4-DCA)	Gesell J.T., 2012b / Report No. 110504 / EU agreed (RAR; Greece, 2014)
	Confirmatory	Not required	-	-

5.3.2.6 Description of methods for the analysis of air (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of 2,4-D in air is given in the following tables. New studies were not provided.

Table 5.3-8: Validated methods for air (if appropriate)

Component of residue definition: 2,4-D			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	4.5 µg/m^3	HPLC-MS/MS	Class, T., 2011 / Report No. P 2166 G / EU agreed (RAR; Greece, 2014)
Confirmatory	Not required	-	-

5.3.2.7 Description of methods for the analysis of body fluids and tissues (KCP 5.2)

Analytical methods for body fluids and tissues are not necessary, because 2,4-D is not classified as toxic or very toxic.
However, the following method was provided and reviewed during the renewal of approval of 2,4-D. It is considered acceptable for the determination of 2,4-D in body fluids (urine or whole blood). New studies were not provided.

Table 5.3-9: Methods for body fluids and tissues (if appropriate)

Component of residue definition: 2,4-D			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.05 mg/kg / mg/L	HPLC-MS/MS	Senciuc M., 2011 / Report No. P 2167 G / EU agreed (RAR; Greece, 2014)
Confirmatory	Not required	-	-

5.3.2.8 Other studies/ information

No other studies are submitted within the framework of this application.

5.3.3 Description of analytical methods for the determination of residues iodosulfuron-methyl-sodium (KCP 5.2)

5.3.3.1 Overview of residue definitions and levels for which compliance is required

Compared to the residue definition proposed in the Draft Assessment Report (incl. its addenda) the current legal residue definition is identical.

Table 5.3-10: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Sum of iodosulfuron-methyl and its salts, expressed as iodosulfuron-methyl-sodium	0.01 mg/kg	MRL Reg. (EU) 289/2014
Plant, high acid content		0.01 mg/kg	MRL Reg. (EU) 289/2014
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	MRL Reg. (EU) 289/2014
Plant, high oil content		0.02 mg/kg	MRL Reg. (EU) 289/2014
Plant, difficult matrices (hops, spices, tea)		0.05 mg/kg	MRL Reg. (EU) 289/2014
Muscle	Not defined (EFSA, 2016)	0.02 mg/kg	MRL Reg. (EU) 289/2014
Milk		0.02 mg/kg	MRL Reg. (EU) 289/2014
Eggs		0.02 mg/kg	MRL Reg. (EU) 289/2014
Fat		0.02 mg/kg	MRL Reg. (EU) 289/2014
Liver, kidney		0.02 mg/kg	MRL Reg. (EU) 289/2014
Soil (Ecotoxicology)	Sum of iodosulfuron-methyl and its salts, expressed as iodosulfuron-methyl	0.021 µg/kg	EFSA Journal 2016;14(4):4453 NOEC < 0.032 g a.s./ha
Drinking water (Human toxicology)	Sum of iodosulfuron-methyl and its salts, expressed as iodosulfuron-methyl and metsulfuron-methyl (AE F075736)	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Sum of iodosulfuron-methyl and its salts, expressed as iodosulfuron-methyl and metsulfuron-methyl (AE F075736)	0.74 µg/L ErC_{50} (*Lemna gibba)*	EFSA Journal 2016;14(4):4453
Air	Sum of iodosulfuron-methyl and its salts, expressed as iodosulfuron-methyl	15 µg/m^3 (AOEL sys: 0.05 mg/kg bw/d)	Calculated according to SANTE/2020/12830, Rev.1 24. February 2021
Tissue (meat or liver)	Sum of iodosulfuron-methyl and its salts, expressed as iodosulfuron-methyl	Not rquired	Not classified as T / T+
Body fluids		Not rquired	Not classified as T / T+

5.3.3.2 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of iodosulfuron-methyl sodium in plant matrices is given in the following tables. New studies were not provided.

Table 5.3-11: Validated methods for food and feed of plant origin (required for all matrix types, "difficult" matrix only when indicated by intended GAP)

Component of residue definition: iodosulfuron-methyl-sodium				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
High water content	Primary	0.01 mg/kg	LC-MS/MS	Stuke, S., Ballmann, C., 2013 / Report No.: MR-13/007 / RAR, Sweden, 2015 / EU agreed
	ILV	0.01 mg/kg	LC-MS/MS	Konrad, S., 2013 / Report No.: 2013/0060/01 / RAR, Sweden, 2015 / EU agreed
	Confirmatory (if required)	Not required	-	-
High acid content	Primary	0.01 mg/kg	LC-MS/MS	Stuke, S., Ballmann, C., 2013 / Report No.: MR-13/007 / RAR, Sweden, 2015 / EU agreed
	ILV	0.01 mg/kg	LC-MS/MS	Konrad, S., 2013 / Report No.: 2013/0060/01 / RAR, Sweden, 2015 / EU agreed
	Confirmatory (if required)	Not required	-	-
High oil content	Primary	0.01 mg/kg	LC-MS/MS	Stuke, S., Ballmann, C., 2013 / Report No.: MR-13/007 / RAR, Sweden, 2015 / EU agreed
	ILV	0.01 mg/kg	LC-MS/MS	Konrad, S., 2013 / Report No.: 2013/0060/01 / RAR, Sweden, 2015 / EU agreed
	Confirmatory (if required)	Not required	-	-
High protein/high starch content (dry)	Primary	0.01 mg/kg	LC-MS/MS	Stuke, S., Ballmann, C., 2013 / Report No.: MR-13/007 / RAR, Sweden, 2015 / EU agreed
	ILV	0.01 mg/kg	LC-MS/MS	Konrad, S., 2013 / Report No.: 2013/0060/01 / RAR, Sweden, 2015 / EU agreed
	Primary	0.01 mg/kg	LC-MS/MS	Stuke, S., 2015 / RAR, Sweden, 2015
	ILV	Not required	-	-
	Confirmatory (if required)	Not required	-	-

Table 5.3-12: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Stuke, S., 2015, RAR, Sweden, 2015, EU agreed / EFSA 2016
Not required, because:	As residues are not expected to be ≥LOQ in cereal grain,

	Method for products of plant origin
	extraction efficiency is not required

5.3.3.3 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No methods for the analysis of residues in food and feed of animal origin were submitted. No residue definition is proposed since no residues in food and feed of animal origin are anticipated.

5.3.3.4 Description of methods for the analysis of soil (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of iodosulfuron-methyl sodium in soil is given in the following tables. New studies were not provided.

Table 5.3-13: Validated methods for soil (if appropriate)

Component of residue definition: iodosulfuron-methyl-sodium			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.01 µg/kg	HPLC-MS/MS	Freitag, T., 2013, / Report No. MR-08/138 / RAR, Sweden, 2015 / EU agreed
Confirmatory	Not required	-	-

5.3.3.5 Description of methods for the analysis of water (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of iodosulfuron-methyl sodium in surface and drinking water is given in the following tables. New studies were not provided.

Table 5.3-14: Validated methods for water (if appropriate)

Component of residue definition: iodosulfuron-methyl-sodium (and metabolite metsulfuron-methyl (AE F075736))				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	0.05 µg/L	HPLC-MS/MS	Krebber, R., Braune, M., 2013 / Report No.: MR-13/085 / RAR, Sweden, 2015 / EU agreed
	ILV	0.05 µg/L	DI-HPLC-MS/MS	Stanislowski, 2013 / Report No.: P3117 G / RAR, Sweden, 2015 / EU agreed
	Confirmatory	Not required	-	-
Surface water	Primary	0.05 µg/L	HPLC-MS/MS	Krebber, R., Braune, M., 2013 / Report No.: MR-13/085 / RAR, Sweden, 2015 / EU agreed

Component of residue definition: iodosulfuron-methyl-sodium (and metabolite metsulfuron-methyl (AE F075736))				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
	ILV	0.05 µg/L	DI-HPLC-MS/MS	Stanislowski, 2013 / Report No.: P3117 G / RAR, Sweden, 2015 / EU agreed
	Confirmatory	Not required	-	-

5.3.3.6 Description of methods for the analysis of air (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of iodosulfuron-methyl sodium in air is given in the following tables. New studies were not provided.

Table 5.3-15: Validated methods for air (if appropriate)

Component of residue definition: iodosulfuron-methyl-sodium			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	1.6 µg/m^3	HPLC-UV	Reichert, N., 2000 / Report No. F-100/21283-00 / Addendum to DAR 2001 / EU agreed
Confirmatory	1.05 µg/m^3	GC-ECD	Everitt, S. L., 1998 / Report No. C001382 / DAR 2000 / EU agreed

5.3.3.7 Description of methods for the analysis of body fluids and tissues (KCP 5.2)

Analytical methods for body fluids and tissues are not necessary, because iodosulfuron-methyl sodium is not classified as toxic or very toxic.

zRMS: Validated methods of analysis for body fluids and tissues are required (data gap; EFSA Journal 2016;14(4):4453).
This deficiency can be supplemented within re-evalution of the product..

5.3.3.8 Other studies/ information

Summary of validation of analytical methods used in dRR section 9 (Ecotoxicology) are provided in Appendix 2.

Appendix 1 Lists of data considered in support of the evaluation

Tables considered not relevant can be deleted as appropriate.

MS to blacken authors of vertebrate studies in the version made available to third parties/public.

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	Owner
KCP 5.1.1/01 5.1.1/02	Ciach J.	2021	JMD-HER 387 OD. Determination of active substrances content of preparation in an COEX bottle. Stage 1: Determination of active substrances content of initial preparation. Stage 2: Determination of physicochemical properties of the preparation stored at temperature 0±2°C for 7 days. Stage 3: Determination of active substrances content of preparation stored at temperature 40±2°C for 8 weeks. Stage 5: Determination of physicochemical properties of preparation stored at temperature 20±2°C for 2 years. Report No 001/DPL/2021 Pestila Spółka z ograniczoną odpowiedzialnością. GLP Yes Unpublished	N	Pestila*
5.1.1/03	Wołoszynowska M.	2021	Determination of physicochemical properties. Report No BA-05/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry GLP Yes Unpublished	N	Pestila*
5.1.1/04	Pstuś J.	2020	GC method for determination of dioxins and furans in JMD-HER 387 OD. Report No RVM/2022/48 Selvita Services Sp. z o.o. GLP: N Published: N	N	Pestila*

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	Owner
KCP 5.1.2/01	Włodarczyk M.	2021	Validation of analytical method for the determination of active substances of the test item JMD-HER 387 OD in 50% sucrose solution Study code: 0005/0099/FA SORBOLAB Research Laboratory LLC GLP Unpublished	N	Pestila*
KCP 5.1.2/02	Włodarczyk M.	2021	Validation of analytical method for the determination of active substances in aqueous solution of the test item JMD-HER 387 OD Study code: 0005/0102/FA SORBOLAB Research Laboratory LLC GLP Unpublished	N	Pestila*
KCP 5.1.2/03 (filed as KCP 10.4.1.1/01)	Arendarczyk A.	2021	JMD-HER 387 OD Earthworm reproduction test (*Eisenia andrei*) STUDY CODE: G-03-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Ecotoxicology Research Group GLP Unpublished	N	Pestila*
KCP 5.1.2/04 (filed as KCP 10.4.2.1/01)	Gierbuszewska A.	2021	JMD-HER 387 OD Collembolan *(Folsomia candida)* Reproduction Test Study code: G-04-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Ecotoxicology Research Group GLP Unpublished	N	Pestila*
KCP 5.1.2/05 (filed as KCP 10.2.1.3/02)	Czarnecka M.	2021	JMD-HER 387 OD *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*) Study code: W-03-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Ecotoxicology Research Group GLP Unpublished	N	Pestila*

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	Owner
KCP 5.1.2/06 (filed as KCP 10.6.2/02)	Arendarczyk A.	2021	JMD – HER 387 OD Terrestrial Plant Test: Vegetative Vigour Test Study code: G-07-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Ecotoxicology Research Group GLP Unpublished	N	Pestila*
KCP 5.1.2/07 (filed as KCP 10.2.1.2/03)	Czarnecka M.	2021	JMD – HER 387 OD *Chironomus* sp., Acute Immobilisation Test Study code: W-01-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Ecotoxicology Research Group GLP Unpublished	N	Pestila*
KCP 5.1.2/08 (filed as KCP 10.2.1.4/01)	Czarnecka M.	2021	JMD – HER 387 OD *Lemna gibba* CPCC 310, Growth inhibition test Study code: W-04-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Ecotoxicology Research Group GLP Unpublished	N	Pestila*
KCP 5.1.2/09 (filed as KCP 10.2.1.4/02)	Turek-Lipka T.	2021	JMD – HER 387 OD Water-sediment *Myriophyllum spicatum* toxicity test Study code: W-05-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Ecotoxicology Research Group GLP Unpublished	N	Pestila*

* Pestila Spółka z ograniczoną odpowiedzialnością.

List of data submitted or referred to by the applicant and relied on, but already evaluated at EU peer review

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
2,4-D					
KCP 5.2	Gesell J.T., Li Q.	2013a	Method validation study for the determination of residues of (2,4-dichlorophenoxy)acetic acid and its esters and conjugates in agricultural commodities using solid-phase extraction and liquid chromatography with tandem mass spectrometry detection. Dow AgroSciences LLC, Indianapolis, USA Report No. 130886 GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Gesell J.T., Li Q.	2013a	Revised Final Report – Method validation study for the determination of residues of (2,4-dichlorophenoxy)acetic acid in agricultural commodities using solid-phase extraction and liquid chromatography with tandem mass spectrometry detection. Dow AgroSciences LLC, Indianapolis, USA Report No. 110357 GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Bendler S.E.	2013b	Independent laboratory validation of an analytical method for the determination of 2,4-D and its esters in crop matrices. Report No. 205G585 EPL Bio Analytical Services GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Bendler S.E.	2013b	Independent laboratory validation of an analytical method for the determination of 2,4-D and its esters in crop matrices Dow AgroSciences LLC, Indianapolis, USA EPL Bio Analytical Services Report No. 205G585 DAS Protocol No. 130888 GLP Unpublished	N	EU 2,4-D Task Force

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2	Langridge G.	2012	Independent laboratory validation of an analytical method for the determination of (2,4-dichlorophenoxy)acetic acid in crops Report No. CEMS-5229 CEM Analytical Services, UK GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Gesell J.T., Li Q.	2013b	Method validation study for the determination of residues of (2,4-dichlorophenoxy)acetic acid and its esters in bovine and poultry tissues using solid-phase extraction and liquid chromatography with tandem mass spectrometry detection. Report No. 130887 Dow AgroSciences LLC, Indianapolis, USA GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Gesell J.T., Li Q.	2013b	Revised Final Report – Method validation study for the determination of residues of (2,4-dichlorophenoxy) acetic acid in bovine and poultry tissues using solid-phase extraction and liquid chromatography with tandem mass spectrometry detection. Dow AgroSciences LLC, Indianapolis, USA Report No. 110468 GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Bendler S.E.	2013a	Independent laboratory validation of an analytical method for the determination of 2,4-D and its esters in bovine and poultry tissues. Report No. 205G584 EPL Bio Analytical Services. GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Garcia-Alix, M	2012a	Independent laboratory validation of an analytical method for the determination of (2,4-dichlorophenoxy)acetic acid in animal matrices. CEMS Analytical Services, UK Study Code CEMS-5230	N	EU 2,4-D Task Force

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			DAS Protocol No. 110763 GLP Unpublished		
KCP 5.2	Gesell J.T.	2012a	Method validation study for the determination of residues of (2,4-dichlorophenoxy) acetic acid and its metabolites in soil. Report No. 110503 Dow AgroSciences LLC, Indianapolis, USA GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Gesell J.T.	2012b	Method validation study for the determination of residues of (2,4-dichlorophenoxy)acetic acid and its metabolites in surface water, ground water and drinking water. Report No. 110504 Dow AgroSciences LLC, Indianapolis, USA GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Garcia-Alix M.	2012	Independent laboratory validation of an analytical method for the determination of (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol, 4-chlorophenol and 2,4-dichloroanisole in water. Report No. CEMS-5324 CEM Analytical Services, UK GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Class T.	2011	2,4-D: Development and validation of an analytical method for the determination of 2,4-D in air. Report No. P 2166 G PTRL Europe GmbH, Germany GLP Unpublished	N	EU 2,4-D Task Force
KCP 5.2	Senciuc M.	2011	Development and validation of an analytical method for the determination of 2,4-D in body fluid(s). Report No. P 2167 G PTRL Europe GmbH, Germany GLP	N	EU 2,4-D Task Force

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Unpublished		
Iodosulfuron-methyl sodium					
KCP 5.2	Stuke S., Ballmann C.	2013	Analytical method 01360 for the determination of amidosulfuron, metsulfuron-methyl, iodosulfuron-methyl-sodium, mesosulfuron-methyl, and foramsulfuron in samples from plant origin by HPLC-MS/MS Bayer CropScience Report No.: MR-13/007 Edition Number: M-455564-01-1 GLP Unpublished	N	Bayer CropScience
KCP 5.2	Konrad S.	2013	Independent lab validation of BCS method 01360 for the determination of residues of amidosulfuron, metsulfuron-methyl, iodosulfuron-methyl-sodium, mesosulfuron-methyl and foramsulfuron in samples from plant origin by HPLC-MS/MS Currenta GmbH & Co. OHG, Leverkusen, Germany BCS Report No.: 2013/0060/01 Edition Number: M-470160-01-1 GLP Unpublished	N	Bayer CropScience
KCP 5.2	Stuke S.	2015	Cross validation of enforcement method 01360 for the determination of sulfonylureas vs. extraction procedure applied in ^{14}C-metabolism studies using incurred residues in plant matrices analysed by HPLC-MS/MS GLP Unpublished	N	Bayer CropScience
KCP 5.2	Freitag T.	2013	Amendment no. 0001 to report no.: MR-08/138 - Analytical Method 01115 for the determination of residues of amidosulfuron,iodosulfuron-methyl-sodium, metsulfuron-methyl, mesosulfuron-methyl and foramsulfuron in soil by HPLC-MS/MS Bayer CropScience Report No.: M-310074-03-1 Edition Number: M-310074-03-1 GLP	N	Bayer CropScience

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Unpublished		
KCP 5.2	Krebber R.; Braune M.	2013	Analytical method 01387 for the determination of various pesticides in drinking and surface water by HPLC-MS/MS Bayer CropScience Report No.: MR-13/085 Edition Number: M-466732-01-1 GLP Unpublished	N	Bayer CropScience
KCP 5.2	Stanislowski T.	2013	Independent laboratory validation of BCS analytical methods 01333 and 01387 for determination of various pesticides in surface water by Di-HPLC-MS/MS PTRL Europe, Ulm, Germany Bayer CropScience Report No.: P3117 G Edition Number: M-470714-02-1 GLP Unpublished	N	Bayer CropScience
KCP 5.2	Reichert N.	2000	Development and validation of an analytical method for the determination of iodosulfuron methyl sodium in air Institut Fresenius Chem.und Biolog. Lab. AG, Taunusstein, Germany Bayer CropScience Report No.: IF-100/21283-00. Edition Number: M-199299-02-1 GLP Unpublished	N	Bayer CropScience
KCP 5.2	Everitt S. L.	1998 Amended: 2000-03-13)	Validation and analytical method for the determination of AE F115008 in air AE F115008 active substance Code: AE F115008 Report No.: C001382 Doc No. M-181311-03-1 GLP Unpublished	N	Bayer CropScience

The following tables are to be completed by MS

List of data submitted by the applicant and not relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner

List of data relied on not submitted by the applicant but necessary for evaluation

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for 2,4-D

A 2.1.1 Methods used for the generation of pre-authorization data (KCP 5.1)

Please refer to the points 5.2.1.1 and 5.2.1.2.

A 2.1.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.1.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.7 A.2.A.9 Other Studies/ Information

A 2.1.2.7.1 HPLC UV-DAD (in 50% sucrose solution)

A 2.1.2.7.1.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/01
Report	Validation of analytical method for the determination of active substances of the test item JMD-HER 387 OD in 50% sucrose solution, Study code: 0005/0099/FA, Włodarczyk M., 2021
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	Yes. The limit of detection (LOD) was determined at a level higher than assumed in the Study Plan (30% of the LOQ value). The analytical method will be used to determine the concentrations of the active substances in solutions of the test item (ecotoxicological study) at a concentration level higher than the LOQ. Therefore, the deviation will not affect the results of determinations in the ecotoxicological study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Validation of analytical method for determination of active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD-HER 387 OD in 50% sucrose solution was conducted. During the validation of the analytical method the following parameters: selectivity, linearity, accuracy, precision (repeatability), limit of detection and limit of quantification were determined.
Determination of test item was performed by high-performance liquid chromatography with UV-DAD diode detection based on the signals originating from the active substances

Test item

Name	JMD-HER 387 OD
Test material description	brown liquid
Batch No	JMD/01/2021
Active substances	2,4-D: 250 g/L in the form of 2,4-D 2 EHE: 377 g/L iodosulfuron methyl sodium: 10 g/L
CAS of active substances	2,4-D: 94-75-7 in the form of 2,4-D 2 EHE: 1928-43-4 iodosulfuron methyl sodium: 144550-36-7
Date of production	02.2021
Expiry date	02.2023
Storage conditions temperature	20±5°C

Reagents

- acetonitrile, HPLC grade, POCH lot number 1076/11/20
- orthophosphoric acid 85% p.a, Chempur, lot number 19/04/08
- sodium hydroxide, p.a, Chempur, lot number 19/12/44
- isopropanol, HPLC grade, Scharlau, lot number 20730109
- methanol, HPLC grade, Avantor, lot number 1225/07/20
- deionized water
- ultrapure water
- hydrolysis solution (obtained by weighing 1.00 g of sodium hydroxide into a 100 mL flask, adding 30 mL of deionized water and filled up to the mark with isopropanol)
- 50% sucrose solution prepared by the Laboratory of Ecotoxicology
- 2,4-D standard, IPO Warszawa, lot number 2E/17

- iodosulfuron methyl sodium standard, CPA Chem, lot number 735806

Equipment

- high performance liquid chromatography Shimadzu Prominence series LC-20 with PDA detector
- deionizer SolPure 78
- volumetric flask class A
- ultrasonic washer Sonic-10
- adjustable automatic pipettes: Transferpette S 1 mL, Acura manual 826 XS
- stopwatch ISOLAB
- system for obtaining ultrapure water Millipore Synergy UV
- analytical balance Radwag XA 82_220.4Y.A and Mettler AT201
- precision balance Ohaus series PA 2102CM-1
- roller shaker RM10V-W
- syringes and syringe filters 0.22 μm

Chromatographic conditions

- Column — Kinetex C18 5 μm 100 Å 150 x 4.60 mm
- Detection — 225 nm
- Mobile phase — acetonitrile : 0.1% (v/v) H3PO4 (45:55)
- Injection volume — 100 μL
- Column thermostat temperature — 40°C
- Flow of mobile phase — 1.3 mL/min

Preparation of 2,4-D standard solution

32.29 mg of 2,4-D standard was weighed into a 10 mL graduated flask. The flask was filled up to the mark with methanol and whole was mixed thoroughly. A 2,4-D standard solution with concentration of 3219.313 mg/L (purity of standard 99.7%) was obtained. The prepared solution was diluted with deionized water.

Preparation of iodosulfuron methyl sodium standard solution

24.88 mg of iodosulfuron methyl sodium standard was weighed into a 10 mL graduated flask. The flask was filled up to the mark with methanol and whole was mixed thoroughly. A iodosulfuron methyl sodium standard solution with concentration of 2485.512 mg/L (purity of standard 99.9%) was obtained. The prepared solution was diluted with deionized water.

Preparation of standards solution in 50% sucrose solution

Appropriate volumes of standards prepared according to section 4.4. and 4.5. were collected into the falcon. Falcon was filled up with 50% sucrose solution to the weight of about 30 g.
The concentration of standards in 50% sucrose solution was calculated according to the formula:

$$C_{mg/kg} = \frac{C_{mg/L} * V}{m_r}$$

where:

- $C_{mg/kg}$ concentration of standard in 50% sucrose solution [mg/kg]
- $C_{mg/L}$ concentration of aqueous solution of standard [mg/L]
- V volume of standard taken for dilution with 50% sucrose solution [mL]
- m_r mass of prepared standard solution in 50% sucrose solution [g]

Preparation of test item solution

52.96 mg of test item was weighed into a falcon, and then 50% sucrose solution was added to weight 40.02790 g. A test item solution in 50% sucrose solution at concentration 1323.07715 mg/kg was obtained. The prepared solution was diluted with 50% sucrose solution.
Concentration of active substances in solution calculated according to formula:

$$C_{SA} = \frac{C_{BM} * C\%}{100\%}$$

where:

C_{SA}	concentration of active substance [mg/kg]
C_{BM}	concentration of test item in 50% sucrose solution [mg/kg]
C%	percentage content of active substance in the test item [% (w/w)]

Preparation of samples for analysis

5 mL of solution in 50% sucrose solution were taken into a 10 mL volumetric flask. The flask was filled up to the mark with deionized water and the whole was mixed thoroughly. 8 mL of the prepared solution were taken into a test tube and 1.95 mL of hydrolysis solution was added. The whole was shaken on a roller shaker for 60 minutes, and then 0.05 mL of orthophosphoric acid was added. The solution prepared in this way was filtered through a syringe filter into a chromatographic vial.

Selectivity

For selectivity analysis, the following analysis of samples were performed: mobile phase, 2,4-D standard solution (at the LOD level), iodosulfuron methyl sodium standard (at the LOD level), test item solution (at LOQ the LOQ level) and 50% sucrose solution. It was confirmed that in the analysis condition in the place of the peak originating from the standards of active substances, there are no peaks originating from other substances with an area exceeding 30% of the area of active substances in the test item solution (at the LOQ level).

Matrix effect

Due to the preparation of standard solutions of the active substance in the same matrix as the samples of the test item (50% sucrose solution), determination of the matrix effect in accordance with the SANTE/2020/12830 guideline, rev.1. was not performed. By preparing the samples for linearity analysis in the same matrix as the test item samples, the matrix effect was compensated.

Linearity

The standards solution in 50% sucrose solution was used to determine the linearity of the method. By serial dilution with 50% sucrose solution, solutions of 2,4-D at concentration: 0.33188 mg/kg; 1.90126 mg/kg and 7.48031 mg/kg and iodosulfuron methyl sodium solutions at concentration: 0.01391 mg/kg; 0.07969 mg/kg and 0.31351 mg/kg were obtained. Next, the samples, each in duplicate, were prepared.
After analysis graph dependence of peaks area from the active substance to standard concentration was plotted and linear correlation coefficient was determined. Functions were linear in full range.
Calibration curves are described by equations:

for 2,4-D:

$$f(x) = 83062.3*x+61.5419$$

for iodosulfuron methyl sodium:

$$f(x) = 94482.3*x+3236.86$$

where:

f(x)	chromatographic peak area
x	concentration of active substance [mg/kg]

For each concentration level, the regression residuals (di) were determined and the dependence of the regression residuals on the level of the calibration curve was plotted separately for each active substance.
Regression residuals were calculated according to the formula:

$$d_i = y_i - yy_i$$

where:

d_i	regression residuals at the level i
y_i	measured value at level i [mg/kg]

yy_i theoretical value at level i [mg/kg]
i level of the calibration curve

Correlation coefficients were equal:
r = 0.999 - for 2,4-D
r = 0.999 - for iodosulfuron methyl sodium.

Criterion of acceptance $r \geq 0.99$ was fulfilled.

A randomly distribution of regression residues (di) were obtained for both active substances.
Range of linearity should ensure the possibility of active substances concentrations determination in the 20% range above the highest nominal active substances concentrations in the analysed samples.

Accuracy
To determine the accuracy of the method, determination of 50% sucrose solution in duplicate and test item solution in 50% sucrose solution at two concentration levels approximate to the lowest and highest point of the calibration curve (in five replicates at each concentration level) were performed.
After determination, recovery (accuracy) of method was calculated by comparing determination results with theoretical value of concentration of active substance in solution according to equation:

$$\text{Recovery} = \frac{C_{ozn}}{C_{teo}} * 100\%$$

where:

Recovery	recovery of analytical method [%]
C_{ozn}	determined concentration of active substance [mg/kg]
C_{teo}	theoretical concentration of active substance [mg/kg]

Theoretical concentration of active substance was calculated according to formula:

$$C_{teo} = \frac{C_{BM} * C\%}{100\%}$$

where:

C_{teo}	theoretical concentration of active substance [mg/kg]
C_{BM}	concentration of the test item in 50% sucrose solution [mg/kg]
C%	percentage of active substance in the test item [% (w/w)]

Accuracy of method equal:
- 85% (level I - 94.6%, level II - 74.9%) for 2,4-D
- 102% (level I - 94.5%, level II - 109.3%) for iodosulfuron methyl sodium.

Precision
To precision determination, samples which prepared in framework of accuracy determination were used.
Precision (repeatability) was calculated by determine the value of the relative standard deviation (RSD [%]) for each of concentration level according to equation:

$$\text{RSD} = 100 \frac{s}{x}$$

where:

s	standard deviation of repeatability [mg/kg]
x	arithmetic mean of the obtained results [mg/kg]

100 units conversion factor

In the method used, precision in the analysed concentration levels were not exceed the value of RSD [%] ≤ 20%.
Outliers of the precision analysis results were identified using the Dixon test.

Limit of detection and limit of quantification
The limit of detection (LOD) and the limit of quantification (LOQ) were determined when determining the linearity and accuracy of the method.
The limit of quantification is the average value of the concentration of the active substance in solutions of the test item at the lower level of the accuracy analysis.
The limit of detection is the lowest point on the calibration curve.

Limit of detection (LOD) equal:
0.33188 mg/kg - for 2,4-D
0.01391 mg/kg - for iodosulfuron methyl sodium.

Limit of quantification (LOQ) equal:
0.50840 mg/kg - for 2,4-D
0.02083 mg/kg - for iodosulfuron methyl sodium.

Results and discussions

Parameter	Required criterion	The result			
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)			
Linearity	r ≥ 0.99 Random distribution of regression residuals di	2,4-D: r = 0.999 (0.33188 mg/kg – 7.48031 mg/kg) iodosulfuron methyl sodium: r = 0.999 (0.01391 mg/kg – 0.31351 mg/kg) For both active substances, a random distribution of regression residues di was obtained			
Accuracy [%]	70-120	2,4-D	level I	94.6	85
			level II	74.9	
		iodosulfuron methyl sodium	level I	94.5	102
			level II	109.3	
Precision [% RSDr]	≤ 20	2,4-D	level I	1.07	
			level II	0.14	
		iodosulfuron methyl sodium	level I	4.86	
			level II	0.94	
Limit of detection LOD [mg/kg]	-	2,4-D		0.33188	
		iodosulfuron methyl sodium		0.01391	
Limit of quantification LOQ [mg/kg]	-	2,4-D		0.50840	
		iodosulfuron methyl sodium		0.02083	

Results of the validation of analytical method was confirmed that this method is suitable for analysis the

content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in 50% sucrose solution.

A 2.1.2.7.2 HPLC UV-DAD (in aqueous solution)

A 2.1.2.7.2.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/02
Report	Validation of analytical method for the determination of active substances in aqueous solution of the test item JMD-HER 387 OD, Study code: 0005/0102/FA, Włodarczyk M., 2021
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	Yes. The limit of detection (LOD) was determined at a level higher than assumed in the Study Plan (30% of the LOQ value). The analytical method will be used to determine the concentrations of the active substances in solutions of the test item (ecotoxicological study) at a concentration level higher than the LOQ. Therefore, the deviation will not affect the results of determinations in the ecotoxicological study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Validation of analytical method for determination of active substances (2,4-D and iodosulfuron methyl sodium) in aqueous solution of the test item JMD-HER 387 OD was conducted. During the validation of the analytical method the following parameters: selectivity, matrix effects, linearity, accuracy, precision (repeatability), limit of detection and limit of quantification were determined.
Determination of active substances (2,4-D and iodosulfuron methyl sodium) in aqueous solution of the test item was performed by high performance liquid chromatography with UV-DAD detection on the basis of signals from active substances. Identification of active substances was made by comparing the UV spectrums and retention times of standards solutions and solution of the test item.

Test item

Name	JMD-HER 387 OD
Test material description	brown liquid
Batch No	JMD/01/2021
Active substances	2,4-D: 250 g/L in the form of 2,4-D 2 EHE: 377 g/L iodosulfuron methyl sodium: 10 g/L
CAS of active substances	2,4-D: 94-75-7 in the form of 2,4-D 2 EHE: 1928-43-4 iodosulfuron methyl sodium: 144550-36-7
Date of production	02.2021
Expiry date	02.2023
Storage conditions temperature	20±5°C

Reagents

- acetonitrile, HPLC grade, POCH lot number 1315/06/20
- orthophosphoric acid 85% p.a, Chempur, lot number 19/04/08

- sodium hydroxide, p.a, Chempur, lot number 19/12/44
- isopropanol, HPLC grade, Scharlau, lot number 18592509
- methanol, HPLC grade, Avantor, lot number 1225/07/20
- deionized water
- ultrapure water
- hydrolysis solution (obtained by weighing 1.00 g of sodium hydroxide into a 100 mL flask, adding 30 mL of deionized water and filled up to the mark with isopropanol)
- 2,4-D standard, IPO Warszawa, lot number 2E/17
- iodosulfuron methyl sodium standard, CPA Chem, lot number 735806

Equipment

- high performance liquid chromatography Shimadzu nexera X2 series LC-30 with PDA detector
- deionizer SolPure 78
- volumetric flask class A
- ultrasonic washer Sonic-10
- adjustable automatic pipettes: Transferpette S 1 mL, Transferpette S 5 mL, Transferpette S 10 mL, Transferpette S 10 µL, Transferpette S 200 µL
- stopwatch ISOLAB
- system for obtaining ultrapure water Millipore Synergy UV
- analytical balance Radwag XA 82_220.4Y.A and Mettler AT201
- precision balance Ohaus series PA 2102CM-1
- roller shaker RM10V-W
- syringes and syringe filters 0.22 µm

Chromatographic conditions

Column	Kinetex C18 5 µm 100 Å 150 x 4.60 mm
Detection	225 nm
Mobile phase	acetonitrile : 0.1% (v/v) H3PO4 (45:55)
Injection volume	50 µL
Column thermostat temperature	30°C
Flow of mobile phase	1.2 mL/min

Preparation of 2,4-D standard solution

32.66 mg of 2,4-D standard was weighed into a 10 mL graduated flask. The flask was filled up to the mark with methanol and whole was mixed thoroughly. A 2,4-D standard solution with concentration of 3256.202 mg/L (purity of standard 99.7%) was obtained. The prepared solution was diluted with deionized water.

Preparation of iodosulfuron methyl sodium standard solution

24.88 mg of iodosulfuron methyl sodium standard was weighed into a 10 mL graduated flask. The flask was filled up to the mark with methanol and whole was mixed thoroughly. A iodosulfuron methyl sodium standard solution with concentration of 2485.512 mg/L (purity of standard 99.9%) was obtained. The prepared solution was diluted with deionized water.

Preparation of test item solution

100.88 mg of test item was weighed into a 10 mL graduated flask. The flask was filled up to the mark with deionized water and whole was mixed thoroughly. A test item solution with concentration of 10088 mg/L was obtained. The prepared solution was diluted with deionized water.

Preparation of samples for analysis

7 mL of the sample for analysis was taken into a test tube, and then 1.95 mL of hydrolysis solution was added. Whole was shaken on a roller shaker for 60 minutes. After the hydrolysis step, 0.05 mL of orthophosphoric acid was added to the test tube, the whole was mixed and filtered through a syringe filter into a chromatography vial.

Concentrations of active substances in the prepared sample of the test item were calculated according to

the formula:

$$C_{SA} = \frac{C_{mg/L} * 7}{9}$$

where:

C_{SA} concentration of the active substance in the test item solution after sample preparation [mg/L]

$C_{mg/L}$ concentration of the active substance in the solution of the test item subjected to the hydrolysis [mg/L]

7 and 9 values resulting from sample preparation [mL].

Selectivity

For selectivity analysis, the following analysis of samples were performed: mobile phase, methanol, deionized water, 2,4-D standard solution (at the LOD level), iodosulfuron methyl sodium standard solution (at the LOD level), test item solution (at the LOQ level) and deionized water. It was confirmed that in the analysis condition in the place of the peak originating from the standards of active substances, there are no peaks originating from other substances with an area exceeding 30% of the area of active substances in the test item solution (at the LOQ level).
Comparison of UV spectrum of standard solution and test item solution is comparable.

Matrix effect

In order to check the matrix effects, solution of the test item and solution of the standards of active substances (2,4-D and iodosulfuron methyl sodium) in deionized water were prepared, so as to the concentrations of active substances in the prepared solutions were comparable.
To calculate the matrix effects, the peak areas corresponding to the active substances contained in the test item solution and solution of standards in deionized water were used.
The matrix effect was calculated from the formula:

$$\text{Matrix effect [\%]} = \frac{100 * P_{BM}}{P_{Wz}} - 100$$

where:

P_{BM} the peak area of the active substance in the test solution

P_{Wz} the peak area of the active substance in standard solution

Matrix effect equal:

- 0.01% - for 2,4-D
- -13.43% - for iodosulfuron methyl sodium

Criterion of acceptance was fulfilled: matrix effect does not exceed ±20%.

Linearity

2,4-D standard solution prepared according to point 4.4. and iodosulfuron methyl sodium standard solution were used to determine the linearity of the method. By serial dilution with deionized water solutions of 2,4-D at concentration: 0.24422 mg/L; 1.22108 mg/L and 7.57067 mg/L (each in duplicate) and solutions of iodosulfuron methyl sodium at concentration: 0.01243 mg/L; 0.07457 mg/L and 0.32312 mg/L were prepared.
After analysis graph dependence of peaks area from the active substance to standard concentration was plotted and linear correlation coefficient was determined. Functions were linear in full range.
Calibration curves are described by equations:

for 2,4-D:

$$f(x) = 86382.3*x+2190.03$$

for iodosulfuron methyl sodium:

$$f(x) = 138182 * x + 343.439$$

where:

f(x)	chromatographic peak area
x	concentration of active substance [mg/kg]

For each concentration level, the regression residuals (di) were determined and the dependence of the regression residuals on the level of the calibration curve was plotted separately for each active substance. Regression residuals were calculated according to the formula:

$$d_i = y_i - yy_i$$

where:

d_i	regression residuals at the level i
y_i	measured value at level i [mg/L]
yy_i	theoretical value at level i [mg/L]
i	level of the calibration curve

Correlation coefficients were equal:
r = 0.999 - for 2,4-D
r = 0.999 - for iodosulfuron methyl sodium.

Criterion of acceptance $r \geq 0.99$ was fulfilled.

A randomly distribution of regression residues (di) were obtained for both active substances.
Range of linearity should ensure the possibility of active substances concentrations determination in the 20% range above the highest nominal active substances concentrations in the analysed samples.

Accuracy
To determine the accuracy of the method, determination of deionized water prepared according to point 4.7 in duplicate and solutions of the test item in deionized wate rat two concentration levels approximate to the lowest and highest point of the calibration curve (in five replicates at each concentration level) were performed.
After determination, recovery (accuracy) of method was calculated by comparing determination results with theoretical value of concentration of active substance in solution according to equation:

$$\text{Recovery} = \frac{C_{ozn}}{C_{teo}} * 100\%$$

where:

Recovery	recovery of analytical method [%]
C_{ozn}	determined concentration of active substance [mg/L]
C_{teo}	theoretical concentration of active substance [mg/L]
100	unit conversion factor

Theoretical concentration of active substance was calculated according to equation:

$$C_{teo} = \frac{C_{BM} * \%SA * 7}{100\% * 9}$$

where:

C_{teo}	theoretical concentration of active substance [mg/L]
C_{BM}	concentration of the test item [mg/L]
%SA	percentage of active substance in the test item [% (w/w)]
7 and 9	values resulting from sample preparation [mL].

Accuracy of method equal:
- 93% (level I - 87.5%, level II - 99.3%) - for 2,4-D
- 89% (level I - 94.3%, level II - 83.6%) - for iodosulfuron methyl sodium.

Precision
To precision determination, samples which prepared in framework of accuracy determination were used. Precision (repeatability) was calculated by determine the value of the relative standard deviation (RSD [%]) for each of concentration level according to equation:

$$RSD = 100 \frac{s}{x}$$

where:

s	standard deviation of repeatability [mg/L]
x	arithmetic mean of the obtained results [mg/L]
100	units conversion factor

In the method used, precision in the analysed concentration levels were not exceed the value of RSD [%] ≤ 20%.
Outliers of the precision analysis results were identified using the Dixon test.

Limit of detection and limit of quantification
The limit of detection (LOD) and the limit of quantification (LOQ) were determined when determining the linearity and accuracy of the method.
The limit of quantification is the average value of the concentration of the active substance in solutions of the test item at the lower level of the accuracy analysis.
The limit of detection is the lowest point on the calibration curve.

Limit of detection (LOD) equal:
0.24422 mg/L - for 2,4-D
0.01243 mg/L - for iodosulfuron methyl sodium.

Limit of quantification (LOQ) equal:
0.40587 mg/L - for 2,4-D
0.01795 mg/L - for iodosulfuron methyl sodium.

Results and discussions

Parameter	Required criterion	The result	
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ) the UV spectrum of active substances in the standard solution and the test item solution is comparable	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ) the UV spectrum of active substances in the standard solution and the test item solution is comparable	
Matrix effect [%]	±20%	2,4-D	0.01
		iodosulfuron methyl sodium	-13.43

Linearity	r ≥ 0.99	2,4-D: r = 0.999 (0.24422 mg/L – 7.57067 mg/L) iodosulfuron methyl sodium: r = 0.999 (0.01243 mg/L – 0.32312 mg/L)			
Accuracy [%]	70-120	2,4-D	level I	87.5	93
			level II	99.3	
		iodosulfuron methyl sodium	level I	94.3	89
			level II	83.6	
Precision [% RSDr]	≤ 20	2,4-D	level I	0.21	
			level II	0.19	
		iodosulfuron methyl sodium	level I	12.09	
			level II	1.41	
Limit of detection LOD [mg/L]	-	2,4-D		0.24422	
		iodosulfuron methyl sodium		0.01243	
Limit of quantification LOQ [mg/L]	-	2,4-D		0.40587	
		iodosulfuron methyl sodium		0.01795	

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) in aqueous solution of the test item JMD-HER 387 OD.

A 2.1.2.7.3 GC with ECD and HPLC with DAD (in artificial soil)

A 2.1.2.7.3.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/03 (filed as KCP 10.4.1.1/01)
Report	JMD-HER 387 OD Earthworm reproduction test (*Eisenia andrei*), Study code: G-03-21, Arendarczyk, A., 2021
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Development and validation analytical method of 2,4-D 2-EHE (GC with ECD)

Materials and methods

The analytical method was developed for the determination of 2,4-D 2-EHE in artificial soil. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of 2,4-D 2-EHE were determined.
The determination was accomplished by the high performance gas chromatography (GC) with ECD detection. Prior to analysis, the samples were concentrated using solid-liquid extraction.

Reagents, solvents and chemicals

- Ethyl acetate, pure p.a., POCH, batch no. 1135/06/20
- Acetone, pure p.a., POCH, batch no. 1062/06/20
- Anhydrous sodium sulphate, pure p.a., J.T.Baker, batch no. 1831301832
- standard solution of 1 mg/mL of 2,4-D 2-EHE in acetone
- working solutions of 2,4-D 2-EHE at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 100.0 µg/mL in acetone

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Balance, WPS 510/C, ZMP RADWAG (Poland)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Bruker 450-GC, BRUKER

Chromatographic conditions

- Chromatographic System: Gas Chromatography (GC, Bruker 450-GC)
- Detector: Electron Capture Detector (ECD)
- Analitycal column: VF 5 ms 30M×0.25 MM ID DF=0.25
- Oven temperature: 200°C (2 minute); gradient 30°C/minute; 300°C (2 minute)
- Intel temperature: 260°C
- Detector temperature: 300°C
- Injection volume: 1 µl

Sample preparation for the chromatographic analysis

Artificial soil

First, 15 mL of ethyl acetate was added to 10 g of artificial soil sample and shaken for 30 minutes. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated with 15 mL of ethyl acetate. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 40°C. The dry residue was dissolved in 10 mL of acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

Fortified Sample

For validation experiments, 10 g of untreated artificial soil were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Sample Type	Sample Weight [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	10	-	-	0.00
Fortification (LOQ)	10	10	0.05	0.05
Fortification (10x LOQ)	10	100	0.05	0.50

This was done to ensure the result fits within the range of the respective standard curve.
Sample of water an untreated (10 g) was spiked with the 2,4-D 2-EHE to achieve fortification levels at the limit of quantification of 0.05 mg/kg and ten times higher i.e. 0.5 mg/kg.

Selectivity and specificity

The analytical method specificity was estimated on the basic of the analysis of the chromatograms ob-

tained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix blank sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.05 µg/mL prepared in solvent to at one at concentration 0.05 µg/mL prepared in blank matrix, for sample of artificial soil.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

Matrix effects [%] = 100 × peak area (matrix) / peak area (solvent) – 100

Matrix effect is 5.2% and not exceed ±20%.

Linearity
The stock solution with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard into a volumetric flask with a capacity of 10 mL, dissolving in acetone, and next the volume was made up to 10 ml with the same solvent. The working solution with a concentration of 100 µg/mL was prepared by dilution of the stock solution with acetone. Calibration and fortification solutions containing of 2,4-D 2-EHE were prepared by dilution of the working solution at concentration 2, 5, 10 and 100 µg/mL in acetone. Further dilutions were conducted with acetone as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 (Stock)	1	10	100
100	1	10	10
100	0.5	10	5
10	2	10	2
10	1	10	1.0[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The standard curve
Working solutions of 2,4-D 2-EHE at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 1.0 µg/mL. The range of calibration curve is equivalent to range from 0.01 mg 2,4-D 2-EHE/kg to 1.0 mg 2,4-D 2-EHE/kg in artificial soil.

The equation of the calibration line was presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear range was given, µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
2,4-D 2-EHE	7205.40031	2.06334	0.9992

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recov-

ery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery ranged for artificial soil is from 92.0 ± 6.8 % to 100.0 ± 1.8 %. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data for 2,4-D 2-EHE – validation method in artificial soil

Matrix	Fortification Level [mg/kg]	Number of Replicates	Mean Recovery [%]	RSD [%]
Artificial soil	0.05	5	92.0	6.8
	0.5	5	100.0	1.8

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the artificial soil is from 1.8% to 6.8%.
The precision is 6.8% at 0.05 mg 2,4-D 2-EHE/kg artificial soil level and 1.8% at 0.5 mg 2,4-D 2-EHE/kg artificial soil level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample. The LoD is 0.01 mg 2,4-D 2-EHE/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.01 µg 2,4-D 2-EHE /mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). The LoQ is 0.05 mg 2,4-D 2-EHE/kg artificial soil and equivalent to the calibration level at concentration 0.05 µg 2,4-D 2-EHE/mL.

Development and validation analytical method of iodosulfuron-methyl sodium and 2,4-D acid (HPLC with DAD

Materials and methods

The analytical method was developed for the determination of iodosulfuron-methyl sodium and 2,4-D acid in artificial soil. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of iodosulfuron-methyl sodium and 2,4-D acid were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. Prior to analysis, the samples were concentrated by solid - liquid extraction.

Reagents, solvents and chemicals
- Water, deionized, Fresh prepared befor analysis
- Acetonitrile, HPLC, J.T.Baker, batch no. 1728501868
- Ortho-phosphoric acid, 85% pure p.a., POCH, batch no. 1077/05/17
- Acetic acid, 99.5%-99.9% pure p.a., POCH, batch no. 1356/09/16
- 0.05% ortho-phosphoric acid solution in deionized artificial soil,
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- mixture of acetonitrile HPLC and deionized water and acetic acid (400:100:3, v/v/v),
- standard solution of 1 mg/mL of iodosulfuron-methyl sodium in acetonitrile for HPLC,
- standard solution of 1 mg/mL of 2,4-D acid in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl sodium at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl sodium at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- working solutions of 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of 2,4-D acid at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of

acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- working solutions of iodosulfuron-methyl sodium and 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl sodium and 2,4-D acid at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v).

Equipment
- Balance, WPS 510/C, ZMP RADWAG (Poland)
- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Laboratory centrifuge, MPW-351e, MPW MED.INSTRUMENTS (Poland)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)

Chromatographic conditions
- Chromatographic System: High Performance Liquid Chromatography (HPLC); Shimadzu, Prominence (Shimadzu Corporation Japan)
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid (45 : 55, v/v)
- Analytical column: Agilent Eclipse 5µm XDB-C8, l = 150 mm, ϕ = 4.6 mm
- Oven temperature: 35°C
- Wavelength: 220 nm
- Flow Rate: 0.6 mL/min
- Injection volume: 30 µl
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis
Artificial soil
First, 10 mL of mixture of acetonitrile HPLC and deionized water and acetic acid (400:100:3, v/v/v), was added to 10 g of artificial soil sample and shaken for 6 minutes by using hand. The organic phases were centrifuged and filtered through paper filter. The extraction was repeated with 10 mL of mixture of acetonitrile HPLC and deionized water and acetic acid (400:100:3, v/v/v).

Fortified Sample
For validation experiments, 10 g of untreated artificial soil were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Active substance	Sample Type	Sample Weight [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
iodosulfuron methyl sodium	Control	10	-	-	0.00
	Fortification (LOQ)	10	10	0.2	0.20
	Fortification (10x LOQ)	10	100	0.2	2.00
2,4-D acid	Control	10	-	-	0.00
	Fortification (LOQ)	10	10	0.2	0.20
	Fortification (10x LOQ)	10	100	0.2	2.00

This was done to ensure the result fits within the range of the respective standard curve.
Sample of artificial soil an untreated (10 g) was spiked with the solution of iodosulfuron-methyl sodium

and 2,4-D acid to achieve fortification levels at the limit of quantification of 0.2 mg/kg and ten times higher i.e. 2.0 mg/kg.

Selectivity and specificity
The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substances was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.2 µg iodosulfuron methyl sodium and 2,4-D acid/mL prepared in solvent to at one at concentration 0.2 µg iodosulfuron-methyl sodium and 2,4-D acid/mL prepared in blank matrix, for sample of artificial soil.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

Matrix effect for iodosulfuron-methyl sodium is -7.4% and not exceed ±20%.
Matrix effect for 2,4-D is -3.9% and not exceed ±20%.

Linearity
The stock solutions of iodosulfuron-methyl sodium and 2,4-D acid with a concentration of 1 mg/mL was prepared separately by weighting 10 mg analytical standards of iodosulfuron-methyl sodium or 2,4-D acid into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 ml with the same solvent. The working solutions of iodosulfuron methyl sodium and 2,4-D with a concentration of 100 µg/mL were prepared common and separately by dilution of the stock solutions with acetonitrile for HPLC. Moreover, the working solution of iodosulfuron methyl sodium and 2,4-D with a concentration of 10 µg/mL were prepared separately by dilution of the working solutions at concentration of 100 µg/mL with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of iodosulfuron methyl sodium and 2,4-D acid were prepared by dilution of the common working solution at concentration 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
100	2	10	20[1)]
100	1	10	10[1)]
100	0.5	10	5[1)]
20	1	10	2[1)]
10	1	10	1[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The first standard curve
Working solutions of iodosulfuron-methyl sodium at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.02 mg iodosulfuron-methyl sodium/kg to 4.0 mg

iodosulfuron-methyl sodium/kg in artificial soil.

Working solutions of 2,4-D acid at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.05 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.1 mg 2,4-D acid/kg to 4.0 mg 2,4-D acid /kg in artificial soil.
The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear range was given, µg/mL.

Analyte	Slope	Intercept	Correlation coefficient
iodosulfuron methyl sodium	183188	264.105	0.9999221
2,4-D acid	114953	166.759	0.9998362

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

The second standard curve
Working solutions of iodosulfuron-methyl sodium and 2,4-D acid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg /mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph range from 0.2 µg/mL to 20.0 µg/mL. The range of calibration curve is equivalent to range from 0.4 mg iodosulfuron-methyl sodium and 2,4-D acid/kg to 40.0 mg iodosulfuron-methyl sodium and 2,4-D acid/kg in artificial soil.

The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear range was given, µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
iodosulfuron methyl sodium	186125	176.781	0.9999933
2,4-D acid	113583	1412.94	0.9998158

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery ranged for iodosulfuron-methyl sodium in artificial soil is from 79.0 ± 6.4 % to 95.6 ± 0.6 %. The mean recovery ranged for 2,4-D acid in artificial soil is from 74.5 ± 5.1 % to 84.4 ± 0.7 %. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data – validation method in artificial soil

Active substance	Matrix	Fortification Level [mg/kg]	Number of Replicates	Mean Recovery [%]	RSD [%]
iodosulfuron-methyl sodium	Artificial soil	0.2	5	79.0	6.4
		2.0	5	95.6	0.6
2,4-D acid		0.2	5	74.5	5.1
		2.0	5	84.4	0.7

In order to study the recovery level, the solutions of the detected substances were added to non-treated artificial soil samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).

The repeatability for detected substance analysed in the artificial soil is from 0.2% to 3.8%.
The precision is 6.4% at 0.2 mg iodosulfuron-methyl sodium/kg artificial soil level and 0.6% at 2.0 mg iodosulfuron-methyl sodium/kg artificial soil level.
The precision is 5.1% at 0.2 mg 2,4-D/kg artificial soil level and 0.7% at 2.0 mg 2,4-D/kg artificial soil level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
The LoD is 0.02 mg iodosulfuron-methyl sodium/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.01 µg iodosulfuron-methyl sodium/mL.
The LoD is 0.1 mg 2,4-D acid/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.05 µg 2,4-D acid/mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The LoQ is 0.2 mg iodosulfuron-methyl sodium and 2,4-D acid/kg artificial soil and equivalent to the calibration level at concentration 0.1 µg iodosulfuron-methyl sodium and 2,4-D acid/mL.

Results and discussions

Parameter	**Required criterion**	**The result**		
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)	no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.		
Matrix effect [%]	±20%	2,4-D 2-EHE		5.2
		2,4-D acid		-3.9
		iodosulfuron methyl sodium		-7.4
Linearity	$r^2 \geq 0.99$	2,4-D 2-EHE: r^2 = 0.9992 (7205.40031 µg/mL + 2.06334 µg/mL)		
		2,4-D acid: r^2 = 0.9998362 (114953 µg/mL + 166.759 µg/mL)		2,4-D acid: r^2 = 0.9998158 (113583 µg/mL + 1412.94 µg/mL)
		iodosulfuron methyl sodium: r^2 = 0.9999221 (183188 µg/mL + 264.105 µg/mL)		iodosulfuron methyl sodium: r^2 = 0.9999933 (186125 µg/mL + 176.781 µg/mL)
Accuracy [%]	70-120	2,4-D 2-EHE	level I	92.0
			level II	100.0
		2,4-D acid	level I	74.5
			level II	84.4
		iodosulfuron methyl sodium	level I	79.0
			level II	95.6
Precision [% RSDr]	≤ 20	2,4-D 2-EHE	level I	6.8
			level II	1.8

		2,4-D acid	level I	5.1
			level II	0.7
		iodosulfuron methyl sodium	level I	6.4
			level II	0.6
Limit of detection LOD [mg/kg]	-	2,4-D 2-EHE		0.01
		2,4-D acid		0.1
		iodosulfuron methyl sodium		0.02
Limit of quantification LOQ [mg/kg]	-	2,4-D 2-EHE		0.05
		2,4-D acid		0.2
		iodosulfuron methyl sodium		0.2

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in artificial soil.

A 2.1.2.7.4 GC with ECD and HPLC with DAD (in artificial soil)

A 2.1.2.7.4.1 Method validation

Comments of evaluator:	Method is accepted

Reference: KCP 5.1.2/04 (filed as KCP 10.4.2.1/01)

Report: JMD-HER 387 Collembolan (*Folsomia candida*) Reproduction Test, Study code: G-04-21, Gierbuszewska, A., 2021

Guideline(s): SANTE/2020/12830, rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

The method is also suitable for the studies:

- JMD-HER 387 OD Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil; STUDY CODE: G-05-21

Development and validation analytical method of 2,4-D 2-EHE (GC with ECD)

Materials and methods

The analytical method was developed for the determination of 2,4-D 2-EHE in artificial soil. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of 2,4-D 2-EHE were determined.
The determination was accomplished by the high performance gas chromatography (GC) with ECD detection. Prior to analysis, the samples were concentrated using solid-liquid extraction.

Reagents, solvents and chemicals

- Ethyl acetate, pure p.a., POCH, batch no. 1135/06/20
- Acetone, pure p.a., POCH, batch no. 1062/06/20
- Anhydrous sodium sulphate, pure p.a., J.T.Baker, batch no. 1831301832
- standard solution of 1 mg/mL of 2,4-D 2-EHE in acetone
- working solutions of 2,4-D 2-EHE at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 100.0 µg/mL in acetone

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Balance, WPS 510/C, ZMP RADWAG (Poland)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Bruker 450-GC, BRUKER

Chromatographic conditions

- Chromatographic System: Gas Chromatography (GC, Bruker 450-GC)
- Detector: Electron Capture Detector (ECD)
- Analytical column: VF 5 ms 30M×0.25 MM ID DF=0.25
- Oven temperature: 200°C (2 minute); gradient 30°C/minute; 300°C (2 minute)
- Intel temperature: 260°C
- Detector temperature: 300°C
- Injection volume: 1 µl

Sample preparation for the chromatographic analysis

Artificial soil

First, 15 mL of ethyl acetate was added to 10 g of artificial soil sample and shaken for 30 minutes. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated with 15 mL of ethyl acetate. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 40°C. The dry residue was dissolved in 10 mL of acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

Fortified Sample

For validation experiments, 10 g of untreated artificial soil were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Sample Type	Sample Weight [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	10	-	-	0.00
Fortification (LOQ)	10	10	0.05	0.05
Fortification (10x LOQ)	10	100	0.05	0.50

This was done to ensure the result fits within the range of the respective standard curve.
Sample of water an untreated (10 g) was spiked with the 2,4-D 2-EHE to achieve fortification levels at the limit of quantification of 0.05 mg/kg and ten times higher i.e. 0.5 mg/kg.

Selectivity and specificity

The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experi-

mental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix blank sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.05 µg/mL prepared in solvent to at one at concentration 0.05 µg/mL prepared in blank matrix, for sample of artificial soil.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

Matrix effects [%] = 100 × peak area (matrix) / peak area (solvent) – 100

Matrix effect is -17.8% and not exceed ±20%.

Linearity
The stock solution with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard into a volumetric flask with a capacity of 10 mL, dissolving in acetone, and next the volume was made up to 10 ml with the same solvent. The working solution with a concentration of 100 µg/mL was prepared by dilution of the stock solution with acetone. Calibration and fortification solutions containing of 2,4-D 2-EHE were prepared by dilution of the working solution at concentration 2, 5, 10 and 100 µg/mL in acetone. Further dilutions were conducted with acetone as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 (Stock)	1	10	100
100	1	10	10
100	0.5	10	5
10	2	10	2
10	1	10	1.0[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The standard curve
Working solutions of 2,4-D 2-EHE at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 1.0 µg/mL. The range of calibration curve is equivalent to range from 0.01 mg 2,4-D 2-EHE/kg to 1.0 mg 2,4-D 2-EHE/kg in artificial soil.

The equation of the calibration line was presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear range was given, µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
2,4-D 2-EHE	7205.40031	2.06334	0.9992

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.

The mean recovery ranged for artificial soil is from 98.0 ± 2.7 % to 96.0 ± 6.1 %. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data for 2,4-D 2-EHE – validation method in artificial soil

Matrix	Fortification Level [mg/kg]	Number of Replicates	Mean Recovery [%]	RSD [%]
Artificial soil	0.05	5	98.0	2.7
	0.5	5	96.0	6.1

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the artificial soil is from 2.7% to 6.1%.
The precision is 2.7% at 0.05 mg 2,4-D 2-EHE/kg artificial soil level and 6.1% at 0.5 mg 2,4-D 2-EHE/kg artificial soil level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample. The LoD is 0.01 mg 2,4-D 2-EHE/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.01 µg 2,4-D 2-EHE /mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). The LoQ is 0.05 mg 2,4-D 2-EHE/kg artificial soil and equivalent to the calibration level at concentration 0.05 µg 2,4-D 2-EHE/mL.

Development and validation analytical method of iodosulfuron-methyl sodium and 2,4-D acid (HPLC with DAD

Materials and methods

The analytical method was developed for the determination of iodosulfuron-methyl sodium and 2,4-D acid in artificial soil. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of iodosulfuron-methyl sodium and 2,4-D acid were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. Prior to analysis, the samples were concentrated by solid - liquid extraction.

Reagents, solvents and chemicals

- Water, deionized, Fresh prepared before analysis
- Acetonitrile, HPLC, J.T.Baker, batch no. 1728501868
- Ortho-phosphoric acid, 85% pure p.a., POCH, batch no. 1077/05/17
- Acetic acid, 99.5%-99.9% pure p.a., POCH, batch no. 1356/09/16
- 0.05% ortho-phosphoric acid solution in deionized artificial soil,
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- mixture of acetonitrile HPLC and deionized water and acetic acid (400:100:3, v/v/v),
- standard solution of 1 mg/mL of iodosulfuron-methyl sodium in acetonitrile for HPLC,
- standard solution of 1 mg/mL of 2,4-D acid in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl sodium at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl sodium at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- working solutions of 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of 2,4-D acid at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- working solutions of iodosulfuron-methyl sodium and 2,4-D acid at concentration 100.0 µg/mL in

acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl sodium and 2,4-D acid at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v).

Equipment
- Balance, WPS 510/C, ZMP RADWAG (Poland)
- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Laboratory centrifuge, MPW-351e, MPW MED.INSTRUMENTS (Poland)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)

Chromatographic conditions
- Chromatographic System: High Performance Liquid Chromatography (HPLC); Shimadzu, Prominence (Shimadzu Corporation Japan)
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid (45 : 55, v/v)
- Analytical column: Agilent Eclipse 5µm XDB-C8, l = 150 mm, ϕ = 4.6 mm
- Oven temperature: 35°C
- Wavelength: 220 nm
- Flow Rate: 0.6 mL/min
- Injection volume: 30 µl
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Artificial soil

First, 10 mL of mixture of acetonitrile HPLC and deionized water and acetic acid (400:100:3, v/v/v), was added to 10 g of artificial soil sample and shaken for 6 minutes by using hand. The organic phases were centrifuged and filtered through paper filter. The extraction was repeated with 10 mL of mixture of acetonitrile HPLC and deionized water and acetic acid (400:100:3, v/v/v).

Fortified Sample

For validation experiments, 10 g of untreated artificial soil were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Active substance	Sample Type	Sample Weight [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
iodosulfuron methyl sodium	Control	10	-	-	0.00
	Fortification (LOQ)	10	10	0.2	0.20
	Fortification (10x LOQ)	10	100	0.2	2.00
2,4-D acid	Control	10	-	-	0.00
	Fortification (LOQ)	10	10	0.2	0.20
	Fortification (10x LOQ)	10	100	0.2	2.00

This was done to ensure the result fits within the range of the respective standard curve.
Sample of artificial soil an untreated (10 g) was spiked with the solution of iodosulfuron-methyl sodium and 2,4-D acid to achieve fortification levels at the limit of quantification of 0.2 mg/kg and ten times higher i.e. 2.0 mg/kg.

Selectivity and specificity
The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substances was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.2 µg iodosulfuron methyl sodium and 2,4-D acid/mL prepared in solvent to at one at concentration 0.2 µg iodosulfuron-methyl sodium and 2,4-D acid/mL prepared in blank matrix, for sample of artificial soil.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

Matrix effect for iodosulfuron-methyl sodium is -7.5% and not exceed ±20%.
Matrix effect for 2,4-D is -2.8% and not exceed ±20%.

Linearity
The stock solutions of iodosulfuron-methyl sodium and 2,4-D acid with a concentration of 1 mg/mL was prepared separately by weighting 10 mg analytical standards of iodosulfuron-methyl sodium or 2,4-D acid into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 ml with the same solvent. The working solutions of iodosulfuron methyl sodium and 2,4-D with a concentration of 100 µg/mL were prepared common and separately by dilution of the stock solutions with acetonitrile for HPLC. Moreover, the working solution of iodosulfuron methyl sodium and 2,4-D with a concentration of 10 µg/mL were prepared separately by dilution of the working solutions at concentration of 100 µg/mL with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of iodosulfuron methyl sodium and 2,4-D acid were prepared by dilution of the common working solution at concentration 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
100	2	10	20[1)]
100	1	10	10[1)]
100	0.5	10	5[1)]
20	1	10	2[1)]
10	1	10	1[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The first standard curve
Working solutions of iodosulfuron-methyl sodium at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.02 mg iodosulfuron-methyl sodium/kg to 4.0 mg iodosulfuron-methyl sodium/kg in artificial soil.

Working solutions of 2,4-D acid at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.05 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.1 mg 2,4-D acid/kg to 4.0 mg 2,4-D acid /kg in artificial soil.
The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear range was given, µg/mL.

Analyte	Slope	Intercept	Correlation coefficient
iodosulfuron methyl sodium	183188	264.105	0.9999221
2,4-D acid	114953	166.759	0.9998362

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

The second standard curve
Working solutions of iodosulfuron-methyl sodium and 2,4-D acid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg /mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph range from 0.2 µg/mL to 20.0 µg/mL. The range of calibration curve is equivalent to range from 0.4 mg iodosulfuron-methyl sodium and 2,4-D acid/kg to 40.0 mg iodosulfuron-methyl sodium and 2,4-D acid/kg in artificial soil.

The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear range was given, µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
iodosulfuron methyl sodium	186125	176.781	0.9999933
2,4-D acid	113583	1412.94	0.9998158

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery ranged for iodosulfuron-methyl sodium in artificial soil is from 81.0 ± 3.8 % to 102.1 ± 0.5 %. The mean recovery ranged for 2,4-D acid in artificial soil is from 81.5 ± 3.6 % to 98.9 ± 0.2 %. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data – validation method in artificial soil

Active substance	Matrix	Fortification Level [mg/kg]	Number of Replicates	Mean Recovery [%]	RSD [%]
iodosulfuron-methyl sodium	Artificial soil	0.2	5	81.0	3.8
		2.0	5	102.1	0.5
2,4-D acid		0.2	5	81.5	3.6
		2.0	5	98.9	0.2

In order to study the recovery level, the solutions of the detected substances were added to non-treated artificial soil samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the artificial soil is from 0.2% to 3.8%.
The precision is 3.8% at 0.2 mg iodosulfuron-methyl sodium/kg artificial soil level and 0.5% at 2.0 mg

iodosulfuron-methyl sodium/kg artificial soil level.
The precision is 3.6% at 0.2 mg 2,4-D/kg artificial soil level and 0.2% at 2.0 mg 2,4-D/kg artificial soil level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
The LoD is 0.02 mg iodosulfuron-methyl sodium/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.01 µg iodosulfuron-methyl sodium/mL.
The LoD is 0.1 mg 2,4-D acid/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.05 µg 2,4-D acid/mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The LoQ is 0.2 mg iodosulfuron-methyl sodium and 2,4-D acid/kg artificial soil and equivalent to the calibration level at concentration 0.1 µg iodosulfuron-methyl sodium and 2,4-D acid/mL.

Results and discussions

Parameter	**Required criterion**	**The result**		
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)	no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.		
Matrix effect [%]	±20%	2,4-D 2-EHE		-17.8
		2,4-D acid		-2.8
		iodosulfuron methyl sodium		-7.5
Linearity	$r^2 \geq 0.99$	2,4-D 2-EHE: r^2 = 0.9992 (7205.40031 µg/mL + 2.06334 µg/mL)		
		2,4-D acid: r^2 = 0.9998362 (114953 µg/mL + 166.759 µg/mL)		2,4-D acid: r^2 = 0.9998158 (113583 µg/mL + 1412.94 µg/mL)
		iodosulfuron methyl sodium: r^2 = 0.9999221 (183188 µg/mL + 264.105 µg/mL)		iodosulfuron methyl sodium: r^2 = 0.9999933 (186125 µg/mL + 176.781 µg/mL)
Accuracy [%]	70-120	2,4-D 2-EHE	level I	98.0
			level II	96.0
		2,4-D acid	level I	81.5
			level II	98.9
		iodosulfuron methyl sodium	level I	81.0
			level II	102.1
Precision [% RSDr]	≤ 20	2,4-D 2-EHE	level I	2.7
			level II	6.1
		2,4-D acid	level I	3.6
			level II	0.2

		iodosulfuron methyl sodium	level I	3.8
			level II	0.5
Limit of detection LOD [mg/kg]	-	2,4-D 2-EHE		0.01
		2,4-D acid		0.1
		iodosulfuron methyl sodium		0.02
Limit of quantification LOQ [mg/kg]	-	2,4-D 2-EHE		0.05
		2,4-D acid		0.2
		iodosulfuron methyl sodium		0.2

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in artificial soil.

A 2.1.2.7.5 HPLC with DAD and GC with ECD (in water)

A 2.1.2.7.5.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/05 (filed as KCP 10.2.1.3/02)
Report	JMD-HER 387 OD *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test; Study code: W-03-21, Czarnecka, M., 2021
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Development and validation analytical method of iodosulfuron-methyl-sodium and 2,4-D acid (HPLC with DAD)

Materials and methods

The analytical method was developed for the determination of iodosulfuron-methyl-sodium and 2,4-D acid in water. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of iodosulfuron-methyl sodium and 2,4-D acid were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. Prior to analysis, the samples were shaken

Reagents, solvents and chemicals

- Water, deionized, Fresh prepared before analysis
- Acetonitrile, HPLC, J.T.Baker, batch no. 1728501868
- Acetone, pure p.a., POCH, batch no. 1062/06/20

- Methanol, pure p.a., POCH, batch no. 1261/01/20
- SUPELCLEAN ENVI-18 SPE, 3 mL, 500 mg, Supelco, batch no. 12546401
- Hydrochloric acid, ACS reagent 37%, Sigma-Aldrich, batch no. STBK 0854
- Ortho-phosphoric acid, 85% pure p.a., POCH, batch no. 1077/05/17
- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- standard solution of 1 mg/mL of iodosulfuron-methyl-sodium in acetonitrile for HPLC,
- standard solution of 1 mg/mL of 2,4-D acid in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v),
- working solutions of 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of 2,4-D acid at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v).

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- SPE vacuum manifold, Visiprep, Supelco (USA)
- SPE cartridges, Supelclean ENVI-18, Supelco (USA)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)
- Rotary vacuum evaporator with water medium bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)

Chromatographic conditions

- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Agilent Eclipse 5µm XDB-C8, l = 150 mm, ϕ = 4.6 mm
- Oven temperature: 35°C
- Flow Rate: 0.6 mL/min
- Wavelength: 220 nm
- Injection volume: 30 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (45 : 55, v/v)
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Each sample of 100 mL volume was acidified by hydrochloric acid to pH ≈ 2 and applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing twice with 5 mL of acetone, twice with 5 mL of methanol, twice with 5 mL of deionised water pH≈2. Following the sample introduction, the column was dried under vacuum for 5 minutes. The analytes were eluted with twice 5 mL of acetone and twice 5 mL methanol. The eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was redissolved in mixture acetonitrile: 0.05% orthophosphoric acid (50:50; v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Fortified Sample

For validation experiments, 100 mL aliquot of untreated water were spiked with appropriate volumes of

fortification solutions. The following fortification scheme was used:

Active substance	Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/L]
iodosulfuron methyl sodium	Control	100	-	-	0.000
	Fortification (LOQ)	100	1	0.1	0.001
	Fortification (10x LOQ)	100	10	0.1	0.01
2,4-D acid	Control	100	-	-	0.000
	Fortification (LOQ)	100	1	0.1	0.001
	Fortification (10x LOQ)	100	10	0.1	0.01

Sample of water an untreated (100 mL) was spiked with the solution of iodosulfuron-methyl-sodium and 2,4-D acid to achieve fortification levels at the limit of quantification of 0.001 mg/L and ten times higher i.e. 0.01 mg/L.
This was done to ensure the result fits within the range of the respective standard curve.

Selectivity and specificity
The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.1 µg iodosulfuron-methyl-sodium and 2,4-D acid/mL prepared in solvent to at concentration 0.1 µg iodosulfuron-methyl-sodium and 2,4-D acid/mL prepared in blank matrix, for sample of water.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

Matrix effect for iodosulfuron-methyl sodium is 0.3% and not exceed ±20%.
Matrix effect for 2,4-D is 5.4% and not exceed ±20%.

Linearity
The stock solutions of iodosulfuron-methyl sodium and 2,4-D acid with a concentration of 1 mg/mL was prepared separately by weighting 10 mg analytical standards of iodosulfuron-methyl-sodium or 2,4-D acid into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 ml with the same solvent. The working solutions of iodosulfuron-methyl-sodium and 2,4-D with a concentration of 100 µg/mL were prepared common and separately by dilution of the stock solutions with acetonitrile for HPLC. Moreover, the working solution of iodosulfuron methyl-sodium and 2,4-D with a concentration of 10 µg/mL were prepared separately by dilution of the working solutions at concentration of 100 µg/mL with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of iodosulfuron-methyl-sodium and 2,4-D acid were prepared by dilution of the common working solution at concentration 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
100	2	10	20[1)]
100	1	10	10[1)]
100	0.5	10	5[1)]
20	1	10	2[1)]
10	1	10	1[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The first standard curve

Working solutions of iodosulfuron-methyl-sodium at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg /mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.0001 mg iodosulfuron-methyl-sodium/L to 0.02 mg iodosulfuron-methyl-sodium/L in water.

Working solutions of 2,4-D acid at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.05 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.0005 mg 2,4-D acid/L to 0.02 mg 2,4-D acid /L in water.

The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear was given in µg/mL.

Analyte	Slope	Intercept	Correlation coefficient
Iodosulfuron methyl sodium	183188	264.105	0.9999221
2,4-D acid	114953	166.759	0.9998362

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

The second standard curve

Working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20.0 µg/mL. The range of calibration curve is equivalent to range from 0.002 mg iodosulfuron-methyl-sodium and 2,4-D acid/L to 0.2 mg iodosulfuron-methyl-sodium and 2,4-D acid/L in water.

The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear was given in µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
Iodosulfuron methyl sodium	186125	176.781	0.9999933
2,4-D acid	113583	1412.94	0.9998158

Linear weighted calibration (1/x weighting) was used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recov-

ery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery range for iodosulfuron-methyl-sodium in water is from 84.0 ± 5.2 % to 94.0 ± 1.2 %. The mean recovery range for 2,4-D acid in water is from 94.0 ± 3.0% to 102.0 ± 3.6%. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data – validation method in water

Active substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
iodosulfuron-methyl sodium	water	0.001	5	84.0	5.2
		0.01	5	94.0	1.2
2,4-D acid		0.001	5	102.0	3.6
		0.01	5	94.0	3.0

In order to study the recovery level, the solutions of the detected substances were added to non-treated water samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the water is from 1.2% to 5.2%.
The precision is 5.2% at 0.001 mg iodosulfuron-methyl-sodium/L water level and 1.2% at 0.01 mg iodosulfuron-methyl-sodium/L water level.
The precision is 3.6% at 0.001 mg 2,4-D/L water level and 3.0% at 0.01 mg 2,4-D/L water level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
The LoD is 0.0001 mg iodosulfuron-methyl-sodium/L water and equivalent to the lowest calibration standard i.e. 0.01 µg iodosulfuron-methyl-sodium /mL.
The LoD is 0.0005 mg 2,4-D acid/L water and equivalent to the lowest calibration standard i.e. 0.05 µg 2,4-D acid /mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The LoQ is 0.001 mg iodosulfuron-methyl-sodium/L water and equivalent to the calibration level at concentration 0.1 µg iodosulfuron-methyl-sodium/mL.
The LoQ is 0.001 mg 2,4-D acid/L water and equivalent to the calibration level at concentration 0.1 µg 2,4-D acid/mL.

Development and validation analytical method of 2,4-D 2-EHE (GC with ECD)

Materials and methods

The analytical method was developed for the determination of 2,4-D 2-EHE in water. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of 2,4-D 2-EHE were determined.
The determination was accomplished by the high performance gas chromatography (GC) with ECD detection. Prior to analysis, the samples were concentrated using liquid-liquid extraction.

Reagents, solvents and chemicals
- Ethyl acetate, pure p.a., POCH, batch no. 1135/06/20
- Acetone, pure p.a., POCH, batch no. 1062/06/20
- Anhydrous sodium chloride, pure p.a., POCH, batch no. 1256/07/18

- Anhydrous sodium sulphate, pure p.a., J.T.Baker, batch no. 1831301832
- standard solution of 1 mg/mL of 2,4-D 2-EHE in acetone
- sodium chloride saturated solution
- working solutions of 2,4-D 2-EHE at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 100.0 µg/mL in acetone

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Bruker 450-GC, BRUKER

Chromatographic conditions

- Chromatographic System: Gas Chromatography (GC, Bruker 450-GC)
- Detector: Electron Capture Detector (ECD)
- Analytical column: VF 5 ms 30M×0.25 MM ID DF=0.25
- Oven temperature: 200°C (2 minute); gradient 30°C/minute; 300°C (2 minute)
- Intel temperature: 260°C
- Detector temperature: 300°C
- Injection volume: 1 µl

Sample preparation for the chromatographic analysis

Water

First, 30 mL of ethyl acetate, 2 mL sodium chloride solution saturated was added to 100 mL of water sample and shaken. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 40°C. The dry residue was dissolved in 1.5 mL of acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

Fortified Sample

For validation experiments, 100 mL aliquot of untreated water were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	100	-	-	0.000
Fortification (LOQ)	100	1	0.1	0.001
Fortification (10x LOQ)	100	10	0.1	0.01

This was done to ensure the result fits within the range of the respective standard curve.
Sample of water an untreated (100 mL) was spiked with the 2,4-D 2-EHE to achieve fortification levels at the limit of quantification of 0.001 mg/L and ten times higher i.e. 0.01 mg/L.

Selectivity and specificity

The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control water, and fortified samples of water. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix blank sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.05 µg/mL prepared in solvent to at one at concentration 0.05 µg/mL prepared in blank matrix, for sample of water.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

Matrix effects [%] = 100 × peak area (matrix) / peak area (solvent) – 100

Matrix effect is 1.6% and not exceed ±20%.

Linearity
The stock solution with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard into a volumetric flask with a capacity of 10 mL, dissolving in acetone, and next the volume was made up to 10 ml with the same solvent. The working solution with a concentration of 100 µg/mL was prepared by dilution of the stock solution with acetone. Calibration and fortification solutions containing of 2,4-D 2-EHE were prepared by dilution of the working solution at concentration 2, 5, 10 and 100 µg/mL in acetone. Further dilutions were conducted with acetone as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 (Stock)	1	10	100
100	1	10	10
100	0.5	10	5
10	2	10	2
10	1	10	1.0[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The standard curve
Working solutions of 2,4-D 2-EHE at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 1.0 µg/mL. The range of calibration curve is equivalent to range from 0.00015 mg 2,4-D 2-EHE /L to 0.015 mg 2,4-D 2-EHE /L in water.

The equation of the calibration line was
presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
2,4-D 2-EHE	7205.40031	2.06334	0.9992

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery ranged for water is from 97.0 ± 12.0 % to 102.0 ± 6.5%. For analyte, the relative

standard deviations (RSD) at each fortification levels were below 20%.

Recovery Data for 2,4-D 2-EHE – validation method in water

Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
Water	0.001	5	102.0	6.5
	0.01	5	97.0	12.0

In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed using the method described above.

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the water is from 6.5% to 12.0%.
The precision is 6.5% at 0.001 mg 2,4-D 2-EHE/L water level and 12.0% at 0.01 mg 2,4-D 2-EHE/L water level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample. The LoD is 0.00015 mg 2,4-D 2-EHE/L water and equivalent to the lowest calibration standard i.e. 0.01 µg 2,4-D 2-EHE /mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). The LoQ is 0.001 mg 2,4-D 2-EHE /L water and equivalent to the calibration level at concentration 0.06 µg 2,4-D 2-EHE/mL.

Results and discussions

Parameter	Required criterion	The result		
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)	no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.		
Matrix effect [%]	±20%	2,4-D 2-EHE		1.6
		2,4-D acid		5.4
		iodosulfuron methyl sodium		0.3
Linearity	$r^2 \geq 0.99$	2,4-D 2-EHE: $r^2 = 0.9992$ (7205.40031 µg/mL + 2.06334 µg/mL)		
		2,4-D acid: $r^2 = 0.9998362$ (114953 µg/mL + 166.759 µg/mL)		2,4-D acid: $r^2 = 0.9998158$ (113583 µg/mL + 1412.94 µg/mL)
		iodosulfuron methyl sodium: $r^2 = 0.9999221$ (183188 µg/mL + 264.105 µg/mL)		iodosulfuron methyl sodium: $r^2 = 0.9999933$ (186125 µg/mL + 176.781 µg/mL)
Accuracy [%]	70-120	2,4-D 2-EHE	level I	102.0
			level II	97.0

		2,4-D acid	level I	102.0
			level II	94.0
		iodosulfuron methyl sodium	level I	84.0
			level II	94.0
Precision [% RSDr]	≤ 20	2,4-D 2-EHE	level I	6.5
			level II	12.0
		2,4-D acid	level I	3.6
			level II	3.0
		iodosulfuron methyl sodium	level I	5.2
			level II	1.2
Limit of detection LOD [mg/L]	-	2,4-D 2-EHE		0. 00015
		2,4-D acid		0. 0005
		iodosulfuron methyl sodium		0. 0001
Limit of quantification LOQ [mg/L]	-	2,4-D 2-EHE		0. 001
		2,4-D acid		0. 001
		iodosulfuron methyl sodium		0. 001

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in water.

A 2.1.2.7.6 HPLC with DAD and GC with ECD (in water)

A 2.1.2.7.6.1 Method validation

Comments of evaluator:	Method is accepted

Reference: KCP 5.1.2/06 (filed as KCP 10.6.2/02)

Report: JMD – HER 387 OD Terrestrial Plant Test: Vegetative Vigour Test, Study code: G-07-21, Arendarczyk, A., 2021

Guideline(s): SANTE/2020/12830, rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

The method is also suitable for the studies:

- JMD-HER 387 OD Terrestial Plant Test: Seedling Emergence and Seedling Growth Test; STUDY CODE: G-08-21

Development and validation analytical method of iodosulfuron-methyl-sodium and 2,4-D acid (HPLC with DAD)

Materials and methods

The analytical method was developed for the determination of iodosulfuron-methyl-sodium and 2,4-D acid in water. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of iodosulfuron-methyl sodium and 2,4-D acid were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. Prior to analysis, the samples were shaken.

Reagents, solvents and chemicals

- Water, deionized, Fresh prepared before analysis
- Acetonitrile, HPLC, J.T.Baker, batch no. 1728501868
- Ortho-phosphoric acid, 85% pure p.a., POCH, batch no. 1077/05/17
- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- standard solution of 1 mg/mL of iodosulfuron-methyl-sodium in acetonitrile for HPLC,
- standard solution of 1 mg/mL of 2,4-D acid in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v),
- working solutions of 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of 2,4-D acid at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v).

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Agilent Eclipse 5µm XDB-C8, l = 150 mm, ϕ = 4.6 mm
- Oven temperature: 35°C
- Flow Rate: 0.6 mL/min
- Wavelength: 220 nm
- Injection volume: 30 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (45 : 55, v/v)
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Water

Each sample of 1 mL volume was transferred into HPLC via for further quantification using HPLC-DAD.

Fortified Sample

For validation experiments, 10 mL aliquot of untreated water were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Active substance	Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/L]
iodosulfuron methyl sodium	Control	10	-	-	0.00
	Fortification (LOQ)	10	10	0.02	0.02
	Fortification (10x LOQ)	10	100	0.02	0.2
2,4-D acid	Control	10	-	-	0.0
	Fortification (LOQ)	10	10	0.1	0.1
	Fortification (10x LOQ)	10	100	0.1	1.0

This was done to ensure the result fits within the range of the respective standard curve.
Sample of water an untreated (10 mL) was spiked with the solution of iodosulfuron-methyl-sodium to achieve fortification levels at the limit of quantification of 0.02 mg/L and ten times higher i.e. 0.2 mg/L.
Sample of water an untreated (10 mL) was spiked with the solution of 2,4-D acid to achieve fortification levels at the limit of quantification of 0.1 mg/L and ten times higher i.e. 1.0 mg/L.

Selectivity and specificity
The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.02 µg iodosulfuron-methyl-sodium 0.1 µg 2,4-D acid/mL prepared in solvent to at one at concentration 0.02 µg iodosulfuron-methyl-sodium and 0.1 µg 2,4-D acid/mL prepared in blank matrix, for sample of water.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

Matrix effect for iodosulfuron-methyl sodium is 2.2% and not exceed ±20%.
Matrix effect for 2,4-D is 4.2% and not exceed ±20%.

Linearity
The stock solutions of iodosulfuron-methyl sodium and 2,4-D acid with a concentration of 1 mg/mL was prepared separately by weighting 10 mg analytical standards of iodosulfuron-methyl-sodium or 2,4-D acid into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 ml with the same solvent. The working solutions of iodosulfuron-methyl-sodium and 2,4-D with a concentration of 100 µg/mL were prepared common and separately by dilution of the stock solutions with acetonitrile for HPLC. Moreover, the working solution of iodosulfuron methyl-sodium and 2,4-D with a concentration of 10 µg/mL were prepared separately by dilution of the working solutions at concentration of 100 µg/mL with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of iodosulfuron-methyl-sodium and 2,4-D acid were prepared by dilution of the common working solution at concentration 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
100	2	10	20[1)]
100	1	10	10[1)]
100	0.5	10	5[1)]
20	1	10	2[1)]
10	1	10	1[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The first standard curve

Working solutions of iodosulfuron-methyl-sodium at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg /mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.01 mg iodosulfuron-methyl-sodium/L to 2.0 mg iodosulfuron-methyl-sodium/L in water.

Working solutions of 2,4-D acid at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.05 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.05 mg 2,4-D acid/L to 2.0 mg 2,4-D acid /L in water.

The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL.

Analyte	Slope	Intercept	Correlation coefficient
Iodosulfuron methyl sodium	183188	264.105	0.9999221
2,4-D acid	114953	166.759	0.9998362

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

The second standard curve

Working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20.0 µg/mL. The range of calibration curve is equivalent to range from 0.2 mg iodosulfuron-methyl-sodium and 2,4-D acid/L to 20.0 mg iodosulfuron-methyl-sodium and 2,4-D acid/L in water.

The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
Iodosulfuron methyl sodium	186125	176.781	0.9999933
2,4-D acid	113583	1412.94	0.9998158

Linear weighted calibration (1/x weighting) was used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recov-

ery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery range for iodosulfuron-methyl-sodium in water is from 105.0 ± 2.1 % to 110.0 ± 3.2 %. The mean recovery range for 2,4-D acid in water is from 99.0 ± 0.6% to 101.0 ± 0.8%. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data – validation method in water

Active substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
iodosulfuron-methyl sodium	water	0.02	5	110.0	3.2
		0.2	5	105.0	2.1
2,4-D acid		0.1	5	101.0	0.8
		1.0	5	99.0	0.6

In order to study the recovery level, the solutions of the detected substances were added to non-treated water samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the water is from 0.6% to 3.2%.
The precision is 3.2% at 0.1 mg iodosulfuron-methyl-sodium/L water level and 2.1% at 0.2 mg iodosulfuron-methyl-sodium/L water level.
The precision is 0.8% at 0.1 mg 2,4-D/L water level and 0.6% at 1.0 mg 2,4-D/L water level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
The LoD is 0.01 mg iodosulfuron-methyl-sodium/L water and equivalent to the lowest calibration standard i.e. 0.01 µg iodosulfuron-methyl-sodium /mL.
The LoD is 0.05 mg 2,4-D acid/L water and equivalent to the lowest calibration standard i.e. 0.05 µg 2,4-D acid /mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The LoQ is 0.02 mg iodosulfuron-methyl-sodium/L water and equivalent to the calibration level at concentration 0.02 µg iodosulfuron-methyl-sodium/mL.
The LoQ is 0.1 mg 2,4-D acid/L water and equivalent to the calibration level at concentration 0.1 µg 2,4-D acid/mL.

Development and validation analytical method of 2,4-D 2-EHE (GC with ECD)

Materials and methods

The analytical method was developed for the determination of 2,4-D 2-EHE in water. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of 2,4-D 2-EHE were determined.
The determination was accomplished by the high performance gas chromatography (GC) with ECD detection. Prior to analysis, the samples were concentrated using liquid-liquid extraction.

Reagents, solvents and chemicals
- Ethyl acetate, pure p.a., POCH, batch no. 1135/06/20
- Acetone, pure p.a., POCH, batch no. 1062/06/20
- Anhydrous sodium chloride, pure p.a., POCH, batch no. 1256/07/18
- Anhydrous sodium sulphate, pure p.a., J.T.Baker, batch no. 1831301832

- standard solution of 1 mg/mL of 2,4-D 2-EHE in acetone
- sodium chloride saturated solution
- working solutions of 2,4-D 2-EHE at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 100.0 µg/mL in acetone

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Bruker 450-GC, BRUKER

Chromatographic conditions

- Chromatographic System: Gas Chromatography (GC, Bruker 450-GC)
- Detector: Electron Capture Detector (ECD)
- Analytical column: VF 5 ms 30M×0.25 MM ID DF=0.25
- Oven temperature: 200°C (2 minute); gradient 30°C/minute; 300°C (2 minute)
- Intel temperature: 260°C
- Detector temperature: 300°C
- Injection volume: 1 µl

Sample preparation for the chromatographic analysis

Water

First, 15 mL of ethyl acetate, 2 mL sodium chloride solution saturated was added to 10 mL of water sample and shaken. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 40°C. The dry residue was dissolved in 5 mL of acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

Fortified Sample

For validation experiments, 10 mL aliquot of untreated water were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	10	-	-	0.00
Fortification (LOQ)	10	1	0.1	0.01
Fortification (10x LOQ)	10	10	0.1	0.1

This was done to ensure the result fits within the range of the respective standard curve.
Sample of water an untreated (10 mL) was spiked with the 2,4-D 2-EHE to achieve fortification levels at the limit of quantification of 0.01 mg/L and ten times higher i.e. 0.1 mg/L.

Selectivity and specificity

The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control water, and fortified samples of water. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix blank sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.02 µg/mL prepared in solvent to at one at concentration 0.02 µg/mL prepared in blank matrix, for sample of water.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

Matrix effects [%] = 100 × peak area (matrix) / peak area (solvent) – 100

Matrix effect is -3.7% and not exceed ±20%.

Linearity
The stock solution with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard into a volumetric flask with a capacity of 10 mL, dissolving in acetone, and next the volume was made up to 10 ml with the same solvent. The working solution with a concentration of 100 µg/mL was prepared by dilution of the stock solution with acetone. Calibration and fortification solutions containing of 2,4-D 2-EHE were prepared by dilution of the working solution at concentration 2, 5, 10 and 100 µg/mL in acetone. Further dilutions were conducted with acetone as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 (Stock)	1	10	100
100	1	10	10
100	0.5	10	5
10	2	10	2
10	1	10	1.0[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The standard curve
Working solutions of 2,4-D 2-EHE at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 1.0 µg/mL. The range of calibration curve is equivalent to range from 0.005 mg 2,4-D 2-EHE /L to 0.5 mg 2,4-D 2-EHE /L in water.

The equation of the calibration line was
presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
2,4-D 2-EHE	7205.40031	2.06334	0.9992

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery ranged for water is from 98.0 ± 7.0 % to 100.0 ± 4.0%. For analyte, the relative standard deviations (RSD) at each fortification levels were below 20%.

Recovery Data for 2,4-D 2-EHE – validation method in water

Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
Water	0.01	5	100.0	4.0
	0.1	5	98.0	7.0

In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed using the method described above.

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the water is from 4.0% to 7.0%.
The precision is 4.0% at 0.01 mg 2,4-D 2-EHE/L water level and 7.0% at 0.1 mg 2,4-D 2-EHE/L water level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample. The LoD is 0.005 mg 2,4-D 2-EHE/L water and equivalent to the lowest calibration standard i.e. 0.01 µg 2,4-D 2-EHE /mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). The LoQ is 0.01 mg 2,4-D 2-EHE /L water and equivalent to the calibration level at concentration 0.02 µg 2,4-D 2-EHE/mL.

Results and discussions

Parameter	Required criterion	The result		
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)	no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.		
Matrix effect [%]	±20%	2,4-D 2-EHE		-3.7
		2,4-D acid		4.2
		iodosulfuron methyl sodium		2.2
Linearity	$r^2 \geq 0.99$	2,4-D 2-EHE: $r^2 = 0.9992$ (7205.40031 µg/mL + 2.06334 µg/mL)		
		2,4-D acid: $r^2 = 0.9998362$ (114953 µg/mL + 166.759 µg/mL)		2,4-D acid: $r^2 = 0.9998158$ (113583 µg/mL + 1412.94 µg/mL)
		iodosulfuron methyl sodium: $r^2 = 0.9999221$ (183188 µg/mL + 264.105 µg/mL)		iodosulfuron methyl sodium: $r^2 = 0.9999933$ (186125 µg/mL + 176.781 µg/mL)
Accuracy [%]	70-120	2,4-D 2-EHE	level I	100.0
			level II	98.0
		2,4-D acid	level I	101.0

			level II	99.0
		iodosulfuron methyl sodium	level I	110.0
			level II	105.0
Precision [% RSDr]	≤ 20	2,4-D 2-EHE	level I	4.0
			level II	7.0
		2,4-D acid	level I	0.8
			level II	0.6
		iodosulfuron methyl sodium	level I	3.2
			level II	2.1
Limit of detection LOD [mg/L]	-	2,4-D 2-EHE		0. 005
		2,4-D acid		0. 05
		iodosulfuron methyl sodium		0. 01
Limit of quantification LOQ [mg/L]	-	2,4-D 2-EHE		0. 01
		2,4-D acid		0.1
		iodosulfuron methyl sodium		0. 02

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in water.

A 2.1.2.7.7 HPLC with DAD and GC with ECD (in Elendt M7 medium)

A 2.1.2.7.7.1 Method validation

Comments of evaluator:	Method is accepted

Reference: KCP 5.1.2/07 (filed as KCP 10.2.1.2/03)

Report: JMD – HER 387 OD *Chironomus* sp., Acute Immobilisation Test, Study code: W-01-21, Czarnecka, M., 2021

Guideline(s): SANTE/2020/12830, rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

The method is also suitable for the study:

- JMD-HER 387 OD *Daphnia magna*, Acute Immobilisation Test, STUDY CODE: W-02-21

Development and validation analytical method of iodosulfuron-methyl-sodium and 2,4-D acid (HPLC with DAD)

Materials and methods

The analytical method was developed for the determination of iodosulfuron-methyl-sodium and 2,4-D acid in Elendt M7 medium. The range of linearity of the analytical graph, the regression residual (di),

selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of iodosulfuron-methyl-sodium and 2,4-D acid were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. Prior to analysis, the samples were shaken.

Reagents, solvents and chemicals

- Water, deionized, Fresh prepared before analysis
- Acetonitrile, HPLC, J.T.Baker, batch no. 1728501868
- Ortho-phosphoric acid, 85% pure p.a., POCH, batch no. 1077/05/17
- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- standard solution of 1 mg/mL of iodosulfuron-methyl-sodium in acetonitrile for HPLC,
- standard solution of 1 mg/mL of 2,4-D acid in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v),
- working solutions of 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of 2,4-D acid at concentration 10.0 µg/mL in acetonitrile for HPLC mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at concentration 100.0 µg/mL in acetonitrile for HPLC,
- working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v).

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Agilent Eclipse 5µm XDB-C8, l = 150 mm, ϕ = 4.6 mm
- Oven temperature: 35°C
- Flow Rate: 0.6 mL/min
- Wavelength: 220 nm
- Injection volume: 30 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (45 : 55, v/v)
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Elendt M7 medium

Each sample of 1 mL volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Fortified Sample

For validation experiments, 10 mL aliquot of untreated Elendt M7 medium were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Active substance	Sample Type	Sample Volume	Concentration of Spiking Solution	Volume of Spiking Solu-	Level of Fortification [mg/L]

		[mL]	[µg/mL]	tion [mL]	
iodosulfuron methyl sodium	Control	10	-	-	0.00
	Fortification (LOQ)	10	10	0.02	0.02
	Fortification (10x LOQ)	10	100	0.02	0.2
2,4-D acid	Control	10	-	-	0.0
	Fortification (LOQ)	10	10	0.1	0.1
	Fortification (10x LOQ)	10	100	0.1	1.0

This was done to ensure the result fits within the range of the respective standard curve.
An untreated sample of Elendt M7 medium (10 mL) was spiked with the solution of iodosulfuron-methyl-sodium to achieve fortification levels at the limit of quantification of 0.02 mg/L and ten times higher i.e. 0.2 mg/L.
An untreated sample of Elendt M7 medium (10 mL) was spiked with the solution of 2,4-D acid to achieve fortification levels at the limit of quantification of 0.1 mg/L and ten times higher i.e. 1.0 mg/L.

Selectivity and specificity
The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.02 µg iodosulfuron-methyl-sodium 0.1 µg 2,4-D acid/mL prepared in solvent to at one at concentration 0.02 µg iodosulfuron-methyl-sodium 0.1 µg 2,4-D acid/mL prepared in blank matrix, for sample of Elendt M7 medium.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

Matrix effect for iodosulfuron-methyl-sodium is 0.2% and not exceed ±20%.
Matrix effect for 2,4-D is -0.4% and not exceed ±20%.

Linearity
The stock solutions of iodosulfuron-methyl-sodium and 2,4-D acid with a concentration of 1 mg/mL were prepared separately by weighting 10 mg analytical standards of iodosulfuron-methyl-sodium or 2,4-D acid into volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solutions of iodosulfuron-methyl-sodium and 2,4-D with a concentration of 100 µg/mL were prepared common and separately by dilution of the stock solutions with acetonitrile for HPLC. Moreover, the working solutions of iodosulfuron-methyl-sodium and 2,4-D with a concentration of 10 µg/mL were prepared separately by dilution of the working solutions at concentration of 100 µg/mL with mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v).
Calibration and fortification solutions containing of iodosulfuron-methyl-sodium and 2,4-D acid were prepared by dilution of the common working solution at a concentration of 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution	Final Concentration

		to a final volume of [mL]	[µg/mL]
100	2	10	20[1)]
100	1	10	10[1)]
100	0.5	10	5[1)]
20	1	10	2[1)]
10	1	10	1[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The first standard curve
Working solutions of iodosulfuron-methyl-sodium at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.01 mg iodosulfuron-methyl sodium/L to 2.0 mg iodosulfuron-methyl-sodium/L in Elendt M7 medium.
Working solutions of 2,4-D acid at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.05 µg/mL to 2.0 µg/mL. The range of calibration curve is equivalent to range from 0.05 mg 2,4-D acid/L to 2.0 mg 2,4-D acid/L in Elendt M7 medium.

The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given as µg/mL.

Analyte	Slope	Intercept	Correlation coefficient
Iodosulfuron methyl sodium	183188	264.105	0.9999221
2,4-D acid	114953	166.759	0.9998362

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

The second standard curve
Working solutions of iodosulfuron-methyl-sodium and 2,4-D acid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20.0 µg/mL. The range of calibration curve is equivalent to range from 0.2 mg iodosulfuron-methyl-sodium and from 0.2 mg 2,4-D acid/L to 20.0 mg iodosulfuron-methyl-sodium and to 20 mg 2,4-D acid/L in Elendt M7 medium.

The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
Iodosulfuron methyl sodium	186125	176.781	0.9999933
2,4-D acid	113583	1412.94	0.9998158

Linear weighted calibration (1/x weighting) was used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recov-

ery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery range for iodosulfuron-methyl-sodium in Elendt M7 medium is from 103.5 ± 1.9 % to 105.0 ± 4.0 %. The mean recovery range for 2,4-D acid in Elendt M7 medium is from 102.0± 0.1 % to 105.0 ± 1.6 %. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data – validation method in Elendt M7 medium

Active substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
iodosulfuron-methyl sodium	Elendt M7 medium	0.02	5	105.0	4.0
		0.2	5	103.5	1.9
2,4-D acid		0.1	5	105.0	1.6
		1.0	5	102.0	0.1

In order to study the recovery level, the solutions of the detected substances were added to non-treated Elendt M7 medium samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the Elendt M7 medium is from 0.1% to 4.0%.
The precision is 4.0% at 0.02 mg iodosulfuron-methyl-sodium/L Elendt M7 medium level and 1.9% at 0.2 mg iodosulfuron-methyl-sodium/L Elendt M7 medium level.
The precision is 1.6% at 0.1 mg 2,4-D/L Elendt M7 medium level and 0.1% at 1.0 mg 2,4-D/L Elendt M7 medium level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
The LoD is 0.01 mg iodosulfuron-methyl-sodium/L Elendt M7 medium and equivalent to the lowest calibration standard i.e. 0.01 µg iodosulfuron-methyl-sodium/mL.
The LoD is 0.05 mg 2,4-D acid/L Elendt M7 medium and equivalent to the lowest calibration standard i.e. 0.05 µg 2,4-D acid/mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The LoQ is 0.02 mg iodosulfuron-methyl-sodium/L Elendt M7 medium and equivalent to the calibration level at concentration 0.02 µg iodosulfuron-methyl-sodium/mL.
The LoQ is 0.1 mg 2,4-D acid/L Elendt M7 medium and equivalent to the calibration level at concentration 0.1 µg 2,4-D acid/mL.

Development and validation analytical method of 2,4-D 2-EHE (GC with ECD)

Materials and methods

The analytical method was developed for the determination of 2,4-D 2-EHE in Elendt M7 medium. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of 2,4-D 2-EHE were determined.
The determination was accomplished by the high performance gas chromatography (GC) with ECD detection. Prior to analysis, the samples were concentrated using liquid-liquid extraction.

Reagents, solvents and chemicals
- Ethyl acetate, pure p.a., POCH, batch no. 1135/06/20

- Acetone, pure p.a., POCH, batch no. 1062/06/20
- Anhydrous sodium chloride, pure p.a., POCH, batch no. 1256/07/18
- Anhydrous sodium sulphate, pure p.a., J.T.Baker, batch no. 1831301832
- standard solution of 1 mg/mL of 2,4-D 2-EHE in acetone
- sodium chloride saturated solution
- working solutions of 2,4-D 2-EHE at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 100.0 µg/mL in acetone

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Bruker 450-GC, BRUKER

Chromatographic conditions

- Chromatographic System: Gas Chromatography (GC, Bruker 450-GC)
- Detector: Electron Capture Detector (ECD)
- Analytical column: VF 5 ms 30M×0.25 MM ID DF=0.25
- Oven temperature: 200°C (2 minute); gradient 30°C/minute; 300°C (2 minute)
- Intel temperature: 260°C
- Detector temperature: 300°C
- Injection volume: 1 µl

Sample preparation for the chromatographic analysis

Elendt M7 medium

First, 15 mL of ethyl acetate, 2 mL sodium chloride solution saturated was added to 10 mL of Elendt M7 medium sample and shaken. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 400C. The dry residue was dissolved in 5 mL of acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

Fortified Sample

For validation experiments, 10 mL aliquot of untreated water were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	10	-	-	0.00
Fortification (LOQ)	10	1	0.1	0.01
Fortification (10x LOQ)	10	10	0.1	0.1

This was done to ensure the result fits within the range of the respective standard curve.
An untreated sample of Elendt M7 medium (10 mL) was spiked with the 2,4-D 2-EHE to achieve fortification levels at the limit of quantification of 0.01 mg/L and ten times higher i.e. 0.1 mg/L.

Selectivity and specificity

The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experi-

mental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix blank sample.

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.02 µg/mL prepared in solvent to at one at concentration 0.02 µg/mL prepared in blank matrix, for sample of Elendt M7 medium.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

Matrix effects [%] = 100 × peak area (matrix) / peak area (solvent) – 100

Matrix effect is -7.0% and not exceed ±20%.

Linearity
The stock solution with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard into a volumetric flask with a capacity of 10 mL, dissolving in acetone for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solution with a concentration of 100 µg/mL was prepared by dilution of the stock solution with acetone. Calibration and fortification solutions containing of 2,4-D 2-EHE were prepared by dilution of the working solution at concentration 2, 5, 10 and 100 µg/mL in acetone. Further dilutions were conducted with acetone as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 (Stock)	1	10	100
100	1	10	10
100	0.5	10	5
10	2	10	2
10	1	10	1.0[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The standard curve
Working solutions of 2,4-D 2-EHE at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 1.0 µg/mL. The range of calibration curve is equivalent to range from 0.005 mg 2,4-D 2-EHE /L to 0.5 mg 2,4-D 2-EHE /L in Elendt M7 medium.

The equation of the calibration line was
presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
2,4-D 2-EHE	7205.40031	2.06334	0.9992

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recov-

ery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery range for Elendt M7 medium is from 109.0 ± 8.8% to 116.0 ± 1.6%.
For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Recovery Data for 2,4-D 2-EHE – validation method in Elendt M7 medium

Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
Elendt M7 medium	0.01	5	109.0	8.8
	0.1	5	116.0	1.6

In order to study the recovery level, the solution of the detected substance was added to non-treated Elendt M7 medium samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the Elendt M7 medium is from 1.6% to 8.8%.
The precision is 8.8% at 0.01 mg 2,4-D 2-EHE/L Elendt M7 medium level and 1.6% at 0.1 mg 2,4-D 2-EHE/L Elendt M7 medium level.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample. The LoD is 0.005 mg 2,4-D 2-EHE/L Elendt M7 medium and equivalent to the lowest calibration standard i.e. 0.01 µg 2,4-D 2-EHE/mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). The LoQ is 0.01 mg 2,4-D 2-EHE/L Elendt M7 medium and equivalent to the calibration level at concentration 0.02 µg 2,4-D 2-EHE/mL.

Results and discussions

Parameter	Required criterion	The result	
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)	no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.	
Matrix effect [%]	±20%	2,4-D 2-EHE	-7.0
		2,4-D acid	-0.4
		iodosulfuron methyl sodium	0.2
Linearity	$r^2 \geq 0.99$	2,4-D 2-EHE: $r^2 = 0.9992$ (7205.40031 µg/mL + 2.06334 µg/mL)	
		2,4-D acid: $r^2 = 0.9998362$ (114953 µg/mL + 166.759 µg/mL)	2,4-D acid: $r^2 = 0.9998158$ (113583 µg/mL + 1412.94 µg/mL)
		iodosulfuron methyl sodium: $r^2 = 0.9999221$ (183188 µg/mL +	iodosulfuron methyl sodium: $r^2 = 0.9999933$ (186125 µg/mL +

		264.105 µg/mL)		176.781 µg/mL)
Accuracy [%]	70-120	2,4-D 2-EHE	level I	109.0
			level II	116.0
		2,4-D acid	level I	105.0
			level II	102.0
		iodosulfuron methyl sodium	level I	105.0
			level II	103.5
Precision [% RSDr]	≤ 20	2,4-D 2-EHE	level I	8.8
			level II	1.6
		2,4-D acid	level I	1.6
			level II	0.1
		iodosulfuron methyl sodium	level I	4.0
			level II	1.9
Limit of detection LOD [mg/L]	-	2,4-D 2-EHE		0.005
		2,4-D acid		0.05
		iodosulfuron methyl sodium		0.01
Limit of quantification LOQ [mg/L]	-	2,4-D 2-EHE		0.01
		2,4-D acid		0.1
		iodosulfuron methyl sodium		0.02

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in Elendt M7 medium.

A 2.1.2.7.8 LC-MS/MS (in 20X AAP medium) and GC with ECD (in water)

A 2.1.2.7.8.1 Method validation

Comments of evaluator:	Method is accepted

Reference: KCP 5.1.2/08 (filed as KCP 10.2.1.4/01)

Report: JMD – HER 387 OD *Lemna gibba* CPCC 310, Growth inhibition test, Study code: W-04-21, Czarnecka, M., 2021

Guideline(s): SANTE/2020/12830, rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

Development and validation analytical method of iodosulfuron-methyl-sodium (LC-MS/MS)

Materials and methods

The analytical method was developed for the determination of iodosulfuron-methyl-sodium in 20X AAP

medium. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, accuracy, and limit of quantification and detection of iodosulfuron-methyl sodium were determined.
The determination was accomplished by the high performance liquid chromatographic method with MS/MS detection. Prior to analysis, the samples were concentrated using solid phase extraction method.

Reagents, solvents and chemicals

- Water, deionized (LC-MS), Fresh prepared before analysis
- 20X AAP medium, Fresh prepared before analysis
- Methanol, HPLC, POCH, batch no. 1252/02/20
- Methanol, pure p.a., POCH, batch no. 1158/11/20
- Methanol, LC-MS, POCH, batch no. 0816/04/19
- SUPELCLEAN ENVI-18 SPE, 3 mL, 500 mg, Supelco, batch no. 12546401
- Formic acid, ≥99%, VWR Chemicals, batch no. DB634419
- mixture deionized water : formic acid (1000:1, v/v),
- mixture of methanol LC-MS : 20xAAP medium (20:80, v/v),
- standard solution of 1 mg/mL of iodosulfuron-methyl sodium in methanol for LC-MS,
- working solutions of iodosulfuron-methyl sodium at concentration 10.0, 1.0, 0.1 and 0.01 µg/mL in methanol for LC-MS,
- working solutions of iodosulfuron-methyl sodium at concentration 5.0, 2.0, 1.0, 0.5, 0.2, 0.1 and 0.05 ng/mL in blank matrix of 20X AAP medium extract.

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- SPE vacuum manifold, Visiprep, Supelco (USA)
- SPE cartridges, Supelclean ENVI-18, Supelco (USA)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Chromatograph, Shimadzu Nexera XR, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: Shimadzu Nexera XR
- Analytical column: Kinetex 2.6µm C18 100A, l=50 mm, Ø=2.1 mm
- Column temperature: 35°C
- Flow Rate: 0.6 mL/min
- Injection volume: 10 µl
- Mobile Phase A: Water deionized : Formic acid (1000 : 1, v/v)
- Mobile Phase B: Methanol for LC-MS
- Gradient (including wash and equilibration):

Time [min]	Phase A [%]	Phase B [%]
0.00	90	10
1.00	90	10
2.00	5	95
2.50	5	95
2.52	90	10
5.00	90	10

- Detection System: Shimadzu LCMS-8045 Mass Spectrometer
- Analyte: Iodosulfuron-methyl sodium
- Transitions: 507.80 --> 167.10[1)]
  507.80 --> 141.10[2)]
- Polarity: positive

1) Quantitation transition. Mass transition used for quantification.
2) Confirmatory transition. The second transition has been monitored but will not reported, except for the validation experiment.

Sample preparation for the chromatographic analysis
Each sample of 20 mL volume was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by washing with 10 mL of methanol pure, 10 mL of deionized water. Following the sample introduction, the column was dried under vacuum for 5 minutes. The analyte was eluted with 15 mL methanol for HPLC. The eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in 2 mL of mixture of methanol LC-MS : 20X AAP medium (20:80, v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC with MS/MS detection.

Fortified Sample
For validation experiments, 20 mL aliquot of untreated 20X AAP medium were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Active substance	Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [ng/mL]	Volume of Spiking Solution [mL]	Level of Fortification [µg/L]
iodosulfuron methyl sodium	Control	20	-	-	0.00
	Fortification (LOQ)	20	10	0.02	0.01
	Fortification (10x LOQ)	20	100	0.02	0.10

Sample of 20X AAP medium an untreated (20 mL) was spiked with the solution of iodosulfuron-methyl sodium to achieve fortification levels at the limit of quantification i.e. 0.01 µg/L and ten times higher of LoQ i.e. 0.10 µg/L.
This was done to ensure the result fits within the range of the respective standard curve.

Selectivity and specificity
The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control of 20X AAP medium, and fortified samples of 20X AAP medium. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.
Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.

Matrix effect
No separate investigations on matrix effects were performed, as matrix-matched standards have been used for quantification.

Linearity
The stock solution of iodosulfuron-methyl sodium with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of iodosulfuron-methyl sodium into a volumetric flask with a capacity of 10 mL, dissolving in methanol for LC-MS, and next the volume was made up to 10 mL with the same solvent. The working solutions and fortification solutions of iodosulfuron-methyl sodium with a concentration of 10, 1.0 and 0.1 µg/mL were prepared by dilution of the stock solution with methanol for LC-MS. Calibration solutions containing of iodosulfuron-methyl sodium were prepared by dilution of the working solutions at concentration 0.1 µg/mL, i.e. 100 ng/mL, 0.01 µg/mL, i.e. 10 ng/mL, and 0.001 µg/mL, i.e. 1 ng/mL in methanol for LC-MS. Further dilutions were conducted with extract of blank 20X AAP medium (2) as described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [ng/mL]

1000	1	10	100
100	1	10	10
10	1	10	1.0
100	0.05	1	5.0[1)]
10	0.2	1	2.0[1)]
10	0.1	1	1.0[1)]
10	0.05	1	0.5[1)]
1	0.2	1	0.2[1)]
1	0.1	1	0.1[1)]
1	0.05	1	0.05[1)]

1) Concentration level used for calibration.
2) 100 mL volume of 20X AAP medium was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by washing with 10 mL of methanol pure, 10 mL of deionized water. Following the sample introduction, the column was dried under vacuum for 5 minutes. The components 20xAAP medium were eluted with 15 mL methanol for HPLC. The eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in 10 mL of mixture of methanol LC-MS : 20X AAP medium (20:80, v/v).

Working solutions at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 ng/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graphs is from 0.05 ng/mL to 5.0 ng/mL.

The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given as ng/mL.

Analyte	Transitions	Slope	Intercept	Coefficient r^2
Iodosulfuron methyl sodium	Quantitation Transition 507.80 → 167.10	77425.98	2016.486	0.9996588
	Confirmatory Transition 507.80 → 141.10	5273.528	82.72816	0.9991359

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation (RSD). The accuracy of the analytical method was tested for the analyte iodosulfuron-methyl sodium in 20X AAP samples using five replicates per fortification level. Control samples (2 replicates) were additionally prepared and handled in exactly the same way as fortified samples, except that no analyte was spiked.
Recovery data was reported for two fortification levels of iodosulfuron-methyl sodium appropriate to level corresponding with LoQ and 10 x LoQ. Mean recoveries for each level is in the range 70-120%.
The mean recovery range for iodosulfuron-methyl sodium in 20X AAP medium is from 95.0 ± 11.5 % to 103.7 ± 4.2%. For analyte, the relative standard deviations (RSD) at each fortification levels were below 20%. All results obtained from measurements of control samples were below the LoD.

Recovery Data – validation method in 20X AAP medium

Active substance	Matrix	Transitions	Fortification Level [µg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
iodosulfuron-methyl sodium	20X AAP medium	Quantitation Transition 507.80 → 167.10	0.01	5	100.0	3.5
			0.10	5	99.7	2.8
		Confirmatory Transition 507.80 → 141.10	0.01	5	95.0	11.5
			0.10	5	103.7	4.2

In order to study the recovery level, the solutions of the detected substances were added to non-treated 20X AAP medium samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The precision is 3.5% at level 0.01 µg/L 20X AAP medium and 2.8% at level 0.1 µg/L 20X AAP medium for Quantitation Transition. The precision is 11.5% at level 0.01 µg/L 20X AAP medium and 4.2% at level 0.1 µg/L 20X AAP medium for Confirmatory Transition.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
The LoD is 0.005 µg/L 20X AAP medium and equivalent to the lowest calibration standard i.e. 0.05 ng/mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The LoQ is 0.01 µg/L 20X AAP medium and equivalent to the calibration level at concentration 0.1 ng/mL.

Development and validation analytical method of 2,4-D 2-EHE (GC with ECD)

Materials and methods

The analytical method was developed for the determination of 2,4-D 2-EHE in water. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection of 2,4-D 2-EHE were determined.
The determination was accomplished by the high performance gas chromatography (GC) with ECD detection. Prior to analysis, the samples were concentrated using liquid-liquid extraction.

Reagents, solvents and chemicals
- Ethyl acetate, pure p.a., POCH, batch no. 1135/06/20
- Acetone, pure p.a., POCH, batch no. 1062/06/20
- Anhydrous sodium chloride, pure p.a., POCH, batch no. 1256/07/18
- Anhydrous sodium sulphate, pure p.a., J.T.Baker, batch no. 1831301832
- standard solution of 1 mg/mL of 2,4-D 2-EHE in acetone
- sodium chloride saturated solution
- working solutions of 2,4-D 2-EHE at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 100.0 µg/mL in acetone

Equipment
- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Bruker 450-GC, BRUKER

Chromatographic conditions
- Chromatographic System: Gas Chromatography (GC, Bruker 450-GC)
- Detector: Electron Capture Detector (ECD)
- Analytical column: VF 5 ms 30M×0.25 MM ID DF=0.25
- Oven temperature: 200°C (2 minute); gradient 30°C/minute; 300°C (2 minute)
- Intel temperature: 260°C

- Detector temperature: 300°C
- Injection volume: 1 µl

Sample preparation for the chromatographic analysis

Water

First, 30 mL of ethyl acetate, 2 mL sodium chloride solution saturated was added to 100 mL of water sample and shaken. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 40°C. The dry residue was dissolved in 1.5 mL of acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

Fortified Sample

For validation experiments, 10 mL aliquot of untreated water were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used:

Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	100	-	-	0.000
Fortification (LOQ)	100	1	0.1	0.001
Fortification (10x LOQ)	100	10	0.1	0.01

This was done to ensure the result fits within the range of the respective standard curve.
Sample of water an untreated (100 mL) was spiked with the 2,4-D 2-EHE to achieve fortification levels at the limit of quantification of 0.001 mg/L and ten times higher.

Selectivity and specificity

The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control water, and fortified samples of water. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the control sample.

Matrix effect

Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.05 µg/mL prepared in solvent to at one at concentration 0.05 µg/mL prepared in blank matrix, for sample of water. Matrix effect, expressed in [%] enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)} / \text{peak area (solvent)} - 100$$

Matrix effect is 1.6% and not exceed ±20%.

Linearity

The stock solution with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard into a volumetric flask with a capacity of 10 mL, dissolving in acetone, and next the volume was made up to 10 mL with the same solvent. The working solution with a concentration of 100 µg/mL was prepared by dilution of the stock solution with acetone. Calibration and fortification solutions containing of 2,4-D 2-EHE were prepared by dilution of the working solution at concentration 2, 5, 10, and 100 µg/mL in acetone. Further dilutions were conducted with acetone as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 (Stock)	1	10	100
100	1	10	10

100	0.5	10	5
10	2	10	2
10	1	10	1.0[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

The standard curve

Working solutions of 2,4-D 2-EHE at the concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.01 µg/mL to 1.0 µg/mL. The range of calibration curve is equivalent to range from 0.00015 mg 2,4-D 2-EHE /L to 0.015 mg 2,4-D 2-EHE /L in water.

The equation of the calibration line was
presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/ml.

Analyte	Slope	Intercept	Correlation coefficient
2,4-D 2-EHE	7205.40031	2.06334	0.9992

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for two fortification levels appropriate to level corresponding with LoQ and 10 x LoQ. Recovery was reported as mean recovery ±relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery ranged for water is from 97.0 ± 12.0 % to 102.0 ± 6.5%. For analyte, the relative standard deviations (RSD) at each fortification levels were below 20%.

Recovery Data for 2,4-D 2-EHE – validation method in water

Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
Water	0.001	5	102.0	6.5
	0.01	5	97.0	12.0

In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed using the method described above.

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the water is from 6.5% to 12.0%.
The precision is 6.5% at 0.001 mg 2,4-D 2-EHE/L water level and 12.0% at 0.01 mg 2,4-D 2-EHE/L water level. The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample. The LoD is 0.00015 mg 2,4-D 2-EHE/L water and equivalent to the lowest calibration standard i.e. 0.01 µg 2,4-D 2-EHE /mL.
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of prefera-

bly ≤ 20%). The LoQ is 0.001 mg 2,4-D 2-EHE /L water and equivalent to the calibration level at concentration 0.06 µg 2,4-D 2-EHE/mL.

Results and discussions

<table>
<tr><th>Parameter</th><th>Required criterion</th><th colspan="4">The result</th></tr>
<tr><td>Selectivity</td><td>at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)</td><td colspan="4">no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.</td></tr>
<tr><td rowspan="2">Matrix effect [%]</td><td rowspan="2">±20%</td><td colspan="2">2,4-D 2-EHE</td><td colspan="2">1.6</td></tr>
<tr><td colspan="2">iodosulfuron methyl sodium</td><td colspan="2">No separate investigations on matrix effects were performed, as matrix-matched standards have been used for quantification</td></tr>
<tr><td rowspan="2">Linearity</td><td rowspan="2">$r^2 \geq 0.99$</td><td colspan="4">2,4-D 2-EHE: $r^2 = 0.9992$
(7205.40031 µg/mL + 2.06334 µg/mL)</td></tr>
<tr><td colspan="2">iodosulfuron methyl sodium:
$r^2 = 0.9996588$
(77425.98 ng/mL + 2016.486 ng/mL)</td><td colspan="2">iodosulfuron methyl sodium:
$r^2 = 0.9991359$
(5273.528 ng/mL + 82.72816 ng/mL)</td></tr>
<tr><td rowspan="4">Accuracy [%]</td><td rowspan="4">70-120</td><td rowspan="2">2,4-D 2-EHE</td><td>level I</td><td colspan="2">102.0</td></tr>
<tr><td>level II</td><td colspan="2">97.0</td></tr>
<tr><td rowspan="2">iodosulfuron methyl sodium</td><td>level I</td><td>100.0</td><td>95.0</td></tr>
<tr><td>level II</td><td>99.7</td><td>103.7</td></tr>
<tr><td rowspan="4">Precision [% RSDr]</td><td rowspan="4">≤ 20</td><td rowspan="2">2,4-D 2-EHE</td><td>level I</td><td colspan="2">6.5</td></tr>
<tr><td>level II</td><td colspan="2">12.0</td></tr>
<tr><td rowspan="2">iodosulfuron methyl sodium</td><td>level I</td><td>3.5</td><td>11.5</td></tr>
<tr><td>level II</td><td>2.8</td><td>4.2</td></tr>
<tr><td rowspan="2">Limit of detection LOD</td><td rowspan="2">-</td><td colspan="2">2,4-D 2-EHE</td><td colspan="2">0.00015 mg/L</td></tr>
<tr><td colspan="2">iodosulfuron methyl sodium</td><td colspan="2">0.005 µg/L</td></tr>
<tr><td rowspan="2">Limit of quantification LOQ</td><td rowspan="2">-</td><td colspan="2">2,4-D 2-EHE</td><td colspan="2">0.001 mg/L</td></tr>
<tr><td colspan="2">iodosulfuron methyl sodium</td><td colspan="2">0.01 µg/L</td></tr>
</table>

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in 20X AAP medium and water.

A 2.1.2.7.9 GC with ECD (in aqueous phase and sediment) and LC-MS/MS (in aqueous phase – Smart and Barko medium)

A 2.1.2.7.9.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/09 (filed as KCP 10.2.1.4/02)
Report	JMD-HER 387 OD Water-sediment *Myriophyllum spicatum* toxicity test, Study code: W-05-21, Turek-Lipka, T., 2021
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Development and validation analytical method of 2,4-D 2-EHE (GC with ECD)

Materials and methods

The analytical method was developed for the determination of 2,4-D 2-EHE in water and sediment. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the gas chromatography (GC) with ECD detection.
The concentrations of active substance of test item were chemically determined using the validated gas chromatography (GC) with ECD detection. The concentration of 2,4-D 2-EHE was chemically determined in aqueous phase of water-sediment system of each test item concentration and the control at exposure initiation. Moreover, the chemical determinations in sediment phase of water-sediment system of the highest test item concentration and the control was performed. The concentration of 2,4-D 2-EHE was chemically determined in aqueous phase and sediment phase of water-sediment system of test item at the lowest and the highest concentration and the control after 7 days exposure. The concentration of 2,4-D 2-EHE was chemically determined in aqueous phase and sediment phase of water-sediment system of each test item concentration and the control at exposure termination.

Reagents, solvents and chemicals

- Ethyl acetate, pure p.a., POCH, batch no. 1135/06/20; 1183/11/20; 1238/05/21
- Acetone, pure p.a., POCH, batch no. 1062/06/20; 1199/03/21
- Anhydrous sodium chloride, pure p.a., POCH, batch no. 1256/07/18
- Anhydrous sodium sulphate, pure p.a., J.T.Baker, batch no. 1831301832 and Acros Organics, batch no. A0410956
- standard solution of 1 mg/mL of 2,4-D 2-EHE in acetone
- working solutions of 2,4-D 2-EHE at concentration 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 100.0 µg/mL in acetone

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Balance, WPS 510/C, ZMP RADWAG (Poland)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Pipettes, Various volumes, Brand (Germany)
- Variable volume pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Bruker 450-GC, BRUKER

Chromatographic conditions

- Chromatographic System: Gas Chromatography (GC, Bruker 450-GC)
- Detector: Electron Capture Detector (ECD)
- Analytical column: VF 5 ms 30M×0.25 MM ID DF=0.25
- Oven temperature: 200°C (2 minute); gradient 30°C/minute; 300°C (2 minute)
- Intel temperature: 260°C
- Detector temperature: 300°C
- Injection volume: 1 µl

Working solutions

Stock and standard solutions

The stock solution with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard into a volumetric flask with a capacity of 10 mL, dissolving in acetone, and next the volume was made up to 10 ml with the same solvent. The working solution with a concentration of 100 µg/mL was prepared by dilution of the stock solution with acetone. Calibration and fortification solutions containing of 2,4-D 2-EHE were prepared by dilution of the working solution at concentration 2, 5, 10 and 100 µg/mL in acetone. Further dilutions were conducted with acetone as exemplarily described in the table below:

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 (Stock)	1	10	100
100	1	10	10
100	0.5	10	5
10	2	10	2
10	1	10	1.0[1)]
5	1	10	0.5[1)]
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.2	1	10	0.02[1)]
0.1	1	10	0.01[1)]

[1)] Concentration level used for calibration.

Fortified Sample

Water phase

For the preparation of procedural recoveries and validation experiments, fortification water were prepared from standard solutions with a concentration of 1 and 10 µg/mL. Standard solutions were added to water as exemplarily described in the table below:

Sample Type	Sample [mL]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	100	-	-	0.000
Fortification (LOQ)	100	10	0.1	0.001
Fortification (10x LOQ)	100	100	0.1	0.01

Sediment phase

For the preparation of procedural recoveries and validation experiments, fortification sediment were prepared from standard solutions with a concentration of 10 and 100 µg/mL. Standard solutions were added to sediment as exemplarily described in the table below:

Sample Type	Sample [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	10	-	-	0.00
Fortification (LOQ)	10	10	0.1	0.05
Fortification (10x	10	100	0.1	0.50

LOQ)				

Sample preparation for the chromatographic analysis

Water phase

First, 30 mL of ethyl acetate, 2 mL sodium chloride solution saturated was added to 100 mL of water sample and shaken. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 40°C. The dry residue was dissolved in acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

Sediment phase

First, 15 mL of ethyl acetate was added to 10 g of artificial soil sample and shaken for 30 minutes. The organic phases were filtered through anhydrous sodium sulphate (VI). The extraction was repeated with 15 mL of ethyl acetate. The extracts were evaporated to dryness using vacuum rotary evaporator at temperature 40°C. The dry residue was dissolved in 10 mL of acetone. An aliquot of the final volume was transferred into a GC vial for further quantification using GC-ECD.

This was done to ensure the result fits within the range of the respective standard curve.

Selectivity and specificity

The analytical methods specificity was estimated on the basic of the analysis of the chromatograms obtained for the control of matrices, and fortified samples of matrices Considering the results of the analysis, no signal of detected substance was overlapping with matrices signal of the control samples in the experiments conditions. Therefore, the specificity of the methods were demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatograms of the matrices control sample.

Matrix effect

Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard prepared in solution to standard preparing in control samples at appropriate concentration. The matrix effects and concentrations are presented in table below.

Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)} / \text{peak area (solvent)} - 100$$

The matrix effect does not exceed ± 20 % in any of the methods:

Method	matrix	Concentration [mg/L]	matrix effect [%]
water phase method	water	0.05	1.6
sediment phase method	sediment	0.05	6.1

Linearity

Working solutions of 2,4-D 2-EHE at all calibration levels were injected at volume 1 µL successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph range from 0.01 µg/mL to 1 µg/mL. The ranges of calibration curve are equivalent to ranges from 0.00015 mg 2,4-D 2-EHE/L to 0.015 mg 2,4-D 2-EHE/L in water and equivalent to ranges from 0.01 mg 2,4-D 2-EHE/kg to 1 mg 2,4-D 2-EHE/kg sediment.

The equation of the calibration line was presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	Slope	Intercept	Correlation coefficient
2,4-D 2-EHE	7205.40031	2.06334	0.9992

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demon-

strated as the regression residual (di).

Accuracy
The accuracy of the methods are reported as mean recovery ± relative standard deviation. Recovery data for water and sediment phase methods were reported for 2 fortification levels appropriate to level corresponding with LOQ and 10 x LOQ. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery ranged for water phase is from 97.0% to 102.0%. The mean recovery for sediment phase is from 88.8% to 111.3%.
A summary of the recovery data of control and fortified samples is presented in the table below.

Method	Matrix	Fortification Level	Number of Repetition	Mean Recovery [%]	RSD [%]
water phase method	water	Control	2	-	-
		0.001 mg/L	5	102.0	6.5
		0.01 mg/L	5	97.0	12.0
sediment phase method	sediment	Control	2	-	-
		0.05 mg/kg	5	111.3	1.7
		0.5 mg/kg	5	88.8	3.4

In order to study the recovery level, the solution of the detected substance was added to non-treated water and sediment samples and then analysed using the methods described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed is:
in the water phase from 6.5% to 12.0%. The repeatability for detected substance analysed in the sediment phase is from 1.7% to 3.4%.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below.

Method	LOQ (limit of quantification)	Equivalent calibration level	LOD (limit of detection)	Equivalent calibration level
water phase method	0.05 mg 2,4-D 2-EHE/kg	0.05 mg/L	0.01 mg 2,4-D 2EHE/kg	0.01 mg/L
sediment phase method	0.001 mg 2,4-D 2-EHE/L	0.06 mg/L	0.00015 mg 2,4-D 2-EHE/L	0.01 mg/L

<u>Development and validation analytical method of iodosulfuron-methyl-sodium (LC-MS/MS)</u>

Materials and methods

The analytical method was developed for the determination of iodosulfuron methyl sodium in aqueous phase – Smart and Barko medium. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatographic method with

MS/MS detection.
The concentration of active substance of test item were chemically determined using the validated high performance liquid chromatographic method with MS/MS detection. The concentration of iodosulfuron methyl sodium was chemically determined in aqueous phase of water-sediment system of all test item concentration and the control at exposure initiation and termination exposure. Moreover, the concentration of iodosulfuron methyl sodium was chemically determined in aqueous phase of water-sediment system of test item at the lowest, the highest concentration and the control after 7 days exposure.

Reagents, solvents and chemicals

- Water, deionized (LC-MS), Fresh prepared before analysis
- Smart & Barko, Fresh prepared before analysis
- Methanol, pure p.a., POCH, batch no. 1158/11/20
- Methanol, LC-MS, POCH, batch no. 0816/04/19 and 0820/08/20
- SUPELCLEAN ENVI-18 SPE, 3 mL, 500 mg, Supelco, batch no. 12546401
- Formic acid, ≥99%, VWR Chemicals, batch no. DB634419
- mixture deionized water : formic acid (1000:1, v/v),
- mixture of methanol LC-MS : Smart Barko (20:80, v/v),
- standard solution of 1 mg/mL of iodosulfuron-methyl sodium in methanol for LC-MS,
- working solutions of iodosulfuron-methyl sodium at concentration 0.1 and 0.01 µg/mL in methanol for LC-MS,
- working solutions of iodosulfuron-methyl sodium at concentration 5.0, 2.0, 1.0, 0.5, 0.2, 0.1 and 0.05 ng/mL in blank matrix of Smart Barko medium extract.

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- SPE vacuum manifold, Visiprep, Supelco (USA)
- SPE cartridges, Supelclean ENVI-18, Supelco (USA)
- Automatic pipettes, Various volume, Eppendorf (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Chromatograph, Shimadzu Nexera XR, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: Shimadzu Nexera XR
- Analytical column: Kinetex 2.6µm C18 100A, l=50 mm, Ø=2.1 mm
- Column temperature: 35°C
- Flow Rate: 0.6 mL/min
- Injection volume: 10 µl
- Mobile Phase A: Water deionized : Formic acid (1000 : 1, v/v)
- Mobile Phase B: Methanol for LC-MS
- Gradient (including wash and equilibration):

Time [min]	Phase A [%]	Phase B [%]
0.00	90	10
1.00	90	10
2.00	5	95
2.50	5	95
2.52	90	10
5.00	90	10

- Detection System: Shimadzu LCMS-8045 Mass Spectrometer
- Ionisation: Electro Spray (ESI)
- Analyte: Iodosulfuron-methyl sodium
- Transitions: 507.80 --> 167.10[1)]

507.80 --> 141.10[2)]

- Polarity: positive

1) Quantitation transition. Mass transition used for quantification.
2) Confirmatory transition. The second transition has been monitored but will not reported, except for the validation experiment.

Sample preparation for the chromatographic analysis

Each sample of 20 mL volume was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by washing with 10 mL of methanol pure, 10 mL of deionized water. Following the sample introduction, the column was dried under vacuum for 5 minutes. The analyte was eluted with 15 mL methanol for LC-MS. The eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in 2 mL of mixture of methanol LC-MS : Smart Barko medium (20:80, v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC with MS/MS detection.
This was done to ensure the result fits within the range of the respective standard curve.

Fortified Sample

Aqueous phase

For the preparation of procedural recoveries and validation experiments, fortification sediment were prepared from standard solutions with a concentration of 10 and 100 ng/mL. Standard solutions were added to Smart Barko medium as exemplarily described in the table below:

Active substance	Sample Type	Sample Volume [mL]	Concentration of Spiking Solution [ng/mL]	Volume of Spiking Solution [mL]	Level of Fortification [µg/kg]
iodosulfuron methyl sodium	Control	20	-	-	0.00
	Fortification (LOQ)	20	10	0.02	0.01
	Fortification (10x LOQ)	20	100	0.02	0.1

Selectivity and specificity

The analytical methods specificity was estimated on the basic of the analysis of the chromatograms obtained for the control of matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the methods were demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatogram of the control sample.

Matrix effect

No separate investigations on matrix effects were performed, as matrix-matched standards have been used for quantification.

Linearity

The stock solution of iodosulfuron-methyl sodium with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of iodosulfuron-methyl sodium into a volumetric flasks with a capacity of 10 mL, dissolving in methanol for LC-MS, and next the volume was made up to 10 mL with the same solvent. The fortification solutions of iodosulfuron-methyl sodium with a concentration of 10, 0.1 and 0.01 µg/mL were prepared by dilution of the stock solution with methanol for LC-MS. Calibration solutions containing of iodosulfuron-methyl sodium were prepared by dilution of the working solutions at concentration 0.1 µg/mL i.e. 100 ng/mL, 0.01 µg/mL i.e. 10 ng/mL in methanol for LC-MS. Further dilutions were conducted with extract of blank Smart & Barko medium (2) as described in the table below:

Take solution [ng/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [ng/mL]
1000	1	10	100
100	1	10	10

10	1	10	1.0
100	0.05	1	5.0[1)]
100	0.02	1	2.0[1)]
10	0.1	1	1.0[1)]
10	0.05	1	0.5[1)]
10	0.02	1	0.2[1)]
1	0.1	1	0.1[1)]
1	0.05	1	0.05[1)]

1) Concentration level used for calibration.
2) 100 mL volume of Smart & Barko medium was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by washing with 10 mL of methanol pure, 10 mL of deionized water. Following the sample introduction, the column was dried under vacuum for 5 minutes. The components 20xAAP medium were eluted with 15 mL methanol for HPLC. The eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in 10 mL of mixture of methanol LC-MS : Smart & Barko medium (20:80, v/v).

Linearity – fortified samples

Working solutions of iodosulfuron methyl sodium at all calibration levels were injected at volume 10 µL successively to the chromatographic column and the chromatograms were recorded.
Working solutions at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 ng/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graphs are from 0.05 ng/mL to 5.0 ng/mL.
The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in ng/mL.

Analyte	Transitions	Slope	Intercept	Coefficient r^2
Iodosulfuron methyl sodium	Quantitation Transition 507.80 → 167.10	243138.3	3359.467	0.9996111
	Confirmatory Transition 507.80 → 141.10	13789.69	146.8471	0.9992795

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Linearity – test samples

Before the each analyses the new calibration curve in range from 0.05 ng/mL to 5 ng/mL was recorded. The results generated during the definitive experiment was calculated on base the three different calibration curves. The standard curve at exposure initiation, after 7 days and in exposure termination in definitive experiment (peak area versus quantity of the standard) are linear.
The equations of the calibration line are presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear was given in ng/mL.

Analyte	Transitions	Date of analysis	Slope	Intercept	Coefficient r^2
Iodosulfuron methyl sodium	Quantitation Transition 507.80 → 167.10[1)]	31.08.2021	195679.6	-2533.537	0.9997373
		07.09.2021	255155.8	-1190.855	0.9981825
		14.09.2021	251377.1	118.4166	0.9989759

1) Quantitation transition. Mass transition used for quantification.

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation (RSD). The accuracy of the analytical method was tested for the analyte iodosulfuron methyl sodium in Smart Barko samples using five replicates per fortification level. Control samples (2 replicates) were additionally prepared and handled in exactly the same way as fortified samples, except that no analyte was spiked. Recovery data was reported for 2 fortification levels of iodosulfuron-methyl sodium appropriate to level corre-

sponding with LoQ and 10 x LoQ. Mean recoveries for each level is in the range 70-120%.
The mean recovery range for iodosulfuron-methyl sodium in Smart Barko medium is from 84.0 ± 3.9 % to 109.9 ± 2.3%. For analyte, the relative standard deviations (RSD) at each fortification levels were below 20%. All results obtained from measurements of control samples were below the LOD.
A summary of the recovery data of control and fortified samples is presented in the table below.

Active substance	Matrix	Transitions	Fortification Level [µg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
iodosulfuron-methyl sodium	Smart & Barko medium	Quantitation Transition 507.80 → 167.10	0.01	5	85.0	9.6
			0.10	5	109.9	2.3
		Confirmatory Transition 507.80 → 141.10	0.01	5	84.0	3.9
			0.10	5	106.0	2.6

In order to study the recovery level, the solution of the detected substance was added to non-treated Smart & Barko medium samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The precision is 9.6% at level 0.01 µg/L 20xAAP medium and 2.3% at level 0.1 µg/L Smart Barko medium for Quantitation Transition. The precision is 3.9% at level 0.01 µg/L Smart Barko medium and 2.6% at level 0.1 µg/L Smart Barko medium for Confirmatory Transition.
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
Limit of quantification (LoQ) and a limit of detection (LoD) are presented in the table below.

Active substance	LOQ (limit of quantification)	Equivalent calibration level	LOD (limit of detection)	Equivalent calibration level
iodosulfuron-methyl sodium	0.01 µg/L	0.1 µg/L	0.005 µg/L	0.05 µg/L

Limit of Quantification (LoQ) was estimated as the lowest concentration of a detected substances at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).

Results and discussions

Parameter	Required criterion	The result	
Selectivity	at the places originating from active substances signals, there is no signals originating from other substances of area exceeding 30% of active substances areas in the test item solution at the level (LOQ)	no signal of detected substance was overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated.	
Matrix effect [%]	±20%	2,4-D 2-EHE (water)	1.6
		2,4-D 2-EHE (sediment)	6.1

		iodosulfuron methyl sodium		No separate investigations on matrix effects were performed, as matrix-matched standards have been used for quantification	
Linearity	$r^2 \geq 0.99$	2,4-D 2-EHE: r^2 = 0.9992 (7205.40031 µg/mL + 2.06334 µg/mL)			
		iodosulfuron methyl sodium: r^2 = 0.9996111 (243138.3 ng/mL + 3359.467 ng/mL)		iodosulfuron methyl sodium: r^2 = 0.9992795 (13789.69 ng/mL + 146.8471 ng/mL)	
Accuracy [%]	70-120	2,4-D 2-EHE (water)	level I	102.0	
			level II	97.0	
		2,4-D 2-EHE (sediment)	level I	111.3	
			level II	88.8	
		iodosulfuron methyl sodium	level I	85.0	84.0
			level II	109.9	106.0
Precision [% RSDr]	≤ 20	2,4-D 2-EHE (water)	level I	6.5	
			level II	12.0	
		2,4-D 2-EHE (sediment)	level I	1.7	
			level II	3.4	
		iodosulfuron methyl sodium	level I	9.6	3.9
			level II	2.3	2.6
Limit of detection LOD	-	2,4-D 2-EHE (water)		0.01 mg/kg	
		2,4-D 2-EHE (sediment)		0.00015 mg/L	
		iodosulfuron methyl sodium		0.005 µg/L	
Limit of quantification LOQ	-	2,4-D 2-EHE (water)		0.05 mg/kg	
		2,4-D 2-EHE (sediment)		0.001 mg/L	
		iodosulfuron methyl sodium		0.01 µg/L	

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances (2,4-D and iodosulfuron methyl sodium) of the test item JMD HER 387 OD in water and sediment and in aqueous phase – Smart and Barko medium.

A 2.2 Analytical methods for iodosulfuron-methyl sodium

A 2.2.1 Methods used for the generation of pre-authorization data (KCP 5.1)

Please refer to the points 5.2.1.1 and 5.2.1.2.

A 2.2.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.2.2.1 Description of analytical methods for the determination of residues in

plant matrices (KCP 5.2)

No new or additional studies have been submitted.

A 2.2.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new or additional studies have been submitted.

A 2.2.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

No new or additional studies have been submitted.

A 2.2.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

No new or additional studies have been submitted.

A 2.2.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted.

A 2.2.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

No new or additional studies have been submitted.

A 2.2.2.7 A.2.A.9 Other Studies/ Information

Please refer to point A 2.1.2.7