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- Flutolanil -

B.5 Methods of analysis

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B.5 Methods of analysis

B.5.1 Methods used for the generation of pre-approval data

B.5.1.1 Methods for the analysis of the active substance as manufactured

Determination of the pure active substance in the active substance as manufactured

The analytical method for the determination of the active ingredient in technical product that was submitted for the first inclusion of flutolanil into Annex I of Council Directive 91/414/EEC was reviewed under uniform principles, and found to be acceptable. Although this GC/FID method is still considered adequate to address this endpoint, a new analytical method based on HPLC/UV has been submitted. In view of this new method, the analytical method evaluated in the DAR has not been summarised in the RAR. Below follows the evaluation of the newly submitted method.

Reference	:	Matsumoto, T. (2016) K-CA 4.1.1/01	GLP statement	:	Yes
Type of study	:	Validation of the analytical method for active ingredient in flutolanil technical	Guideline	:	SANCO/3030/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3079

The validation of the analytical method for the active ingredient is summarised below.

Principle of the method

The contents of the active ingredient in Flutolanil technical were determined by HPLC internal standard methods (HPLC analysis and UV-absorption spectrum).

An internal standard solution was prepared by dissolving an accurate amount of Diethyl Phthalate in methanol. An accurate weight of Flutolanil technical was dissolved in methanol using an ultrasonic cleaning device. The solution is spiked with the internal solution before analysis by HPLC/UV.

HPLC/UV conditions:

Column: Inertsil ODS-P, 4.6 x 150 mm, 5 µm

Mobile phase: A = ultrapure water containing 0.085% phosphoric acid

B = methanol

Time (min.)	A (%)	B (%)	Gradient
0	65	35	Linear gradient
15	30	70	Linear gradient
20	0	100	Retain
25	0	100	

Column temperature: 50°C

Flow rate: 1.0 mL/min

Detection: 230 nm (SPD-20A), 210-400 nm (SPD-M20A)
Injection volume: 5 µL
Analysis time: 25 min.

UV-absorption and ^{1}H-NMR spectra:

UV-absorption spectra were obtained by the photodiode array detector connected to HPLC. The characterization (purity and storage stability) and identification of Flutolanil technical used in this study were performed by ^{1}H-NMR and HPLC.

Validation:

The method validation data are summarised in the table below:

Parameters		**Flutolanil**
Linearity	**Concentration Range (mg/L)**	5 calibration points: 30-70 mg (corresponding to 600-1700 mg/L)
	Intercept (a)	0.0356
	Slope of the line (b)	0.0226
	Correlation Coefficient (r)	1.000
Limit of Quantification (LOQ)	**% w/w (in samples based on % recovery)**	n.a.
Precision	**Mean Content (% w/w)**	97.0%
	% RSD	0.12%
	Acceptance criteria	<1%
Accuracy (% Recovery)	**Level-I**	50 mg
	Mean Recovery (n=6)	99.9%
	% RSD	0.1%
	Acceptance criteria	100 ± 2%
Stability	The test solution was proved to be stable for 37.3 hours at ambient temperature (99.9%).	
1H-NMR	There was no change in the 1H-NMR spectrum from the starting to the end of the experimentation, proving that it was stable.	
Specificity (Non-analyte interference)	No interference.	
	UV spectrum and retention time of the active ingredient in Flutolanil technical were identical with those of Flutolanil standards.	

n.a. = not applicable

Conclusion:

The method was successfully validated in compliance with SANCO/3030/99 rev. 4, and is suitable for the determination of flutolanil in the technical material.

Applicability of existing CIPAC methods

No CIPAC methods are available for the determination of flutolanil in technical material and formulations.

Determination of significant and relevant impurities and additives in the active substance as manufactured

There are no impurities considered to be of toxicological or environmental significance in flutolanil as manufactured that would justify the submission of enforcement methods. No stabilizers or other additives are included in the active substance as manufactured.

B.5.1.2 Methods for risk assessment

B.5.1.2.1 Methods used in support of environmental fate studies

Reference	:	Van de Ruit, A.N.R. (1998) K-CA 4.1.2/01	GLP statement	:	Yes
Type of study	:	Method validation study for the analysis of flutolanil in soil and in soil/potato mixture by gas chromatography/mass spectrometry	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3039

The objective of the study was to validate a method for the analysis of flutolanil in soil by Gas Chromatography – Mass Spectrometry (GC/MS). An additional matrix, consisting of a soil/potato mixture, was included in the validation experiments.

Principle of the method:

Sample were homogenized, extracted with acetone and centrifuged. Acetone was evaporated and the remaining water phase back-extracted with petroleum ether. After dehydration and solid-phase extraction the sample extracts were dissolved in methanol and flutolanil was analysed by fused silica capillary gas chromatography with MSD and internal standardization. Monitoring of two characteristic ions was used, m/z 173 for quantification and m/z 145 for confirmation.

Soil details:

Code	UK	BGN1 (topsoil)	BGN2 (topsoil)	BGN3
Origin of the soil	Manningtree, UK	Ottersum, The Netherlands	Goch, Northern Germany	Niederkirchen, Southern Germany
Reception date	April 17, 1997	May 8, 1997	May 8, 1997	May 8, 1997

GC-MS conditions:

Instrument:	GC: HP 5890a
Injection:	1 µL, on-column
Column:	CP-Sil 5 CB fused silica WCOT, length 25 m.
Pre-column:	uncoated fused silica, length ca. 2m.
Carrier gas:	Helium, ca 1.0 ml/min

Detector: MS: HP 5972-1

Mass range: target ion 173 m/z, product ion (Q1) 145 m/z

Assessment of method validation in soil

Validation data are presented in the table below.

Parameters		Flutolanil				
Linearity	Concentration Range (ng/mL)	4 calibration points – 0.1 – 1.0 mg/L		5 calibration points – 1.0 – 20 mg/L		
	Remark	Linearity covering the concentration range from the LOQ to 10xLOQ ± at least 20%.				
	Intercept (a)	-		-0.139		
	Slope of the line (b)	0.254		0.375		
	Correlation Coefficient (r)	0.9985		0.9995		
Limit of Detection (LOD)		0.0005 mg/kg (n=4)				
Limit of Quantification (LOQ)		0.005 mg/kg				
Precision and Accuracy (%)	Soil code	UK	UK	BGN 1 (NL)	BGN 2 (DE)	BGN 3 (DE)
	Intra-assay or inter-assay	intra-assay	inter-assay	intra-assay		
	Fortification Level-I (µg/kg)	5	5	5	5	5
	Mean Recovery	106% (n=5)	96% (n=4)	83% (n=3)	91% (n=3)	92% (n=3)
	% RSD	3%	7%	9%	6%	11%
	Fortification Level-II (µg/kg)	250	250	250	250	250
	Mean Recovery	95% (n=5)	110% (n=5)	108% (n=3)	101% (n=3)	114% (n=3)
	% RSD	4%	13%	4%	12%	9%
	Fortification Level-III (µg/kg)	1000	1000	1000	1000	1000
	Mean Recovery	85% (n=5)	95% (n=5)	108% (n=3)	97% (n=3)	104% (n=3)
	% RSD	4%	8%	9%	11%	4%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%				
Confirmatory		Additional confirmatory analysis is not required as the primary method is a highly specific method (GC/MS).				
Specificity		Control samples were analysed and the average measured concentration of flutolanil detected is 0.00014 mg/kg which is less than 30% of the LOQ (0.0015 mg/kg). So the specificity of the method has been proven.				

Assessment of method validation in soil/potato

An additional matrix, consisting of a soil/potato mixture, was included in the validation experiments. The validation are not fully validated following the criteria of SANCO/3029/99 rev.4 but are presented here for the sake of completeness. Validation data are presented in the table below.

Parameters		Flutolanil	
Limit of Detection (LOD)		0.0008 mg/kg (n=4)	
Limit of Quantification (LOQ)		0.01 mg/kg	
Precision and Accuracy (% Recovery)	Matrix	Soil/potato (5/1, w/w) - UK	Soil/potato (5/1, w/w) - Germany
	Level-I	0.01 mg/kg	0.01 mg/kg
	Mean Recovery (n=2)	103%	90.5%
	Level-II	60 mg/kg	60 mg/kg
	Mean Recovery (n=2)	77.5%	84.6%
	Level-III	120 mg/kg	120 mg/kg
	Mean Recovery (n=2)	81.7%	77.8%
	Acceptable Limit % [SANCO]	70-110%	

Discussion:

Method Van de Ruit (1998) was not fully validated in compliance with SANCO/3029/99 rev. 4. For UK soil, 5 recovery determinations have been made at two different fortification levels. For NL and DE soil, only 3 recovery determinations have been made. As the matrices are sufficiently similar, these reduced validation data are acceptable. This method has been evaluated to be acceptable in the original DAR, and validation is still acceptable for soil matrix.

For the soil/potato mixture, insufficient validation data has been presented. The submitted validation data for this mixture can be regarded as informational only.

Conclusion:

As determined in the original DAR, method Van de Ruit (1998) is validated in compliance with SANCO/3029/99 rev. 4 for the matrix soil, with an LOQ of 0.005 mg/kg.

Reference	:	Ihara, T. (2007a) K-CA 4.1.2/02	GLP statement	:	Yes
Type of study	:	Validation of analytical method for flutolanil in soil	Guideline	:	SANCO/3029/99 rev. 4 SANCO/825/00 rev. 7
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3048

The analytical method described in this study has been evaluated in the original DAR. Since it is also used as monitoring method, it is summarized in section B.5.2.3.

Reference	:	Castro, L. (1994) K-CA 4.1.2/03	GLP statement	:	Yes
Type of study	:	Dissipation of flutolanil on bare soil following application of flutolanil 50 WP, USA, 1989	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	E-3018

Principle of the method:

Flutolanil and desisopropyl flutolanil were extracted from soil by shaking with an acetone/water mixture. The extract was recovered by vacuum filtration and the acetone was removed using rotary evaporation. Flutolanil was extracted from the aqueous concentrate using hexane and the desisopropyl flutolanil was extracted from the concentrate using dichloromethane. Each extract was filtered through a sodium sulfate column before drying using a rotary evaporator. Flutolanil residues were re-dissolved and purified through a Florisil column. The eluant was dried and reconstituted in a known amount of solvent. Residues of flutolanil were then quantified using gas chromatography with nitrogen-phosphorus detection. (GC-NPD)

The extract containing the desisopropyl flutolanil has been extracted following a different treatment. No summary and validation data for desisopropyl flutolanil are reported in this summary as desisopropyl flutolanil is not part of the residue definition.

Characteristics of the soil used in this study (Cantonment, FL) are presented in the table below.

Sampling location	Cantonment, FL, USA					
Horizon (cm)	0-15	15-30	30-45	45-60	60-75	75-90
pH	6.4	6.4	5.6	5.4	5.3	5.2
CEC [a]	6.3	8.6	5.5	4.9	5.4	4.7
MHC [b]	17	18	19	23	26	25
Sand (%)	45	47	43	45	41	39
Silt (%)	36	30	32	28	30	30
Cray (%)	19	23	25	27	29	31
Organic	2.2	2.4	N/A	N/A	N/A	N/A

[a] Cation exchange capacity (meq/100g)

[b] Moisture holding capacity (%) at 0.33 bar

N/A= not applicable (not in normal growth zone)

GC-NPD conditions:

Injection:	4 µL
Column:	DB-1; 15 m x 0.53 mm; 1.5 µm film thickness
Carrier gas:	Helium (26-29 mL/min.)
Gas flows:	Hydrogen (4 mL/min)
	Air (100 mL/min)
Temperatures:	Oven: 180°C
	Injector: 205°C
	Detector: 300°C
Detection mode:	NPD

Assessment of method validation in soil

Validation data are presented in the table below.

Parameters		Flutolanil
Linearity	**Concentration Range (ng/mL)**	0.05 to 5.0 µg/mL
	Intercept (a)	Not reported
	Slope of the line (b)	Not reported
	Correlation Coefficient (r)	Not reported
Limit of Quantification (LOQ)		0.01 mg/kg
Precision and Accuracy (% Recovery) Data extracted from table AV-1, Appendix V (considering only the data from ABC labs)	**Level-I**	0.01 mg/kg
	Mean Recovery (n=26)	97.8%
	% RSD	15.7%
	Level-II	0.05 mg/kg
	Mean Recovery (n=7)	100.1%
	% RSD	7.9%
	Level-III	0.2 mg/kg
	Mean Recovery (n=17)	100.8%
	% RSD	9.7%
	Level-IV	0.5 mg/kg
	Mean Recovery (n=3)	93.7%
	% RSD	7.6%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		No additional confirmatory is required as the GC-NPD is highly specific.
Specificity		No interference above 30% of the LOQ were observed in the control soil samples at the retention time of Flutolanil.

Discussion:

The method is not sufficiently validated according to SANCO/3029/99 rev. 4. Linearity of the method has not been determined. Since the available recovery data is based on extraction of soil samples, this is insufficient information to establish linearity. However, as the purpose of this study is only to determine whether leaching takes place, this analytical method can be considered fit for purpose.

Reference	:	Castro, L. (1993) K-CA 4.1.2/04	GLP statement	:	Yes
Type of study	:	Long-term field dissipation of flutolanil under conditions of peanut cultivation initiated 1989, USA	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	E-3023

Principle of the method:

Soil samples were extracted with an acetone/water mixture with subsequent removal of the acetone using a rotary evaporator. Flutolanil residue were extracted with hexane and, afterwards

desisopropyl flutolanil (DIP) residues were extracted with dichloromethane. The flutolanil residues were subsequently purified through Florisil and quantified using gas chromatography with nitrogen-phosphorus detection (GC-NPD).

The extract containing the desisopropyl flutolanil has been extracted following a different treatment. No summary and validation data for desisopropyl flutolanil are reported in this summary as desisopropyl flutolanil is not part of the residue definition.

Characteristics of the soil used in this study are presented in the table below.

Sampling location	Molino, FL, USA					
Horizon (cm)	0-15	15-30	30-45	45-60	60-75	75-90
pH	6.3	6.2	5.4	5.4	5.4	5.3
CEC [a]	7.6	7.2	4.8	4.6	5.5	4.5
Sand (%)	45.2	47.2	93.2	47.2	56.2	41.2
Silt (%)	34	32	32	28	20	28
Cray (%)	20.8	20.8	28.8	24.8	24.8	30.8
OMC [c]	1.96	N/A	N/A	N/A	N/A	N/A

[a] Cation exchange capacity (meq/100g)
[b] Moisture holding capacity (%) at 0.33 bar
[c] Organic matter content as percent weight
N/A= not applicable (not in normal growth zone)

GC-NPD conditions:

Injection:	4 or 5 µL, constant
Column:	DB-1; 5 or 12 or 15 m x 0.53 mm; 1.5 µm film thickness
Carrier gas:	Helium (26-92 mL/min.)
Gas flows:	Make-up (1-4 mL/min)
	Air (100 mL/min)
	Hydrogen (4 mL/min)
Temperatures:	Oven: 145 or 175 or 180°C
	Injector: 195 or 205°C
	Detector: 300°C
Detection mode:	NPD

Assessment of method validation in soil

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range (ng/mL)**	0.05 to 5.0 µg/mL
	Intercept (a)	Not reported
	Slope of the line (b)	Not reported
	Correlation Coefficient (r)	Not reported
Limit of Quantification (LOQ)		0.01 mg/kg
Precision and Accuracy (% Recovery) Data extracted	**Level-I**	0.01 mg/kg
	Mean Recovery (n=23)	97.0%
	% RSD	14.6%
	Level-II	0.05 mg/kg

from table AV-1, Appendix V (considering only the data from ABC labs)	**Mean Recovery (n=6)**	96.0%
	% RSD	5.3%
	Level-III	0.2 mg/kg
	Mean Recovery (n=13)	102.3%
	% RSD	9.4%
	Level-IV	0.5 mg/kg
	Mean Recovery (n=2)	105.0%
	% RSD	-
	Level-V	1 mg/kg
	Mean Recovery (n=5)	95.2%
	% RSD	5.8%
	Level-VI	2 mg/kg
	Mean Recovery (n=1)	95%
	% RSD	-
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		No additional confirmatory is required as the GC-NPD is highly specific.
Specificity		No interference above 30% of the LOQ were observed in the control soil samples at the retention time of Flutolanil.

Discussion:

The method is not sufficiently validated according to SANCO/3029/99 rev. 4. Linearity of the method has not been determined. Since the available recovery data is based on extraction of soil samples, this is insufficient information to establish linearity. However, as the purpose of this study is only to determine whether leaching takes place, this analytical method can be considered fit for purpose.

Reference	:	Bourgade, C., Yslan, F. (1998a) K-CA 4.1.2/05	GLP statement	:	Yes
Type of study	:	Flutolanil: Analytical method for the determination of residues in drinking water and surface water	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3040

Principle of the method:

Water samples (600 ml) were purified using a polystyrene divinylbenzene cartridge, the cartridge was washed by using 40 ml water-acetonitrile (50:50) mixture. The eluate was discarded. The cartridge was then eluted with 50 ml water-acetonitrile (40:60) mixture. The collected eluates were evaporated to dryness and the residue redissolved in toluene and flutolanil analysed by semi-capillary column gas chromatography with thermoionic detector (TID) and external standardisation.

GC-TID conditions:

Injection: 5 µL, septum programmable injector (SPI)

Temp (°C)	Rate (°C/min)	Hold time (min)
90	-	0.20
240	180	14.37

Column: AT-PESTICIDE; 20 m x 0.53 mm; 0.6 µm film thickness

Column temp.:

Temp (°C)	Rate (°C/min)	Hold time (min)
90	-	1
200	25	0
240	10	6

Carrier gas: Helium (9 mL/min.)

Detection mode: Thermoionic detector (TID)

Detector Temperature: 300°C

Gas flows: Nitrogen (25 mL/min)
Hydrogen (5 mL/min)
Air (183 mL/min)

Assessment of method validation in drinking water and surface water

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range**	4 calibration points (in duplicate) - 20 to 1000 µg/L (corresponding to 0.07 – 3.3 mg/kg in sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	16.68127
	Slope of the line (b)	21.73588
	Correlation Coefficient (r)	0.999968
Limit of Quantification (LOQ)		0.1 µg/L for drinking water and 1.0 µg/L for surface water
Mineral water: **Precision and Accuracy (% Recovery)**	**Level-I**	0.1 µg/L
	Mean Recovery (n=5)	101%
	% RSD	5%
	Level-II	1.0 µg/L
	Mean Recovery (n=5)	103%
	% RSD	2%
Tap water: **Precision and Accuracy (% Recovery)**	**Level-I**	0.1 µg/L
	Mean Recovery (n=5)	96%
	% RSD	7%
	Level-II	1.0 µg/L
	Mean Recovery (n=5)	100%

	% RSD	2%

Surface water: **Precision and Accuracy (% Recovery)**	**Level-I**	1.0 µg/L
	Mean Recovery (n=5)	102%
	% RSD	2%
	Level-II	10 µg/L
	Mean Recovery (n=5)	102%
	% RSD	1%
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		A separate confirmatory method has been developed and validated (see CA 4.1.2/06)
Specificity		No interference above 30% of the LOQ were observed in the water soil samples at the retention time of flutolanil.

Conclusion

As determined in the original DAR, Method Bourgade (1998a) is validated in compliance with SANCO/3029/99 rev. 4 for drinking and surface water, with an LOQ of 0.1 µg/L for drinking water and 1.0 µg/L for surface water.

Reference	:	Bourgade, C., Yslan, F. (1998b) K-CA 4.1.2/06	GLP statement	:	Yes
Type of study	:	Flutolanil: Confirmatory chromatographic method for the determination of residues in water	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3041

Principle of the method:

Water samples (600 ml) were purified using a polystyrene divinylbenzene cartridge, the cartridge was washed by using 40 ml water-acetonitrile (50:50) mixture. The eluate was discarded. The cartridge was then eluted with 50 ml water-acetonitrile (40:60) mixture. The collected eluates were evaporated to dryness and the residue redissolved in toluene and flutolanil analysed by fused silica capillary column gas chromatography with tandem mass spectrometer detector (GC/MS/MS) and external standardisation.

GC/MS/MS conditions:

GC instrument: VARIAN 3800 equipped with an 8200 autosampler

Injection: 5 µL, Septum Programmable Injector (SPI)

Temp (°C)	Rate (°C/min)	Hold time (min)
90	-	0.20
250	180	13.10

Injection mode: split/splitless

Column: SP-2250; 30 m x 0.25 mm; 0.2 µm film thickness

Column temp.:

Temp (°C)	Rate (°C/min)	Hold time (min)
100	-	0.25
250	30	9

Carrier gas: Helium (9 mL/min.)

Detector: SATURN 2000 MS/MS

Parent ion: mass fragment m/z 173

Daughter ion: mass fragment m/z 145

Assessment of method validation in drinking water and surface water

Validation data are presented in the table below.

Parameters		**Flutolanil**	
Linearity	**Concentration Range**	3 calibration points 20 to 100 µg/L (corresponding to 0.07 – 0.3 mg/kg in sample)	3 calibration points 100 to 500 µg/L (corresponding to 0.3 – 1.7 mg/kg in sample)
	Note	The linearity is covering the concentration range from the LOQ to 10xLOQ ± at least 20%.	
	Intercept (a)	-91275	3000000
	Slope of the line (b)	61421	36268
	Correlation Coefficient (r)	0.9999	0.9921
Limit of Quantification (LOQ)		0.1 µg/L for drinking water and 1.0 µg/L for surface water	
Precision and Accuracy (% Recovery) – drinking water	**Level-I**	0.1 µg/L	
	Mean Recovery (n=2)	109%	
	% RSD	-	
Precision and Accuracy (% Recovery) – surface water	**Level-II**	1.0 µg/L	
	Mean Recovery (n=2)	120%	
	% RSD	-	
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%	

Specificity	No interference above 30% of the LOQ were observed in the water soil samples at the retention time of flutolanil.

Conclusion:

As determined in the original DAR GC/MS/MS analytical method A-3041 was successfully validated following SANCO/3029/99 rev 4 as confirmatory method to Method A-3040 for the determination of residues of flutolanil in drinking water and surface water.

Reference	:	Dorn, U. (1999) K-CA 4.1.2/07	GLP statement	:	Yes
Type of study	:	Development and validation of an analytical method for the determination of flutolanil in air	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3042

The analytical method described in this study has been evaluated in the original DAR. Since it is also used as monitoring method, it is summarized in section B.5.2.5.

B.5.1.2.2 Methods used in support of efficacy studies

No new specific methods were developed for the support of efficacy studies.

B.5.1.2.3 Methods used in support of toxicological studies

No new specific methods were developed for the support of toxicological studies.

B.5.1.2.4 Methods used in support of operator, worker, resident and bystander exposure studies

No new specific methods were developed for the support of operator, worker, resident and bystander exposure studies.

B.5.1.2.5 Methods used in support of residues studies

Reference	:	Wouters, G.A.J.M. (2000) K-CA 4.1.2/08	GLP statement	:	Yes
Type of study	:	Method validation study for the analysis of flutolanil in potato by GC/MS	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3037

Principle of the method:

Ground soil, potato or a mixture of soil and potato is extracted with acetone. The acetone is evaporated and the remaining water layer is back-extracted with petroleum ether. After dehydration and soli-phase extraction the sample extract is dissolved in methanol and analysed onto the GC-MS.

Only validation data on the matrix, potato, is summarised below.

GC-MSD conditions:

Instrument:	Hewlett-Packard (HP) 5890a GC
Detector:	Hewlett-Packard (HP) 5972-1 MS
Injection:	2 µL, on-column injection
Column:	CP-Sil 5 CB fused silica WCOT; 25 m x 0.25 mm; 0.4 µm
Pre-column:	uncoated fused silica, 2m x 0.5 mm
Carrier gas:	Helium (1 mL/min.)
Temperatures:	Oven: 150°C, hold 2 min
	20ºC/min to 230ºC
Target ion:	173 m/z (Flutolanil), 188 m/z (D10-anthracene)
Qualifier ion:	145 m/ z (Flutolanil), 189 m/z (D10-anthracene)
Detector temperature:	280ºC

Assessment of method validation in potato

Validation data are presented in the table below.

Parameters		**Flutolanil**	
Linearity	**Concentration Range (ng/mL)**	4 calibration points: 0.1 – 1.0 mg/L	5 calibration points: 1.0 – 20 mg/L
	Intercept (a)	-	-0.133
	Slope of the line (b)	0.284	0.378
	Correlation Coefficient (r)	0.9995	0.9975
Limit of Quantification (LOQ)		0.01 mg/kg	
Precision and Accuracy (% Recovery)	**Level-I**	0.01 mg/kg	
	Mean Recovery	108% (intra-assay n=5), 100% (inter-assay n=2)	
	% RSD	7.8%	
	Level-II	60 mg/kg	

	Mean Recovery	108% (intra-assay n=5), 95.3% (inter-assay n=2)
	% RSD	4.8%
	Level-III	120 mg/kg
	Mean Recovery	109% (intra-assay n=5), 98.9% (inter-assay n=2)
	% RSD	4.6%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		No additional confirmatory is required as the GC-MS is highly specific.
Specificity		No interference above 30% of the LOQ were observed in the control potato samples at the retention time of Flutolanil.

Conclusion

As determined in the original DAR, method Wouters (2000) is validated in compliance with SANCO/3029/99 rev. 4 for potato, with an LOQ of 0.01 mg/kg.

Reference	:	Fuchsbichler, G. (2001) K-CA 4.1.2/09	GLP statement	:	Yes
Type of study	:	Determination of flutolanil in potatoes: independent laboratory validation of the method described in report CRLD 97-82	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3038

This method is an independent laboratory validation of study Wouters (2000).

Principle of the method:

Potato samples are extracted with acetone. The acetone is evaporated, and the remaining water layer is back-extracted with petroleum ether. The petroleum ether extract is purified on an aluminium oxide column with diethyl ether as eluent. The analysis is carried out using GC-MS. The quantification is done by internal standardisation.

Minor modifications were applied compared to the original method however, the impact was considered as non-significant and acceptable.

GC-MSD conditions:

Instrument:	Hewlett-Packard (HP) 6890
Injection:	1 µL, splitless
Column:	DB-5, fused silica : 30 m x 0.25 mm; 0.25 µm
Carrier gas:	Helium (0.8 mL/min.)
Temperatures:	Oven: 100°C, hold 1 min

	10ºC/min to 260ºC
Target ion:	145, 173, 323 m/z (Flutolanil), 187, 188, 189 m/z (anthracene-d_{10})
Detector temperature:	280ºC

Assessment of method validation in potato

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range (ng/mL)**	6 calibration points: 0.05 – 2.0 µg/mL
	Intercept (a)	-0.0042
	Slope of the line (b)	0.1993
	Correlation Coefficient (r)	0.9986
Limit of Quantification (LOQ)		0.01 mg/kg
Precision and Accuracy (% Recovery)	**Level-I**	0.01 mg/kg
	Mean Recovery (n=5)	93%
	% RSD	11.7%
	Level-II	1.0 mg/kg
	Mean Recovery (n=5)	97%
	% RSD	0.4%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		No additional confirmatory is required as the GC-MS is highly specific.
Specificity		No interference above 30% of the LOQ were observed in the control potato samples at the retention time of Flutolanil.

Conclusion

As determined in the original DAR, method Fuchsbichler (2001) is validated in compliance with SANCO/3029/99 rev. 4 for potato. It is a suitable ILV for method Wouters (2000). The LOQ of 0.01 mg/kg is supported by the ILV.

Reference	:	Ihara, T. (2007b) K-CA 4.1.2/10	GLP statement	:	Yes
Type of study	:	Validation of analytical method for flutolanil and its metabolites in potato	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-2 and M-4	Method reference	:	A-3050

Principle of the method

Residues of flutolanil and the metabolites M-2 and M-4 were extracted and homogenised with acetonitirile. Extracts were purified with graphite carbon/aminopropyl silica gel cartridge column, the sample was determined by LC/MS/MS analysis.

Analysis by LC-MS/MS:

HPLC Conditions				
Column	Cadenza CD-C18 (2×50mm, 3µm ODS)			
Injection Volume	10 µL			
Mobile Phase A	Distilled water + 0.1% (v/v) formic acid			
Mobile Phase B	Methanol + 0.1% (v/v) formic acid			
Flow Rate	0.20 mL/min			
Column temperature	40ºC			
Gradient	Initial: 50% solvent B 0 - 0.5min: 50-70% solvent B 0.5 – 5.5min: 70-100% solvent B 5.5 - 7.5min: 100% solvent B hold			
Mass Spectrometer and General Instrument Conditions				
Instrument	3200Q trap triple quadrupole mass spectrometer (Applied Biosystems/MSD Sciex)			
Ion Source	ESI			
Source Temperature	600°C			
Needle voltage	5.5kV			
Nebulizer gas pressure	60psi			
Turbo gas pressure	80psi			
Collision gas pressure	3psi			
Analytes	Quantification LC-MS/MS transitions	1st Confirmation LC-MS/MS transitions	2nd Confirmation LC-MS/MS transitions	Dwell time
Flutolanil	324.1 > 262.1	324.1 > 242.0	324.1 > 282.1	100
M-2	340.1 > 258.0	340.1 > 278.2	340.1 > 81.2	100
M-4	282.0 > 242.1	282.0 > 65.0	282.0 > 145.3	100

Assessment of method validation:

Validation data are presented in the table at the end of this study summary.

Linearity: Linearity was determined by injection of six calibration standards. Good linearity was obtained in the range 0.2 – 50 µgm/L, covering from the 30% of LOQ to the highest nominal concentration of the analyte + at least 20%.

Analyte	Typical calibration line (forced to origin)	Quantification LC-MS/MS transitions	Correlation coefficient (R^2)
Flutolanil	y = 18500.5 x	324 > 262	0.9999
M-2	y = 8533.2 x	340 > 258	1.0000
M-4	y = 5754.1 x	282 > 242	0.9996

Specificity: No interference/contamination peak above 30% of the LOQ was detected at the retention time of flutolanil, M-2 and M-4 in any blank or control samples. The specificity of the method has been confirmed by the quantification of three LC-MS/MS transitions for each analytes.

Repeatability: Repeatability was determined as the relative standard deviation (RSD) calculated from five determinations at each fortification level. Repeatability for

flutolanil, M-2 and M-4 was within the required specification (i.e. RSD ≤ 20%) in all cases.

Accuracy: Recovery was determined at two fortification levels (LOQ and the highest expected residues level). Mean recovery values for flutolanil, M-2 and M-4 were within the required specification (i.e. between 70 and 110% of the amount added) in all cases.

Limit of determination: The limit of quantification (LOQ), defined as the lowest fortification level at which acceptable recovery data (between 70 and 110%) and RSD (≤20%) are obtained, was 0.01 mg/kg for flutolanil and metabolites M-2 and M-4 in potato.

Summary of the validation data from Ihara, T (2007b)

Analyte	Level of fortification (mg/kg)	Number of replicates (n)	Average recovery ± standard deviation (%)	R.S.D (%)
Flutolanil	Blank	2	4.9	-
	0.01	5	98.8 ± 3.7	3.8
	0.1	5	93.9 ± 3.1	3.3
M-2	Blank	2	-	-
	0.01	5	70.5 ± 5.8	7.9
	0.1	5	70.9 ± 4.0	5.7
M-4	Blank	2	-	-
	0.01	5	102.4 ± 2.3	2.3
	0.1	5	98.7 ± 2.3	2.3

Conclusion

The analytical method described in this study has been sufficiently validated according to SANCO/3029/99 rev. 4. It is therefore suitable for the determination of flutolanil and metabolites M-2 and M-4 in potato tubers with an LOQ of 0.01 mg/kg for all analytes.

Reference	:	Ihara, T. (2008) K-CA 4.1.2/11	GLP statement	:	Yes
Type of study	:	Validation of analytical method for flutolanil and its metabolites in potato (revalidation)	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-2 and M-4	Method reference	:	A-3051

The analytical method described in Ihara (2008) is identical to that described in Ihara (2007b). The later validation report contains a second validation, supporting the results of the first validation report. The study and validation results of the 2008 study are therefore not summarised in this document, as the method was already found to be sufficiently validated. The study is therefore not relied on and is not included in the references relied on.

Reference	:	Burton, D. (2011) K-CA 4.1.2/12	GLP statement	:	Yes
Type of study	:	Validation of methodology for the determination of residues of flutolanil and metabolites M-2 and M-4 in potato	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-2 and M-4	Method reference	:	A-3056

The methodology used was based on the LC-MS/MS method reported by Ihara, 2007b (see CP 4.1.2/10) and Ihara, 2008 (see CP 4.1.2/11) but the procedure included an acid hydrolysis step to hydrolyse any conjugated metabolites, to allow metabolites to be measured as the sum of free and conjugated forms.

Principle of method

Flutolanil and its metabolites M-2 and M-4 are extracted from potato by blending twice with acetonitrile. The extract is then evaporated and the residue incubated with 4N hydrochloric acid for 24 hours to hydrolyse any M-2 and M-4 conjugates. The hydrolysed extract is cleaned-up by liquid-liquid partition. Quantitation was performed using liquid chromatography with mass spectrometric detection (LC-MS/MS).

Analysis by LC-MS/MS:

<table>
<tr><td colspan="2">Mass Spectrometer and General Instrument Conditions</td></tr>
<tr><td>Instrument</td><td>Waters Quattro</td></tr>
<tr><td>Ion mode</td><td>Positive electrospray</td></tr>
<tr><td>Source Temperature</td><td>120°C</td></tr>
<tr><td>Ion monitoring details</td><td>Quantitation
<table>
<tr><td>Analyte</td><td>m/z</td><td>Cone V</td><td>Collision energy (eV)</td></tr>
<tr><td>Flutolanil</td><td>324>262</td><td>25</td><td>20</td></tr>
<tr><td>M-2</td><td>340>278</td><td>27</td><td>17</td></tr>
<tr><td>M-4</td><td>282>242</td><td>25</td><td>20</td></tr>
</table>
Confirmation
<table>
<tr><td>Analyte</td><td>m/z</td><td>Cone V</td><td>Collision energy (eV)</td></tr>
<tr><td>Flutolanil</td><td>324>242</td><td>25</td><td>27</td></tr>
<tr><td>M-2</td><td>340>258</td><td>27</td><td>27</td></tr>
<tr><td>M-4</td><td>282>93</td><td>25</td><td>30</td></tr>
</table></td></tr>
<tr><td colspan="2">HPLC Conditions</td></tr>
<tr><td>Column</td><td>Luna C_8 µm (15cm×2mm), Phenomenex</td></tr>
<tr><td>Injection Volume</td><td>20 µL</td></tr>
<tr><td>Mobile Phase A</td><td>Water:Methanol (90:10 v/v) with 0.1% formic acid and 0.01M ammonium formate</td></tr>
<tr><td>Mobile Phase B</td><td>Methanol + 0.1% (v/v) formic acid</td></tr>
<tr><td>Flow Rate</td><td>0.20 mL/min</td></tr>
<tr><td>Column temperature</td><td>Ambient</td></tr>
</table>

Retention time	Flutolanil M-2 M-4	6.1 min. 5.1 min. 4.5 min.	
Gradient	Time (mins) 0 6 10 11 15	A (%) 50 0 0 50 50	B (%) 50 100 100 50 50

Assessment of method validation:

Linearity: Linearity was determined by injection of eight calibration standards. Good linearity was obtained in the range 0.2 – 50 µgm/L, covering from the 30% of LOQ to the highest nominal concentration of the analyte + at least 20%.

Analyte	Typical calibration line	Quantification LC-MS/MS transitions	Correlation coefficient (R)
Flutolanil	y = 582.203 x + 38.4112	324 > 262	0.9986
M-2	y = 565.236 x + 55.9567	340 > 278	0.9975
M-4	y = 254.818 x + 19.4857	282 > 242	0.9971
Confirmatory			
Flutolanil	y = 642.151 x + 68.5369	324 > 242	0.9980
M-2	y = 577.077 x + 42.1557	340 > 258	0.9973
M-4	y = 103.120 x + 3.91979	282 > 93	0.9967

Specificity: No interference/contamination peak above 30% of the LOQ was detected at the retention time of flutolanil, M-2 and M-4 in any blank or control samples. The specificity of the method has been confirmed by the quantification of two LC-MS/MS transitions for each analyte.

Repeatability: Repeatability was determined as the relative standard deviation (RSD) calculated from five determinations at each fortification level. Repeatability for flutolanil, M-2 and M-4 was within the required specification (i.e. RSD $\leq$ 20%) in all cases. This limit was achieved for both quantitation and confirmation MRM transitions.

Accuracy: Recovery was determined at two fortification levels (LOQ and the highest expected residues level). Mean recovery values for flutolanil, M-2 and M-4 were within the required specification (i.e. between 70 and 110% of the amount added) in all cases. This limit was achieved for both quantitation and confirmation MRM transitions.

Limit of determination: The limit of quantification (LOQ), defined as the lowest fortification level at which acceptable recovery data (between 70 and 110%) and RSD (≤20%) are obtained, was 0.01 mg/kg for flutolanil and metabolites M-2 and M-4 in potato. The limit of detection (LOD) for the method was shown to be 0.2 ng/mL (equivalent to 0.001mg/kg in potato).

Storage stability: Flutolanil and it metabolites, M-2 and M-4, were all found to be stable in the potato final extract when stored for 7 days at approximately -20°C.

Summary of the validation data from Burton, D (2011)

Analyte	Transition	Level of fortification (mg/kg)	Number of replicates (n)	Average recovery ± standard deviation (%)	R.S.D (%)
Flutolanil	324>262	Blank	2	-	-
		0.01	5	79 ± 4.0	5.1
		0.1	5	73 ± 2.8	3.8
	324>242	Blank	2	-	-
		0.01	5	81 ± 3.0	3.8
		0.1	5	73 ± 3.3	4.5
M-2	340>278	Blank	2	-	-
		0.01	5	97 ± 7.8	8.1
		0.1	5	89 ± 3.8	4.2
	340>258	Blank	2	-	-
		0.01	5	99 ± 6.8	6.9
		0.1	5	90 ± 5.7	6.4
M-4	282>242	Blank	2	-	-
		0.01	5	84 ± 7.7	9.1
		0.1	5	82 ± 3.7	4.5
	282>93	Blank	2	-	-
		0.01	5	88 ± 8.0	9.1
		0.1	5	82 ± 4.3	5.3

Discussion

The analytical method described in this study report is sufficiently validated in compliance with SANCO/3029/99 rev. 4. However, it has not been sufficiently demonstrated that this method determines both conjugated and free forms of the metabolites. The efficiency of the hydrolysis step has not been addressed, and spiking of the samples has only been done with the free forms. It can be assumed that a hydrolysis step of 24 h with 4N HCL is sufficient to break up all conjugated forms of M-2 and M-4.

Conclusion

The analytical method described in this study report is considered to be fit for purpose with respect to determining the total content of flutolanil and its metabolites M-2 and M-4 (both free form and conjugate) in potato with an LOQ of 0.01 mg/kg for all analytes.

Reference	:	Bernal, J. (2016) K-CA 4.1.2/13	GLP statement	:	Yes
Type of study	:	Flutolanil - Validation of the analytical method for the determination of flutolanil and its metabolites M-2, M-4, M-101 and M-102 in potato tubers	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-2, M-4, M-101 and M-102	Method reference	:	A-3070

Principle of the method:

Samples of potato tubers were extracted twice with acetonitrile. The extract is centrifuged, and the supernatant filtered. An aliquot is concentrated by evaporation then diluted with acidified water prior to quantification by LC-MS/MS.

LC-MS/MS conditions:

Pump + autosampler: LC20AD, Shimadzu + SIL20AC, Shimadzu

Detector: API 4000 Sciex

Column: Cadenza C18 50 x 2 mm

Column Temperature: 40°C

Injection volume: 10 µL

Flow column HPLC: 0.3 mL/min

Mobile phase: Solvent A: Ultra-pure water + 0.1% AF

Solvent B: Methanol + 0.1% AF

Gradient:

Time (min.)	% A	% B
0.0	80	20
0.5	80	20
4.5	0	100
5.5	0	100
5.6	80	20
8.0	80	20

Analyte	Ionisation mode	Transition	CE (V)	
Flutolanil	ESI+	324.1 / 262.1	23	Quantification
		324.1 / 242.2	35	Confirmation
M-2	ESI+	340.1 / 258.1	33	Quantification
		340.1 / 278.2	25	Confirmation
M-4	ESI+	282.1 / 262.1	19	Quantification
		282.1 / 242.2	25	Confirmation
M-101	ESI+	190.1 / 102.2	41	Quantification
		190.1 / 170.1	15	Confirmation
M-102	ESI-	188.9 / 145.1	-16	Quantification
		188.9 / 68.9	-50	Confirmation

Assessment of method validation

Parameters		**Flutolanil, M-2, M-4, M-101 and M-102**					
Linearity	**Concentration Range**	8 calibration points: 0.75 ng/mL to 150 ng/mL (corresponding to 0.003 mg/kg to 0.6 mg/kg) covering the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample extract					
Linearity	**Compound**	**Flutolanil**		**M-2**		**M-4**	
		Quant.	Conf.	Quant.	Conf.	Quant.	Conf.
	Intercept	-6766	-4581	2170	1753	-1705	-1166
	Slope	51993	38866	32601	46631	26929	13824

	Correlation Coefficient (r)	0.9995	0.9994	0.9995	0.9998	0.9996	0.9995
	Compound	**M-101**			**M-102**		
	Intercept	983		2350	-4153		-207
	Slope	14660		36404	28384		1310
	Correlation Coefficient (r)	0.9995		0.9997	0.9991		0.9991
Limit of Quantification (LOQ)		0.01 mg/kg					
Precision and Accuracy (% Recovery)		See table below (Table B.5.1.2.5-1)					
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%					
Matrix effect		Matrix effects on the detection of flutolanil, M-2 and M-4in extracts of potato were found to be significant (≥ 20 %). Matrix effects on the detection of, M-101 and M-102 in extracts of potato were found to be insignificant (< 20 %). Therefore without any significant impact on the results, matrix-matched standards were used for quantification.					
Specificity		Quantification was performed by use of LC-MS/MS detection. Two mass transitions were evaluated for each analyte in order to demonstrate that the method achieves a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the control sample extracts of potato, so that a high level of selectivity was demonstrated.					

Table B.5.1.2.5-1 Precision and Accuracy (% Recovery)

Flutolanil							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 324→262 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	100, 101, 98, 95, 97	98	2	5	96	3
	0.10	94, 95, 95, 95, 94	95	1	5		
Mass Transition 324→242 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	99, 99, 98, 98, 97	98	1	5	96	3
	0.10	93, 94, 94, 93, 94	94	1	5		
Flutolanil metabolite M-2 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 340→258 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	100, 93, 100, 98, 91	96	4	5	97	3
	0.10	95, 97, 99, 98, 96	97	2	5		
Mass Transition 340→278 *m/z* (Proposed for Confirmation)							

Potato tuber	0.01	99, 92, 98, 96, 93	96	3	5	96	2
	0.10	94, 97, 98, 97, 94	96	2	5		
Flutolanil metabolite M-4 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 282→262 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	100, 99, 99, 97, 93	98	3	5	96	3
	0.10	94, 93, 94, 95, 92	94	1	5		
Mass Transition 282→242 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	101, 98, 96, 97, 92	97	3	5	95	3
	0.10	95, 94, 94, 94, 92	94	1	5		
Flutolanil metabolite M-101 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 190→02 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	93, 91, 91, 91, 94	92	2	5	91	2
	0.10	89, 93, 91, 92, 87	90	3	5		
Mass Transition 190→170 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	94, 89, 91, 89, 90	91	2	5	90	2
	0.10	89, 90, 91, 89, 86	89	2	5		
Flutolanil metabolite M-102 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 190→145 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	99, 98, 100, 98, 99	99	1	5	95	4
	0.10	92, 93, 91, 92, 90	92	1	5		
Mass Transition 190→69 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	95, 93, 106, 89, 98	96	7	5	94	5
	0.10	92, 90, 93, 90, 90	91	2	5		

Conclusion:

The analytical method described in Bernal (2016) is sufficiently validated in compliance with SANCO/3029/99 rev. 4. It is suitable for the determination of flutolanil and its metabolites M-2, M-4, M-101 and M-102 in potato tubers with an LOQ of 0.01 mg/kg for all analytes.

Reference	:	Merdian, H. (2016) K-CA 4.1.2/14	GLP statement	:	Yes
Type of study	:	Validation of the analytical method for the determination of flutolanil and its metabolites M-2, M-4, M-101 and M-102 in potato after hydrolysis	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-2, M-4, M-101 and M-102	Method reference	:	A-3081

Principle of the method:

Samples of potato were extracted with acetonitrile/1M HCl (4/1, v/v) and evaporated to remove acetonitrile under vacuum. A 24-hour hydrolysis step was performed following further acidification of the specimens, to allow de-conjugation of metabolites. A liquid-liquid partition was performed, followed by a solid phase extraction 'SPE' clean-up procedure using carbon cartridges. After the SPE, specimens were evaporated to low volume under vacuum and re-dissolved for a final solvent ratio of acetonitrile/water (50/50, v/v). This extract was used for determination of M-102 and a separate extract was further diluted by factor 5 and used for quantification of flutolanil, M-2, M-4 and M-101.

HPLC-MS/MS Analysis parameters:

HPLC system	Shimadzu Nexera X2 LC-30AD HPLC pump and SIL-30ACMP auto-sampler			
Column	Phenomenex Kinetex PFP (100 x 3.0 mm, 2.6 µm, 100A)			
Column oven temperature	40 °C			
Injection volume	50 µL			
Mobile phases	Eluent A: Water containing 0.1 % (v/v) formic acid Eluent B: Acetonitrile containing 0.1 % (v/v) formic acid			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.01	80	20	500
	0.20	80	20	500
	1.00	20	80	500
	6.00	20	80	500
	6.60	80	20	500
	10.00	80	20	500

MS system	AB Sciex 5500 QTrap LC/MS/MS System			
Analyte	Ionisation mode	Transition	CE (V)	
Flutolanil	ESI+	324 / 242	33	Quantification
		324 / 262	25	Confirmation
M-2	ESI+	340 / 258	37	Quantification
		340 / 278	27	Confirmation
M-4	ESI+	282 / 262	19	Quantification
		282 / 242	31	Confirmation
M-101	ESI+	190 / 170	15	Quantification
		190 / 130	29	Confirmation
M-102	ESI-	189 / 145	-18	Quantification
		189 / 69-18	-46	Confirmation

Assessment of method validation

Parameters		
Linearity	**Concentration Range**	5-6 calibration points: 1.0 ng/mL to 100 ng/mL for M-102 0.20 ng/mL to 20 ng/mL for M-101

		0.20 ng/mL to 16 ng/mL for flutolanil 0.20 ng/mL to 16 ng/mL (or 20 ng/mL) for M-2 and M-4 corresponding to 0.0025 mg/kg to 0.20 mg/kg (or 0.25 mg/kg) and covering the range from no more than 25 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample extract					
Linearity	**Compound**	**Flutolanil**		**M-2**		**M-4**	
		Quant.	Conf.	Quant.	Conf.	Quant.	Conf.
	Intercept	22700	23000	26000	-26800	216	1480
	Slope	408000	400000	605000	524000	302000	233000
	Correlation Coefficient (r)	0.9989	0.9989	0.9991	0.9991	0.9999	1.0000
	Compound	**M-101**		**M-102**			
	Intercept	118	-7060	1210	8380		
	Slope	214000	288000	85100	-307		
	Correlation Coefficient (r)	0.9999	1.0000	0.9998	0.9999		
Limit of Quantification (LOQ)		0.01 mg/kg					
Precision and Accuracy (% Recovery)		See table below (Table B.5.1.2.5-2)					
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%					
Matrix effect		Matrix effects on the detection of flutolanil, M-2, M-4, M-101 and M-102 in extracts of potato were found to be significant (≥ 20 %). Therefore, matrix-matched standards were used for quantification.					
Specificity		Quantification was performed by use of LC-MS/MS detection. Two mass transitions were evaluated for each analyte in order to demonstrate that the method achieves a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the control sample extracts of potato, so that a high level of selectivity was demonstrated.					

Table B.5.1.2.5-2 Precision and Accuracy (% Recovery)

Flutolanil							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 324→242 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	87, 91, 93, 99, 88	92	5	5	90	7
	0.10	92, 100, 86, 80, 82	88	9	5		
Mass Transition 324→262 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	84, 87, 90, 98, 89	90	6	5	88	7
	0.10	89, 97, 85, 80, 80	86	8	5		
Flutolanil metabolite M-2 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 340→258 *m/z* (Proposed for Quantification)							

Potato tuber	0.01	87, 91, 92, 90, 95	91	3	5	93	4
	0.10	93, 98, 95, 92, 98	95	3	5		
Mass Transition 340→278 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	86, 91, 92, 91, 93	91	3	5	93	3
	0.10	95, 96, 94, 95, 96	95	1	5		
Flutolanil metabolite M-4 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 282→262 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	89, 91, 92, 90, 97	92	3	5	92	3
	0.10	91, 93, 94, 90, 94	92	2	5		
Mass Transition 282→242 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	87, 88, 89, 88, 95	89	4	5	91	3
	0.10	90, 93, 93, 92, 94	92	2	5		
Flutolanil metabolite M-101 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 190→170 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	87, 93, 88, 98, 90	91	5	5	89	6
	0.10	88, 92, 91, 78, 86	87	6	5		
Mass Transition 190→130 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	84, 94, 92, 101, 88	92	7	5	90	7
	0.10	88, 93, 93, 79, 86	88	7	5		
Flutolanil metabolite M-102 (expressed as flutolanil)							
Matrix	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Mass Transition 189→145 *m/z* (Proposed for Quantification)							
Potato tuber	0.01	91, 96, 68, 89, 97	88	13	5	93	11
	0.10	94, 96, 106, 88, 100	97	7	5		
Mass Transition 189→69 *m/z* (Proposed for Confirmation)							
Potato tuber	0.01	93, 94, 67, 86, 96	87	14	5	91	11
	0.10	91, 94, 103, 85, 101	95	8	5		

The fortification levels are defined as parent equivalent

No observable peak was detected in any control sample extracts

Discussion

The analytical method described in this study report is sufficiently validated in compliance with SANCO/3029/99 rev. 4. However, it has not been sufficiently demonstrated that this method

determines both conjugated and free forms of the metabolites. The efficiency of the hydrolysis step has not been addressed, and spiking of the samples has only been done with the free forms. However, it can be assumed that a hydrolysis step of 24 h with 4N HCL is sufficient to break up all conjugated forms of the metabolites.

Conclusion:
The analytical method described in this study report is considered to be fit for purpose with respect to determining the total content of flutolanil and its metabolites M-2, M-4, M-101 and M-102 (both free form and conjugate) in potato with an LOQ of 0.01 mg/kg for all analytes.

Reference	:	Robinson, J.D. (1999) K-CA 4.1.2/15	GLP statement	:	Yes
Type of study	:	Validation of an analytical method for the determination of residues n products of animal origin	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3025

Principle of the method:
The analytical method was validated for the determination of flutolanil in products of animal origin: bovine muscle, liver, kidney and fat, milk, eggs. The method of analysis involved the homogenisation of the samples in acetone, filtration of the extracts through celite and concentration of the extracts by evaporation. Sample clean-up was performed by liquid partition into dichloromethane, dehydrated and evaporated to dryness, re-suspended in hexane and cleaned up by a silica gel cartridge. After evaporation to dryness the residue was re-suspended in toluene and analysed by fused silica capillary gas chromatography with MSD and external standardisation.
Samples of bovine liver, kidney, muscle, fat, milk and eggs from hens, obtained from untreated control animals were supplied by the Department of Large Animal and Avian Studies, Huntingdon Life Sciences.

GC/MS Analysis parameters:

Instrument: GC8000 TOP with Voyager MS detector, Quadrapole, EI+ mode

Injection: 2 µL, split/splitless

Column: OV-5; 30 m x 0.32 mm; 0.25 µm film thickness

Carrier gas: Helium

Time(min)	Pressure KPa
0	10
4	10
12	50
19	50

Temperatures gradient:

Time(min)	Temperature (°C)
0	150
2	150
6	230

11	230
12	250
19	250

Mass range: m/z 323 (quantitation) and m/z 281 and 173 (confirmation)

Assessment of method validation

Parameters		**Flutolanil**
Linearity	**Concentration Range (ng/mL)**	6 calibration points 20 to 800 ng/mL (corresponding to 0.02 – 0.8 mg/kg in muscle, liver, kidney, fat and eggs sample or corresponding to 0.004 – 0.16 mg/L in milk sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	-1519.5
	Slope of the line (b)	1147.7
	Correlation Coefficient (r)	0.9990
Limit of Quantification (LOQ)		0.05 mg/kg in muscle, liver, kidney, fat and eggs 0.01 mg/L in milk
Precision and Accuracy (% Recovery)		See table below (Table B.5.1.2.5-3)
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		Additional confirmatory analysis is not required as the primary method is a highly specific method (GC-MS).
Specificity		No interference above 30% of the LOQ were observed in the control soil samples at the retention time of flutolanil.

Table B.5.1.2.5-3 Precision and Accuracy (% Recovery)

Substrate	**Fortification level (mg/kg)**	**LOQ (mg/kg)**	**Recovery (%) mean**	**range**	**RSD (%)**	**N**
Bovine liver	0.05	0.05	108	102-118	6	5
	0.50		96	93-100	4	5
	overall		102	93-118	8	10
Bovine kidney	0.05	0.05	91	83-99	6	5
	0.50		88	78-108	14	5
	overall		90	78-108	10	10
Bovine muscle	0.05	0.05	103	94-112	7	5
	0.50		87	75-95	9	5
	overall		95	75-112	12	10
Bovine Fat	0.05	0.05	89	75-111	19	5
	0.50		89	73-96	10	5
	overall		89	73-111	15	10
Milk	0.01	0.01	82	70-98	16	5
	0.10		104	92-115	8	5
	overall		93	70-115	16	10
Eggs	0.05	0.05	90	85-96	5	5
	0.50		88	81-98	9	5
	overall		89	81-98	7	10

Conclusion:

As determined in the original DAR, method Robinson (1999) is validated in compliance with SANCO/3029/99 rev. 4 for the determination of flutolanil in muscle, liver, kidney, fat, eggs and milk, with an LOQ of 0.05 mg/kg in liver, kidney, muscle, fat and eggs, and 0.01 mg/kg in milk.

Reference	:	Wouter, G.A.J.M. (1999) K-CA 4.1.2/16	GLP statement	:	Yes
Type of study	:	Independent laboratory validation of Rhone-Poulenc analytical method AR 192-99 for the determination of flutolanil in products of animal origin	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3027

The analytical method described in this study is an ILV to the method described in Robinson (1999).

Principle of the method:

Samples were extracted with acetone. The acetone layer was filtered on Celite 545 and evaporated. Aqueous sodium chloride was added and the solution was back-extracted with dichloromethane. The dichloromethane was dehydrated with Na2SO4 and evaporated. The residue was transferred to silica gel with hexane. After clean-up the flutolanil was eluted using ethyl acetate/hexane. The solvent was evaporated and the residue was re-suspended in toluene. Analysis of the toluene solution was carried out by GC/MS and quantification performed by monitoring the ion fragments at 173 m/z for flutolanil.

GC/MS Analysis parameters:

Instrument: HP 5890a GC, with HP 5972-1 MS

Injection: 2 µL, split/splitless

Column: CP-Sil 5 CB fused silica WCOT; 25

Carrier gas: Helium (1.0 mL/min

Temperatures gradient:

Time(min)	Temperature (°C)
0	150
2	150
6	230
11	230

Mass range: m/z 173 (quantitation) and m/z 145 (confirmation)

Assessment of method validation

Parameters		**Flutolanil**
Linearity	**Concentration Range (ng/mL)**	6 calibration points 20 to 800 ng/mL (corresponding to 0.004 – 0.16 mg/kg in sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	63.1
	Slope of the line (b)	51377
	Correlation Coefficient (r)	0.9953

Limit of Quantification (LOQ)	0.05 mg/kg in beef meat 0.01 mg/kg in milk
Precision and Accuracy (% Recovery)	See table below (Table B.5.1.2.5-4)
Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory	Additional confirmatory analysis is not required as the primary method is a highly specific method (GC-MS).
Specificity	No interference above 30% of the LOQ were observed in the control soil samples at the retention time of flutolanil.

Table B.5.1.2.5-4 Precision and Accuracy (% Recovery)

Substrate	Fortification level (mg/kg)	LOQ (mg/kg)	Recovery (%) mean	Recovery (%) range	RSD (%)	N
Beef meat	0.05	0.05	105	101-116	6	5
ILV	0.50		102	93-115	8	5
	overall		103	93-116	7	10
Milk	0.01	0.01	119	97-135	12	5
ILV	0.10		107	97-115	8	5
	overall		113	97-135	11	10

Conclusion:

As determined in the original DAR, study Wouter (1999) describes an acceptable ILV to method Robinson (1999). The method is validated in compliance with SANCO/3029/99 rev. 4 for the determination of flutolanil in beef meat and milk, with an LOQ of 0.05 mg/kg in beef meat and 0.01 mg/kg in milk.

Reference	:	Airs, D. (2015) K-CA 4.1.2/17	GLP statement	:	Yes
Type of study	:	Flutolanil: Validation of methodology for the determination of residues of flutolanil and metabolites in bovine liver, kidney, muscle, fat, whole milk, skimmed milk and cream	Guideline	:	SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-2, M-4, M-7 and M-101	Method reference	:	A-3073

This method is also validated and used as monitoring method. Therefore, it is summarized in section B.5.2.2.

Conclusion:

The method was sufficiently validated according to the guidance document SANCO/3029/99 rev. 4 for the determination of Flutolanil and metabolites (M-2, M-4, M-7 and M-101 (including after de-conjugation step for M-2 and M-4)) in bovine whole milk, skimmed milk cream, liver, kidney, muscle and fat. The analytical method is therefore suitable for pre-registration purposes.

Reference	:	Dias, N.A. (2016) K-CA 4.1.2/18	GLP statement	:	Yes
Type of study	:	Flutolanil: Residues of flutolanil and its metabolites in eggs and tissues of laying hens	Guideline	:	SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-2, M-4, M-7, M-101 and M-102	Method reference	:	A-3075

This method is also validated and used as monitoring method. Therefore, it is summarized in section B.5.2.2.

Conclusion:

The method was sufficiently validated according to the guidance document SANCO/3029/99 rev. 4 for the determination of flutolanil and metabolites (M-2, M-4, M-7, M-101 and M-102 (including after de-conjugation step for M-2 and M-4)) in poultry liver, muscle, fat and eggs. The analytical method is therefore suitable for pre-registration purposes.

B.5.1.2.6 Methods used in support of ecotoxicology studies

Reference	:	Brekelmans, M.J.C. (2003a) K-CA 4.1.2/19	GLP statement	:	Yes
Type of study	:	Development and validation of an analytical method for the analysis of flutolanil in sediment samples from the sediment water chironomid toxicity test	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3035

Principle of the method:

Soil samples were extracted with acetonitrile using a vortex for 15 seconds. The upper solution was then diluted with Milli-Q water in a 1:1 (v:v) ratio. The extract was analysed by HPLC/UV.

HPLC/UV Analysis parameters:

Column:	LiChrospher 100 RP-18, 125 x 4 mm, 5 µm
Mobile phase:	Acetonitrile/Milli-Q water (55:45 v/v) - isocratic
Flow rate:	1.0 mL/min
Injection volume:	100 µL
UV wavelength:	208 nm

Assessment of method validation in sediment

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range**	7 calibration points – 0.0298 – 4.97 mg/L (corresponding to 0.3 – 49.7 mg/kg in sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	-2750
	Slope of the line (b)	1130000
	Correlation Coefficient (r)	0.999928
Limit of Quantification (LOQ)		0.5 mg/kg
Precision and Accuracy (% Recovery)	**Level-I**	0.5 mg/kg
	Mean Recovery (n=6)	104%
	% RSD	2.2%
	Level-II	20 mg/kg
	Mean Recovery (n=6)	105%
	% RSD	1.6%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%

Confirmatory	No additional confirmatory method was

	conducted in this study. However the method validation from the DAR (**CA 4.1.2/02**) can be considered as the confirmatory method for this study
Specificity	No interference above 30% of the LOQ were observed in the control soil samples at the retention time of MU-466.

Conclusion:

The analytical method described in this study report is sufficiently validated in compliance with SANCO/3029/99 rev. 4. It is suitable as preregistration method for the determination of flutolanil in sediment with an LOQ of 0.5 mg/kg.

Reference	:	Brekelmans, M.J.C. (2003b) K-CA 4.1.2/20	GLP statement	:	Yes
Type of study	:	Development and validation of an analytical method for the analysis of flutolanil in iso-medium samples from the sediment water chironomid toxicity test	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3036

Principle of the method:

Test medium samples were diluted with 50/50 (v/v) Acetonitrile/ISO-medium and analyse by HPLC/UV. The test medium was ISO-medium (Medium formulated using Milli-Ro water).

HPLC/UV Analysis parameters:

Column: LiChrospher 100 RP-18, 125 x 4 mm, 5 µm

Mobile phase: A:ACN

B: Milli-Q water

Flow rate: 1.0 mL/min

Injection volume: 200µL

Gradient:

Time (min.)	A (%)	B (%)
0	40	60
5	40	60
7	55	45
13	55	45
15	100	0
18	100	0
20	40	60
22	40	60

UV wavelength: 208 nm

Assessment of method validation in aqueous test medium

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range**	9 calibration points – 0.002 – 10 mg/L (corresponding to 0.004 – 20 mg/L in sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	5.20
	Slope of the line (b)	1150000
	Correlation Coefficient (r)	0.99996
Limit of Quantification (LOQ)		0.00508 mg/L
Precision and Accuracy (% Recovery)	**Level-I**	0.00508 mg/L
	Mean Recovery (n=6)	101%
	% RSD	9.2%
	Level-II	5.08 mg/L
	Mean Recovery (n=6)	98%
	% RSD	1.9%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		No additional confirmatory method was conducted in this study.
Specificity		No interference above 30% of the LOQ were observed in the control soil samples at the retention time of flutolanil.

Conclusion:

The analytical method described in this study report is sufficiently validated in compliance with SANCO/3029/99 rev. 4. It is suitable as preregistration method for the determination of flutolanil in iso-medium with an LOQ of 0.00508 mg/L.

Reference	:	Kendall, T.Z., Nixon, W.B. (2011) K-CA 4.1.2/21	GLP statement	:	Yes
Type of study	:	Analytical method verification for the determination of flutolanil in freshwater	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3059

Principle of the method:

The test solutions were diluted with freshwater, as necessary and analysed by HPLC/UV. Freshwater was obtained from a well, approximately 40 meters deep, located on the Wildlife International Ltd. site. The well water was characterized as moderately-hard water.

HPLC/UV Analysis parameters:

Column:	YMC PACK ODS-AM column (150 x 4.6 mm, 3 µm particle size)		
Mobile phase:	A: 0.1% H_3PO_4		
	B: CH_3CN		
Flow rate:	1.0 mL/min		
Gradient	Time (min)	A (%)	B (%)
	0.01	90	10
	1.0	90	10
	9.0	2	98
	10.0	2	98
	10.1	90	10
	14.0	90	10
Injection volume:	100 µL		
UV wavelength:	220 nm		

Assessment of method validation in aqueous test medium

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range**	5 calibration points – 0.01 – 0.3 mg/L (corresponding to 0.01 – 0.3 mg/L in sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	2.58547
	Slope of the line (b)	434.63
	Correlation Coefficient (r)	0.9999
Limit of Quantification (LOQ)		0.02 mg/L
Precision and Accuracy (% Recovery)	**Level-I**	0.02 mg/L
	Mean Recovery (n=5)	107%
	% RSD	1.7%
	Level-II	0.2 mg/L
	Mean Recovery (n=5)	100%
	% RSD	0.9%
	Level-III	2.0 mg/L
	Mean Recovery (n=5)	99.8%
	% RSD	1.6%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		No additional confirmatory method was conducted in this study.
Specificity		No interference above 30% of the LOQ were observed in the control samples at the retention time of flutolanil.

Conclusion:

The analytical method described in this study report is sufficiently validated in compliance with SANCO/3029/99 rev. 4. It is suitable as preregistration method for the determination of flutolanil in fresh water with an LOQ of 0.02 mg/L.

Reference	:	Bowman, J.H. (1987a) K-CA 4.1.2/22	GLP statement	:	Yes
Type of study	:	Acute toxicity of flutolanil technical to rainbow trout (Salmo gairdneri)	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	W-3008

Reference	:	Bowman, J.H. (1987b) K-CA 4.1.2/23	GLP statement	:	Yes
Type of study	:	Acute toxicity of flutolanil technical to Bluegill Sunfish (Lepomis macrochirus)	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	W-3009

Reference	:	Bowman, J.H., Bussard, J. (1990) K-CA 4.1.2/24	GLP statement	:	Yes
Type of study	:	Acute toxicity of flutolanil technical to Fathead Minnow (Pimephales promelas)	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	W-3010

Reference	:	Forbis, A.D. (1991) K-CA 4.1.2/25	GLP statement	:	Yes
Type of study	:	Acute toxicity of flutolanil to *Mysidopsis bahia*	Guideline	:	SANCO/3029/99 rev. 4
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	W-3015

Principle of the method:

An aliquot of the test solution samples was extracted with toluene by shaking for 1 minutes. The toluene phase was collected and further diluted with toluene before analysis by GC-ECD.

GC-ECD Analysis parameters:

Column:	HP-1 (Methyl-Silicone) 5 m x 0.53 mm x 2.65 µm
Instrument:	HP 5890 gas liquid chromatograph equipped with ECD
Temperature:	Column: 190-200°C
	Injector: 250°C
	Detector: 350°C
Flow rate:	10.6 mL/min (N_2)
Injection volume:	3 µL

Assessment of method validation in aqueous test medium

Validation data are presented in the table below.

Parameters		Flutolanil
Linearity	Concentration Range (ng/mL)	4 calibration points – 53 – 215 ng/mL[1)] covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	0.566876 [2)]
	Slope of the line (b)	0.0321864 [2)]
	Correlation Coefficient (r)	1.0 [2)]
Limit of Quantification (LOQ)		0.01 mg/L
Precision and Accuracy (% Recovery)		See table below (Table B.5.1.2.6-1)
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		No additional confirmatory method was conducted in this study.
Specificity		No interference above 30% of the LOQ were observed in the control samples at the retention time of flutolanil.

[1)] Concentration range obtained in the study CA 4.1.2-23.
[2)] Calibration curve determined in the study CA 4.1.2-23.

Table B.5.1.2.6-1 Precision and Accuracy (% Recovery)

Fortification level (mg/L)	Individual Recoveries* (%)	Mean Recovery (%)	RSD (%)
0.01	109, 100, 118	109	8.2
0.03	104, 106	105	-
0.06	106, 109	107.5	-
0.1	100, 109, 109, 100, 105	104.6	4.3
0.4	108	108	-
1	100, 101	100.5	-
5	107, 109, 109, 106, 102, 102	105.8	3.0
10	101, 99	100	-
11	100, 100, 118, 100	104.5	8.6

* from fresh fortification samples obtained through the 4 ecotoxicological studies

Conclusion:

The analytical method described in these four studies is not sufficiently validated with respect to SANCO/3029/99 rev. 4. For the linearity determination, only four data points are used, where five are required. In addition, the LOQ of 0.01 mg/L is not supported, since only three recovery determinations have been made. From the collected data, the analytical method can be considered fit for purpose with an LOQ of 0.1 mg/L for the determination of flutolanil in aqueous test medium.

Reference	:	Scheller, K. (2016) K-CA 4.1.2/26	GLP statement	:	Yes
Type of study	:	Repeated exposure of Flutolanil 40 SC to honey bee (*Apis mellifera*) larvae under laboratory conditions (*in vitro*)	Guideline	:	Not stated
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	N-3079

The purpose of this study was to validate an analytical method for the determination of flutolanil in the feeding stock solutions and the feeding solutions used in support in the ecotoxicological study on bees.

Principle of the method:

The stock solutions samples were diluted into two different media:

- for Diet B: 15% (w/v) glucose, 15% (w/v) fructose, 3% (W/v) yeast
- for Diet C: 15% (w/v) glucose, 15% (w/v) fructose, 3% (W/v) yeast

Consecutively, the feeding solution samples were diluted in 50/50 (w/w) Royal Jelly / stock solution from Diet B or C.

A known weight of Diet C was spiked with Flutolanil. An aliquot of 1 g was then analysed following a QuEChERS extraction procedure, involving extraction with water and acetonitrile as well as QuEChERS citrate extraction mix. After shaking and centrifugation, the acetonitrile-QuEChERS extracts were diluted with a mixture of acetonitrile/water (50/50) and the determination was conducted by an In-house developed method using reverse – high performance liquid chromatographic (MS-MS) detection.

LC-MS/MS Analysis parameters:

Column:	Zorbax Eclipse Plus C18 (50 x 2.1 mm, 1.8 µm particle size)		
Mobile phase:	A: Water containing 5 mM ammonium formate, 0.1% (v/v) formic acid B: Methanol containing 5 mM ammonium formate, 0.1% (v/v) formic acid		
Flow rate:	0.4 mL/min		
Gradient	Time (min)	A (%)	B (%)
	0.0	95	5
	1.5	50	50
	8.5	0	100
	9.0	0	100
Detector:	ESI positive, MRM: m/z 324/262, 323/242		

Assessment of method validation in the feeding stock solutions and the feeding solutions

Validation data are presented in the table below.

Parameters		Flutolanil
Linearity	**Concentration Range**	6 calibration points – 7.37 – 516.2 mg/L (corresponding to 0.28 – 192 mg/L in sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Equation	$y = -0.013223 x^2 + 44.276899 x + 40.850491$
	Correlation Coefficient (r)	0.99941425
Limit of Quantification (LOQ)		0.771 mg/kg (in Diet C in undiluted samples) 1.576 mg/kg (in stock solution for Diet C)
Precision and Accuracy (% Recovery) **Test medium of feeding stock solution**	**Level-I**	1.576 mg/kg
	Mean Recovery (n=5)	98%
	% RSD	6.1%
	Level-II	66.73 mg/kg
	Mean Recovery (n=5)	98%
	% RSD	0.1%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Precision and Accuracy (% Recovery) **Test medium of feeding solution**	**Level-I**	0.771 mg/kg
	Mean Recovery (n=5)	100%
	% RSD	2.0%
	Level-II	33.37 mg/kg
	Mean Recovery (n=5)	96%
	% RSD	3.2%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory	The specificity of the method was assured by multiple reaction monitoring (MRM)-detection with two transitions and the absence of interfering peaks. The ratio of quantifier and qualifier ions was recorded and was constant within 70 ± 10%	
Specificity	No interference above 30% of the LOQ were observed in the control samples at the retention time of flutolanil.	
Stability	Recoveries of flutolanil in the stability samples were between 87 – 117% in the sample of the final diet. No active ingredient was detection in the control samples. Thus, the stability of the flutolanil in the test medium Diet B and Diet C for 24 h under honeybee larvae test conditions was verified.	

Conclusion:

The analytical method described in this study is considered to be fit for purpose for the determination of flutolanil in test item feeding solutions at the relevant concentrations.

To fully comply with the requirements, the confirmatory transition should have been validated.

Reference	:	Ruhland, S. (2016) K-CA 4.1.2/27	GLP statement	:	Yes
Type of study	:	Chronic toxicity of Flutolanil SC to the honey bee *Apis mellifera* L. under laboratory conditions.	Guideline	:	Not stated
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	N-3078

The purpose of this study was to validate an analytical method for the determination of flutolanil in the test item feeding solutions (sucrose solution containing 50% (w/v) sucrose) used in support in the ecotoxicological study on bees.

Principle of the method:

Test item feeding solutions (sucrose solution containing 50% (w/v) sucrose) were spiked with the test item Flutolanil 40 SC.

The spiked test item feeding solutions were diluted with dilution medium (methanol/water (v/v)) and the determination was conducted by an In-house developed method using reverse phase – high performance liquid chromatographic (HPLC) with UV-detection.

HPLC/UV Analysis parameters:

Column:	Macherey Nagel Nucleoshell RP18 (100 x 2.7 mm, 2.7 µm particle size)		
Mobile phase:	A: Water with 0.1% (v/v) phosphoric acid		
	B: Acetonitrile with 0.1% (v/v) phosphoric acid		
Flow ra:	0.4mL/min		
Gradient	Time (min)	A (%)	B (%)
	0.0	50	50
	3.0	20	80
	3.01	10	90
	5.0	10	90
	5.01	50	50
	7.0	50	50
UV wavelength:	208 nm		

Assessment of method validation in the test item feeding solutions

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range**	5 calibration points – 6.18 – 23.78 mg/L (corresponding to 77.25 – 297 mg/L in sample) covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Intercept (a)	-12871.4
	Slope of the line (b)	143563
	Correlation Coefficient (r)	0.9999918

Limit of Quantification (LOQ)		112.4 mg/L
Precision and Accuracy (% Recovery)	**Level-I**	112.4 mg/L
	Mean Recovery (n=5)	101%
	% RSD	0.2%
	Level-II	3645 mg/L
	Mean Recovery (n=5)	99%
	% RSD	0.2%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory	The specificity of the method was assured by the following method: UV spectra from 200-300 nm were continuously recorded by diode-array detector (DAD). Spectra of the peaks were compared to those of the reference. Similar spectra with approximately equal absorption maxima, a constant chromatographic retention time and no interfering peaks were observed.	
Specificity	No interference above 30% of the LOQ were observed in the control samples at the retention time of flutolanil.	

Conclusion:

The analytical method is considered sufficiently validated to be fit for purpose for the determination of flutolanil in the test item feeding solutions. Results of this study are considered supported.

To fully comply with the requirements, the confirmatory transition should have been validated.

B.5.1.2.7 Methods in water, buffer solutions, organic solvents and any additional matrices resulting from the physical and chemical properties tests

No new methods have been submitted in support of physical and chemical properties tests.

B.5.2 Methods for post-approval control and monitoring purposes

B.5.2.1 Methods for the determination of residues in plant matrices

Reference	:	Fuchsbicher, G. (2002) K-CA 4.2/01	GLP statement	:	Yes
Type of study	:	Development and validation of the multi-residue method DFG S19 modified, for the determination of the residue of flutolanil in potatoes	Guideline	:	SANCO/825/00 rev. 6
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3034

Principle of the method:

The samples were extracted with acetone/water (2/1, v/v) with subsequent extraction with cyclohexane/ethyl acetate (1:1, v/v) and partition into acetone/cyclohexane/ethyl acetate. The extracts were cleaned up by gel permeation chromatography on a Bio Beads SX-3 column. The residues of flutolanil were determined by GC-MS and external standardisation.

GC-MS conditions:

Instrument:	gas chromatograph Hewlett-Packard (HP) 6890 with MS
Injection:	1 µL, splitless injection
Column:	DB 5, 30 m x 0.25 mm; 0.25 µm
Carrier gas:	Helium (0.8 mL/min.)
Temperatures:	Oven: 100°C, hold 1 min
	10ºC/min to 260ºC, hold 10 min
Target ion:	145, 173, 323 m/z (Flutolanil)
Detector temperature:	280ºC

Assessment of method validation in potato

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range**	4 calibration points (in duplicate): 0.01 – 0.10 µg/mL (corresponding to 0.0025 – 0.025 mg/kg in the sample) covering from 30% of the LOQ to at least 10xLOQ + 20%
	Intercept (a)	1683.4
	Slope of the line (b)	3000000
	Correlation Coefficient (r)	0.9979
Limit of Quantification (LOQ)		0.01 mg/kg

Precision and	**Mass used**	TIC (m/z 145, 173, 323)

Accuracy (% Recovery)	**Level-I**	0.01 mg/kg
	Mean Recovery (n=5)	99%
	% RSD	3.0%
	Level-II	0.10 mg/kg
	Mean Recovery (n=5)	91%
	% RSD	7.1%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-120% with RSD ≤ 20%
Confirmatory	Quantification method was done and assessed using the TIC mode (combination of the signals from the 3 m/z). Therefore the confirmatory method is already included in the quantification method. However, separate quantification and confirmatory method has been conducted in the ILV (see Torn, 2016). No additional confirmatory method is therefore required.	
Specificity	No interference above 30% of the LOQ were observed in the control potato samples at the retention time of Flutolanil.	

Conclusion:

As determined in the original DAR, the analytical method DFG S19 is validated in compliance with SANCO/825/00 rev. 6 for the determination of flutolanil residues in potato with an LOQ of 0.01 mg/kg. In order to comply with SANCO/825/00 rev. 8.1, additional methods have been submitted to cover all relevant matrices.

Reference	:	Taoudi, M. (2016a) K-CA 4.2/02	GLP statement	:	Yes
Type of study	:	Method validation – determination of residues of flutolanil in crops by LC-MS/MS	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	XG/16/002

Principle of the method:

The objective of this study was to validate a method for the determination of residues of Flutolanil in Crops (representing dry, high acid and high oil crop commodities) according to the EU guidance document SANCO 825/00 rev. 8.1.

Each homogenised sample (5 g for split pea and olive, 10 g for grape) was weighed into a 50 mL centrifuge tube. Recovery samples were fortified. Water was added to split pea and olive samples only (8.5 mL for split pea and 4 mL for olive) and acetonitrile (10 mL) was added. Samples were left to stand for 2 minutes and then homogenised. A pre-mixed QuEChERS buffer-salt mixture was then added to each sample, followed by shaking and centrifugation. For olive samples only, a 1 mL aliquot of the supernatant was then transferred into a tube containing 25 mg PSA, 150 mg $MgSO_4$ and 25 mg C18 [roQ^{TM}; KS0-8913]. A portion of the final extracts were then filtered through a nylon 13 mm 0.45 µm syringe filter into an autosampler vial. The samples were then analysed by LC-MS/MS, monitoring two mass transitions.

LC-MS/MS conditions:

	HPLC Conditions					
Column:	Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 x 50 mm					
Column Temperature:	20ºC					
Injection Volume:	10 µL					
Flow Rate:	500 µL/min					
Mobile Phase A:	0.1% Formic acid in water					
Mobile Phase B:	0.1% Formic acid in acetonitrile					
Gradient:	Time (min)		%A		%B	
	0.0		95		5	
	4.0		5		95	
	5.0		5		95	
	5.1		95		5	
	6.0		95		5	
	Mass Spectrometer Conditions					
Mass Spec:	API 5500 / API 6500					
Ion Source:	Turbo Ion Spray					
Polarity:	Positive					
Analyte	Transition	Ion Mass Transitions (*m/z*)	Dwell Time (msec)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)	Declustering Potential (DP)
Flutolanil	1	324.1/242.0	100	23	16	80 V
	2	324.1/262.0	100	15	12	80 V

Assessment of method validation in split pea, grape and olive

Validation data are presented in the table below.

Parameters		**Flutolanil**	
Linearity	**Concentration Range (ng/mL)**	1 to 120 ng/mL (equivalent to 0.001 – 0.12 mg/kg for grape and 0.002 to 0.24 mg/kg for split pea and olive), covering a range from 30% of the LOQ to 120% above the highest concentration level. 8 single determinations	
	Transition No. (m/z)	**324.1/242**	**324/262**
	Typical calibration curve	$y = -271 x^2 + 133000 x - 13500$	$y = -278 x^2 + 131000 x - 11100$
	Correlation Coefficient (r)	0.9999	0.9999
	Note	Quadratic regression with a weighting of 1/x	
Limit of Quantification (LOQ)		0.01 mg/kg	

Precision and Accuracy (% Recovery)	**Transition No. (m/z)**	**324.1/242**	**324/262**
	Level-I	0.01 mg/kg	0.01 mg/kg

- **Split Pea**	**Mean Recovery (n=6)**	105.0	104.4
	% RSD	1.1	1.9
	Level-II	0.10 mg/kg	0.10 mg/kg
	Mean Recovery (n=6)	101.4	101.4
	% RSD	2.4	2.5
	Acceptable Limit % [SANCO]	70-120% with RSD < 20%	
Precision and Accuracy (% Recovery) - **Grape**	**Transition No. (m/z)**	**324.1/242**	**324/262**
	Level-I	0.01 mg/kg	0.01 mg/kg
	Mean Recovery (n=6)	101.1	101.0
	% RSD	2.1	2.3
	Level-II	0.10 mg/kg	0.10 mg/kg
	Mean Recovery (n=6)	99.6	99.5
	% RSD	2.9	3.0
	Acceptable Limit % [SANCO]	70-120% with RSD < 20%	
Precision and Accuracy (% Recovery) - **Olive**	**Transition No. (m/z)**	**324.1/242**	**324/262**
	Level-I	0.01 mg/kg	0.01 mg/kg
	Mean Recovery (n=6)	101.3	102.5
	% RSD	1.9	1.2
	Level-II	0.10 mg/kg	0.10 mg/kg
	Mean Recovery (n=6)	98.8	100.2
	% RSD	2.2	2.7
	Acceptable Limit % [SANCO]	70-120% with RSD < 20%	
Matrix effect		No significant matrix effects (<20%) was observed on both transition.	
Specificity		Chromatographic interferences at the retention time of Flutolanil were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples, demonstrating good selectivity	
Extraction efficiency		In the plant metabolism studies (B.7.2.1.2 to B.7.2.1.6), the majority of extractable residual radio-activity was recovered in rinse fraction and acetonitrile/water (4/1=20% water) extract fraction (87.6-100.0% of extractable). Dominant residual radioactivity was parent compound. As futolanil is a low polarity compound, it is expected that an extraction with acetonitrile and acetonitrile/water will	

	be effective for monitoring purpose.

Conclusion
The primary validation of DFG-S19 was successful and complies with the requirements of SANCO/825/00 rev 8.1. Please refer to the overall conclusion under the ILV for overall conclusions of the method.

Reference	:	Torn, J. (2016) K-CA 4.2/03	GLP statement	:	Yes
Type of study	:	Independent laboratory validation for the determination of residues of flutolanil in crops by GC/MS and LC-MS/MS	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	100075555

Principle of the method:

The objective of this study was to independently validate two previously reported methods Aventis Crop Sciences Study No. 02-13 “Development and Validation of the Multi-Residue Method DFG S19 Modified, for the Determination of the Residues of Flutolanil in Potatoes” and Battelle UK Limited (BUKL) Study No. XG/16/002 “Method Validation – Determination of Residues of Flutolanil in Crops by LC-MS/MS” according to the EU Guidance Documents SANCO/825/00 rev. 8.1, 16/11/2010 and SANCO/3029/99 rev. 4, 11/07/2000.

In method 1, residues of flutolanil were extracted from potato using a modified DFG S19 procedure with gel permeation chromatography (GPC) clean-up. Final determination was by gas chromatography with mass spectrometry (GC/MS), in selected ion monitoring (SIM) mode monitoring three ions.

In method 2, residues of flutolanil were extracted from split peas, grapes, and olives using the QuEChERS procedure. Final determination was by liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two ion mass transitions.

GC/MS conditions (Method 1):

Column:	Agilent DB-5 30 m x 0.25 mm (diameter) X 0.25 µm (film thickness)
Injection Temperature:	260 °C
Injection Volume:	1.0 µL splitless mode
Carrier gas:	Helium
Flow Rate:	0.8 mL/minute constant flow mode
Initial Oven Temperature:	100 °C hold for 1 minute
Ramp Rate:	10 °C/minute to 260 °C hold for 10 minutes
MS Transfer Line:	280 °C
MS Source:	280 °C
Acquisition Mode:	SIM
Group 1 ions	145, 173, 323
Group 1 dwell time	100

LC-MS/MS conditions (Method 2):

Column:	Water Acquity UPLC BEH C_{18}, 50 x 2.1 mm, 1.7 µm
Column Temperature:	20 ºC
Injection Volume[1]:	10 µL
Flow Rate:	500 µL/min
Mobile Phase A:	HPLC grade water containing 0.1% formic acid
Mobile Phase B:	HPLC grade acetonitrile containing 0.1% formic acid

Time – minutes	% Mobile Phase A	% Mobile Phase B
0.00	95	5
4.00	5	95
5.00	5	95
5.10	95	5
6.00	95	5

Approximate Retention Time: Flutolanil 3.4 minutes

Ion Source: Turbo Ion Spray

Polarity: Positive

Ion Spray Voltage (IS): 5500 v

Analyte	Ion Mass Transitions (*m/z)*	Dwell Time (msec)	Declustering Potential (DP)	Collision Energy[2] (CE)	Collision Cell Exit Potential (CXP)
Flutolanil	324.1 →242	100	80	23	16
	324.1→262	100	80	15	12

Assessment of method validation – Method 1

Validation data are presented in the table below.

Parameters		**Flutolanil**		
Linearity	**Transition No. (m/z)**	**323**	**145**	**173**
	Concentration Range (ng/mL)	10 to 600 ng/mL matrix-match standards, covering a range from 30% of the LOQ to 120% above the highest concentration level. 6 single determinations.		
	Intercept (a)	-	-	-
	Slope of the line (b)	53800	244000	730000
	Correlation Coefficient (r)	1.000	1.000	1.000
Limit of Quantification (LOQ)		0.01 mg/kg		

Precision and	**Transition No. (m/z)**	**323**	**145**	**173**

Accuracy (% Recovery) - **Potato**	**Level-I**	0.01 mg/kg	0.01 mg/kg	0.01 mg/kg
	Mean Recovery (n=5)	84	101	108
	% RSD	6.4	6.8	4.3
	Level-II	0.10 mg/kg	0.10 mg/kg	0.10 mg/kg
	Mean Recovery (n=5)	73	77	78
	% RSD	1.7	1.7	1.5
	Acceptable Limit % [SANCO]	70-120% with RSD < 20%		
Specificity	Chromatographic interferences at the retention time of Flutolanil were less than 30% of the limit of quantification in control samples, demonstrating good selectivity.			

Assessment of method validation – Method 2

Validation data are presented in the table below.

Parameters		**Flutolanil**	
Linearity	**Concentration Range (ng/mL)**	1.5 to 120 ng/mL solvent standards, 1.5 to 75 mg/mL for olive matrix-matched standards covering a range from 30% of the LOQ to 120% above the highest concentration level. A minimum of 9 single determinations	
	Transition No. (m/z)	**324.1/242**	**324/262**
	Typical calibration curve	$y = -709.947 x^2 + 29130.59221 x + 8274.84706$	$y = -999.014 x^2 + 30124.7 x + 8869.86906$
	Correlation Coefficient (r)	0.99885	0.99910
	Note	Quadratic regression with a weighting of 1/x	
Limit of Quantification (LOQ)		0.01 mg/kg	
Precision and Accuracy (% Recovery) - **Split Pea**	**Transition No. (m/z)**	**324.1/242**	**324/262**
	Level-I	0.01 mg/kg	0.01 mg/kg
	Mean Recovery (n=5)	89	91
	% RSD	4.9	3.3
	Level-II	0.10 mg/kg	0.10 mg/kg
	Mean Recovery (n=5)	91	92
	% RSD	4.5	4.1
	Acceptable Limit % [SANCO]	70-120% with RSD < 20%	

Precision and Accuracy (%	**Transition No. (m/z)**	**324.1/242**	**324/262**
	Level-I	0.01 mg/kg	0.01 mg/kg

Recovery) - **Grape**	**Mean Recovery (n=5)**	86	86
	% RSD	5.0	5.2
	Level-II	0.10 mg/kg	0.10 mg/kg
	Mean Recovery (n=5)	95	94
	% RSD	2.3	1.6
	Acceptable Limit % [SANCO]	70-120% with RSD < 20%	
Precision and Accuracy (% Recovery) - **Olive**	**Transition No. (m/z)**	**324.1/242**	**324/262**
	Level-I	0.01 mg/kg	0.01 mg/kg
	Mean Recovery (n=5)	91	92
	% RSD	5.1	3.3
	Level-II	0.10 mg/kg	0.10 mg/kg
	Mean Recovery (n=5)	87	88
	% RSD	4.2	2.6
	Acceptable Limit % [SANCO]	70-120% with RSD < 20%	
Matrix effect	No significant matrix effects (< 20%) were observed for the split pea and grape matrices, however, significant matrix effects (>20%) where observed in the olive matrix. Matrix-matched standards were used for quantification of the olive matrix..		
Specificity	Chromatographic interferences at the retention time of Flutolanil were less than 30% of the limit of quantification in control samples, demonstrating good selectivity.		
Extraction efficiency	In the plant metabolism studies (B.7.2.1.2 to B.7.2.1.6), the majority of extractable residual radio-activity was recovered in rinse fraction and acetonitrile/water (4/1=20% water) extract fraction (87.6-100.0% of extractable). Dominant residual radioactivity was parent compound. As flutolanil is a low polarity compound, it is expected that an extraction with acetonitrile and acetonitrile/water will be effective for monitoring purposes.		

Conclusion:

The DFG S19 method was sufficiently validated for the determination of flutolanil residues in potato. Combined with the QuEChER method for the remaining plant matrices sufficient validation in compliance with SANCO/825/00 rev. 8.1 is available, with an LOQ of 0.01 mg/kg for all matrices.

The ILV report is not final. A final report should be submitted.

B.5.2.2 Methods for the determination of residues in animal matrices

Following the EFSA Conclusion on flutolanil (EFSA Scientific Report (2008) 126), an analytical method for food of animal origin is not required due to the fact that no residue definition is proposed. However, following MRL conclusion (EFSA Journal 2013;11(9):3360), it was recommended to conduct a

validation as well as its ILV for the determination of flutolanil and all metabolites containing the 2-trifluoromethylbenzoic acid moiety in animal commodities.
As a common moiety method is not recommended, the notifier has validated an analytical method allowing to analyse individually flutolanil, M-2, M-4 and their conjugates, M-7 and M-101.

Note RMS: Based on the available data two separate residue definitions for poultry and ruminants are proposed. For poultry the residue definition is proposed as the parent compound flutolanil. For ruminants the residue definition for monitoring is proposed to be the sum of flutolanil, metabolite M-4 (free and conjugated), expressed as flutolanil.

Reference	:	Airs, D. (2015) K-CA 4.2/04	GLP statement	:	Yes
Type of study	:	Flutolanil: Validation of methodology for the determination of residues of flutolanil and metabolites in bovine liver, kidney, muscle, fat, whole milk, skimmed milk and cream	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil, M-4	Method reference	:	A-3073

The objective of this study was to validate methodology for the determination of Flutolanil and metabolites (M-2, M-4, M-7 and M-101 (including after de-conjugation step for M-2 and M-4)) in bovine whole milk, skimmed milk cream, liver, kidney, muscle and fat in accordance of SANCO/3029/99 rev. 4 and SANCO/825/00 rev 8.1.

Principle of the method:

Samples (except fat) were extracted with acetonitrile and acidic acetonitrile and clean-up with a C18 solid phase extraction (SPE) cartridge. Fat samples were extracted with acetonitrile/hexane (50/50, v/v) and acidic acetonitrile/hexane (50/50, v/v) and cleaned-up by liquid-liquid partition. An enzyme hydrolysis step was also included for M-2 and M-4 for all matrices except fat. Quantification was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Control matrices used were from stocks held within the Environmental Analysis Department, Envigo.

HPLC-MS/MS Analysis parameters:

HPLC system	Applied Biosystems Sciex API 4000 coupled with Waters Acquity UPLC system			
Column	Acquity UPLC® BEH C_{18} (2.1 cm x 50 mm, 1.7 µm)			
Injection volume	10 µL			
Mobile phases	Eluent A: Methanol:Water (10/90, v/v) + 0.01M ammonium formate + 0.1 % formic acid Eluent B: Methanol + 0.1 % formic acid			
For Flutolanil, M-2, M-4 and M-7:				
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0	70	30	0.4
	0.2	70	30	0.4
	3.5	5	95	0.4

	3.6	5	95	0.4
	3.8	70	30	0.4
	5	70	30	0.4

For M-101:				
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0	70	30	0.5
	0.2	70	30	0.5
	2	5	95	0.5
	2.5	5	95	0.5
	3	70	30	0.5
	4	70	30	0.5

Analyte	Ionisation mode	Transition	
Flutolanil	ESI+	324 / 262	Quantification
		324 / 242	Confirmation
M-2	ESI+	340 / 278	Quantification
		340 / 258	Confirmation
M-4	ESI+	282 / 262	Quantification
		282 / 242	Confirmation
M-7	ESI+	312 / 292	Quantification
		312 / 272	Confirmation
M-101	ESI+	190 / 170	Quantification
		190 / 130	Confirmation

Assessment of method validation

Parameters		Flutolanil and M-2, M-4 and their conjugates, M-7 and M-101
Linearity	**Concentration Range (ng/mL)**	> 5 calibration points 0.01 to 1 ng/mL (corresponding to 0.002 – 0.2 mg/kg in sample) for flutolanil, M-2, M-4, and their conjugates, and M-7. 0.05 to 5 ng/mL (corresponding to 0.002 – 0.2 mg/kg in sample) for M-101 covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Typical calibration	See table below (Table B.5.2.2-1)
Limit of Quantification (LOQ)		0.01 mg/kg
Precision and Accuracy (% Recovery)		See table below (Table B.5.2.2-2)
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		Additional confirmatory analysis is not required as the primary method is a highly specific method (LC-MS/MS).
Specificity		No interference above 30% of the LOQ were observed in the control soil samples at the retention time of flutolanil and its metabolites.

Table B.5.2.2-1 Typical calibration curves and correlation coefficient

	Flutolanil	M-2

Quantitation	y = 162923 x + 1246.81	r = 0.9963	y = 55700 x – 197.726	r = 0.9972
Confirmation	y = 133379 x + 1311.28	r = 0.9972	y = 51583.4 x – 172.244	r = 0.9968
	M-4		M-7	
Quantitation	y = 161410.9 x – 28.1652	r = 0.9973	y = 49220 x – 228.983	r = 0.9975
Confirmation	y = 12453.1 x + 6.97711	r = 0.9979	y = 38366.4 x – 154.732	r = 0.9962
	M-101			
Quantitation	y = 28780.4 x + 2181.54	r = 0.9983		
Confirmation	y = 30201.4 x + 2951.87	r = 0.9984		

Table B.5.2.2-2 Precision and Accuracy (% Recovery)

Matrix	Ion transition	Spike level (mg/kg)	Mean Recovery (RSD) (%) n=5						
			Flutolanil	M-2	M-2 conj	M-4	M-4 conj	M-7	M-101
Whole milk	Quant.	0.01	89 (16.5)	89 (13.2)	95 (3.4)	95 (14.1)	85 (9.6)	87 (7.4)	81 (8.3)
		0.1	101 (5.5)	105 (8.8)	111 (5.1)	103 (4.6)	93 (6.3)	105 (5.0)	81 (5.0)
	Conf.	0.01	88 (15.4)	84 (12.9)	95 (7.7)	85 (13.6)	91 (3.8)	94 (5.0)	86 (6.9)
		0.1	100 (4.4)	103 (12.1)	107 (1.4)	104 (4.8)	85 (3.5)	107 (4.2)	84 (1.9
Skimmed milk	Quant.	0.01	92 (10.0)	86 (8.7)	108 (7.7)	82 (13.1)	104 (7.6)	92 (11.6)	84 (18.6)
		0.1	98 (8.0)	95 (6.3)	99 (8.5)	97 (5.1)	93 (14.5)	98 (5.7)	81 (5.2)
	Conf.	0.01	92 (13.3)	91 (6.9)	110 (7.4)	88 (11.7)	104 (11.3)	93 (10.2)	88 (16.7)
		0.1	97 (5.8)	97 (8.0)	102 (9.7)	99 (6.0)	94 (13.2)	99 (8.3)	83 (5.6)
Cream	Quant.	0.01	97 (12.2)	97 (7.7)	101 (9.2)	98 (8.8)	96 (12.0)	101 (3.9)	76 (4.7)
		0.1	110 (6.3)	108 (7.2)	108 (4.3)	107 (5.2)	100 (9.8)	103 (7.9)	96 (5.5)
	Conf.	0.01	91 (13.9)	96 (9.3)	98 (6.4)	96 (13.8)	91 (10.6)	100 (11.8)	78 (4.1)
		0.1	104 (7.2)	108 (6.4)	106 (4.8)	105 (8.1)	105 (7.8)	105 (6.8)	95 (5.9)
Liver	Quant.	0.01	84 (7.7)	86 (8.4)	82 (15.0)	88 (14.0)	79 (10.1)	95 (11.6)	77 (1.7)
		0.1	97 (8.3)	107 (8.1)	87 (4.3)	102 (3.6)	88 (3.3)	103 (3.2)	102 (6.2)
	Conf.	0.01	81 (9.1)	84 (10.5)	82 (6.4)	94 (11.0)	76 (16.4)	95 (6.4)	92 (5.4)
		0.1	102 (6.2)	107 (8.7)	89 (3.3)	96 (6.4)	92 (3.6)	107 (7.3)	103 (5.5)
Kidney	Quant.	0.01	102 (6.0)	89 (15.1)	97 (11.8)	90 (5.6)	78 (12.6)	95 (6.8)	88 (8.4)
		0.1	94 (7.9)	106 (7.0)	86 (10.0)	107 (4.7)	88 (7.8)	107 (4.3)	102 (2.2)
	Conf.	0.01	93 (8.8)	95 (2.5)	95 (11.7)	91 (8.8)	78 (5.7)	98 (9.6)	98 (13.6)
		0.1	95 (5.4)	104 (6.8)	89 (10.0)	107 (3.2)	87 (9.3)	108 (3.4)	109 (5.7)
Muscle	Quant.	0.01	92 (13.5)	95 (19.5)	84 (10.8)	93 (13.9)	81 (12.5)	89 (12.6)	97 (10.1)
		0.1	98 (4.3)	99 (16.0)	88 (11.4)	103 (6.2)	106 (10.3)	104 (5.7)	101 (6.4)
	Conf.	0.01	99 (5.4)	89 (15.4)	98 (7.4)	91 (12.8)	95 (5.4)	93 (8.0)	96 (11.1)
		0.1	100 (4.8)	97 (13.4)	92 (14.5)	102 (4.9)	103 (8.4)	106 (4.0)	99 (4.8)
Fat	Quant.	0.01	102 (8.3)	98 (10.7)	-	96 (9.7)	-	95 (12.7)	94 (6.6)
		0.1	110 (1.3)	110 (3.2)	-	109 (1.2)	-	109 (1.6)	93 (3.5)
	Conf.	0.01	100 (11)	100 (9.6)	-	97 (3.3)	-	97 (11.3)	89 (6.4)
		0.1	110 (0.9)	107 (2.0)	-	108 (3.4)	-	108 (3.1)	93 (3.9)

Conclusion

The analytical method is sufficiently validated in compliance with SANCO/825/00 rev. 8.1 to determine the sum of flutolanil and metabolite M-4 (free and conjugated) in ruminants (bovine whole milk, skimmed milk cream, liver, kidney and muscle). All metabolites can be determined with an LOQ of 0.01 mg/kg.

Reference	:	Dias, N.A. (2016) K-CA 4.2/05	GLP statement	:	Yes
Type of study	:	Flutolanil: residues of flutolanil and its metabolites in eggs and tissues of laying hens	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3075

One objective of this study was to validate methodology for the determination of Flutolanil and metabolites (M-2, M-4, M-7, M-101 and M-102 (including after de-conjugation step for M-2 and M-4)) in eggs and tissues of laying hens in accordance of SANCO/3029/99 rev. 4 and SANCO/825/00 rev 8.1.

Principle of the method:

Original method:

Samples (except fat) were extracted with acetonitrile and clean-up with a C18 solid phase extraction (SPE) cartridge. Fat samples were extracted with acetonitrile/hexane (50/50, v/v) and cleaned-up by liquid-liquid partition. Quantification was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

Modified method (with acid extraction and de-conjugation step):

Samples (except fat) were extracted with acetonitrile and acidic acetonitrile and clean-up with a C18 solid phase extraction (SPE) cartridge. Fat samples were extracted with acetonitrile/hexane (50/50, v/v) and acidic acetonitrile/hexane (50/50, v/v) and cleaned-up by liquid-liquid partition. An enzyme hydrolysis step with ß-glucorinidase was also included for M-2 and M-4 for all matrices except fat, followed by a clean-up with a C_{18} solid phase extraction (SPE) cartridge. Quantification was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

HPLC-MS/MS Analysis parameters:

HPLC system	Applied Biosystems Sciex API 4000 coupled with Waters Acquity UPLC system
Column	Acquity UPLC® BEH C_{18} (2.1 cm x 50 mm, 1.7 µm)
Injection volume	10 µL
Mobile phases	Eluent A: Methanol:Water (10/90, v/v) + 0.01M ammonium formate + 0.1 % formic acid Eluent B: Methanol + 0.1 % formic acid

For Flutolanil, M-2, M-4 and M-7:				
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0	70	30	0.4
	0.2	70	30	0.4
	3.5	5	95	0.4
	3.6	5	95	0.4
	3.8	70	30	0.4
	5	70	30	0.4
For M-101:				

	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
Gradient	0	70	30	0.5
	0.2	70	30	0.5
	2	5	95	0.5
	2.5	5	95	0.5
	3	70	30	0.5
	4	70	30	0.5

For M-102:				
Mobile phases	Eluent A: Water/acetic acid (100/0.1, v/v) Eluent B: Acetonitrile/acetic acid (100/0.1, v/v)			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0	100	0	0.5
	0.2	100	0	0.5
	2	10	90	0.5
	2.5	10	90	0.5
	3	100	0	0.5
	4	100	0	0.5

Analyte	Ionisation mode	Transition	
Flutolanil	ESI+	324 / 262	Quantification
		324 / 242	Confirmation
M-2	ESI+	340 / 278	Quantification
		340 / 258	Confirmation
M-4	ESI+	282 / 262	Quantification
		282 / 242	Confirmation
M-7	ESI+	312 / 292	Quantification
		312 / 272	Confirmation
M-101	ESI+	190 / 170	Quantification
		190 / 130	Confirmation
M-102	ESI-	189 / 145	Quantification
		189 / 68.9	Confirmation

Assessment of method validation

Parameters		Flutolanil, M-2, M-4, M-7, M-101 and M-102
Linearity	**Concentration Range (ng/mL)**	>5 calibration points 0.01 to 1 ng/mL (corresponding to 0.002 – 0.2 mg/kg in sample) for flutolanil, M-2, M-4, and their conjugates and M-7 0.05 to 5 ng/mL (corresponding to 0.002 – 0.2 mg/kg in sample) for M-101 0.05 to 5 ng/mL – standards in matrix - (corresponding to 0.002 – 0.2 mg/kg in sample) for M-102 covering the concentration range from the LOQ to 10xLOQ ± at least 20%.
	Typical calibration	See table below (Table B.5.2.2-3)
Limit of Quantification (LOQ)		0.01 mg/kg
Precision and Accuracy (% Recovery)		See table below (Table B.5.2.2-4) See table below (Table B.5.2.2-5)
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%
Confirmatory		Additional confirmatory analysis is not required as the primary method is a highly specific method (LC-MS/MS).

Specificity	No interference above 30% of the LOQ were observed in the control soil samples at the retention time of flutolanil and its metabolites.

Table B.5.2.2-3 Typical calibration curves and correlation coefficient

	Flutolanil		M-2	
Quantitation	y = 269812 x + 3.69005	r = 0.9974	y = 106127 x + 1.29795	r = 0.9974
Confirmation	y = 294406 x + 2776.33	r = 0.9997	y = 115659 x + 0.236673	r = 0.9995
	M-4		M-7	
Quantitation	y = 38794.6 x + 0.426367	r = 0.9977	y = 96741.5 x + 0.859452	r = 0.9983
Confirmation	y = 35097 x + 0.19471	r = 0.9993	y = 93403.4 x – 0.032963	r = 0.9999
	M-101			
Quantitation	y = 96655.7 x + 4481.72	r = 0.9991		
Confirmation	y = 94857.3 x + 90.336	r = 0.9993		

	M-101		M-102	
	Standard in matrix (muscle)		Standard in matrix (fat)	
Quantitation	y = 27743.4 x + 962.171	r = 0.9973	y = 136409 x + 14167.4	r = 0.9999
Confirmation	y = 29402 x + 2048.93	r = 0.9975	y = 9518.22 x + 143.017	r = 0.9971

Table B.5.2.2-4 Precision and Accuracy (% Recovery) – Original method

Matrix	Ion transition	Spike level (mg/kg)	Mean Recovery (RSD) (%) n=5					
			Flutolanil	M-2	M-4	M-7	M-101	M-102
Liver	Quant.	0.01	80 (11.4)	89 (12.1)	99 (12.2)	99 (13.6)	83 (5.6)	99 (15.6)
		0.1	93 (2.8)	102 (3.6)	104 (4.7)	101 (4.3)	92 (2.1)	105 (3.4)
	Conf.	0.01	80 (13.3)	93 (14.6)	97 (13.7)	96 (13.1)	102 (4.9)	94 (19.9)
		0.1	95 (2.4)	101 (3.1)	102 (2.2)	102 (4.9)	95 (2.3)	103 (4.2)
Muscle	Quant.	0.01	84 (10.4)	89 (9.6)	90 (7.6)	87 (7.9)	92 (6.9)	93 (17.5)
		0.1	92 (2.4)	96 (5.6)	95 (4.5)	96 (3.7)	92 (4.1)	94 (5.2)
	Conf.	0.01	83 (5.1)	85 (7.2)	93 (3.8)	88 (7.8)	99 (7.5)	96 (15.8)
		0.1	91 (3.4)	94 (3.8)	94 (3.6)	96 (3.5)	92 (4.7)	92 (4.5)
Fat	Quant.	0.01	77 (5.4)	77 (8.1)	89 (7.1)	75 (8.0)	81 (19.8)	79 (6.5)
		0.1	89 (6.9)	91 (8.5)	91 (9.4)	91 (7.8)	85 (5.6)	88 (4.9)
	Conf.	0.01	75 (7.8)	80 (8.2)	81 (9.1)	76 (8.5)	84 (17.7)	80 (9.6)
		0.1	89 (8.4)	93 (8.4)	90 (6.6)	93 (9.1)	86 (5.4)	88 (3.8)
eggs	Quant.	0.01	88 (10.6)	95 (3.5)	82 (4.5)	99 (8.0)	86 (8.0)	75 (6.4)
		0.1	85 (7.0)	98 (4.1)	85 (8.8)	102 (6.2)	94 (2.0)	93 (3.9)
	Conf.	0.01	85 (16.4)	97 (4.3)	87 (3.4)	102 (7.7)	89 (8.0)	74 (1.1)
		0.1	84 (6.7)	96 (6.7)	87 (6.7)	100 (5.8)	93 (3.2)	94 (4.4)

Table B.5.2.2-5 Precision and Accuracy (% Recovery) – modified method

Matrix	Ion transition	Spike level	Mean Recovery (RSD) (%)

		(mg/kg)	n=5						
			Flutolanil	M-2	M-2 conj	M-4	M-4 conj	M-7	M-101
Liver	Quant.	0.01	99 (8.3)	82 (6.8)	98 (12.6)	95 (3.7)	82 (2.3)	101 (5.1)	74 (5.9)
		0.1	95 (3.0)	97 (3.3)	100 (10.0)	102 (1.9)	95 (7.7)	93 (3.3)	71 (2.7)
	Conf.	0.01	97 (6.3)	99 (10.6)	104 (7.5)	92 (13.8)	89 (13.7)	96 (5.1)	76 (7.2)
		0.1	97 (5.0)	97 (1.7)	101 (10.3)	102 (3.9)	95 (6.9)	94 (3.3)	70 (5.1)
Muscle	Quant.	0.01	97 (3.4)	107 (3.8)	82 (7.6)	90 (7.5)	91 (8.5)	97 (5.9)	96 (6.3)
		0.1	92 (6.4)	98 (6.3)	85 (9.2)	102 (5.1)	94 (9.0)	103 (4.5)	103 (4.1)
	Conf.	0.01	99 (5.3)	107 (3.9)	80 (6.7)	96 (5.4)	92 (7.4)	102 (3.8)	102 (7.6)
		0.1	95 (5.4)	103 (7.2)	58 (10.9)	100 (6.5)	94 (10.8)	105 (4.8)	103 (3.9)
Fat	Quant.	0.01	79 (13.4)	90 (12.8)	-	101 (8.3)	-	80 (9.9)	76 (8.6)
		0.1	86 (13.4)	92 (9.6)	-	109 (8.2)	-	96 (4.8)	78 (8.8)
	Conf.	0.01	79 (17.6)	88 (14.3)	-	107 (5.2)	-	90 (9.1)	81 (7.8)
		0.1	88 (14.9)	92 (9.8)	-	105 (10.0)	-	96 (3.8)	78 (10.0)
eggs	Quant.	0.01	99 (4.6)	87 (8.2)	-	95 (5.8)	-	83 (13.1)	96 (6.8)
		0.1	105 (7.0)	99 (6.1)	-	102 (5.6)	-	95 (5.4)	99 (3.4)
	Conf.	0.01	100 (5.2)	84 (10.8)	-	99 (3.0)	-	82 (12.9)	95 (11.1)
		0.1	106 (6.3)	96 (6.9)	-	101 (5.3)	-	95 (8.2)	101 (2.2)

Comparisons of original method and modified method

Both sets of validation results are considered to be equivalent (except for those following the de-conjugation step). This is demonstrated by the similar results obtained for Flutolanil and metabolites (except for the results following the de-conjugation step for M-2 and M-4) which were obtained using both methods.

Conclusion

The analytical method is sufficiently validated in compliance with SANCO/825/00 rev. 8.1 to determine flutolanil in poultry with an LOQ of 0.01 mg/kg.

An independent laboratory validation (ILV) of the analytical method for the determination of flutolanil in milk, liver, kidney, muscle, fat and egg was conducted. A full description and its validation follows.

Reference	:	Ihara, T. (2016) K-CA 4.2/06	GLP statement	:	Yes
Type of study	:	Flutolanil: ILV (independent laboratory validation) study of analytical method for flutolanil and its metabolites in foodstuffs of animal origin (bovine and hen)	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3074

The objective of this study was to independently validate the analytical method for the determination of flutolanil and metabolites (M-2, M-4, M-7, M-101 and M-102) in milk, liver, kidney, muscle, fat and egg in accordance of SANCO/825/00 rev 8.1. As the proposed residue definition for monitoring purposes is flutolanil, only the results for flutolanil are reported below.

Principle of the method:

Modified method (with acid extraction and de-conjugation step):

Samples (except fat) were extracted with acetonitrile and acidic acetonitrile and clean-up with a C18 solid phase extraction (SPE) cartridge. Fat samples were extracted with acetonitrile/hexane (50/50, v/v) and acidic acetonitrile/hexane (50/50, v/v) and cleaned-up by liquid-liquid partition. An enzyme hydrolysis step with ß-glucorinidase was also included for M-2 and M-4 for all matrices except fat, followed by a clean-up with a C_{18} solid phase extraction (SPE) cartridge. Quantification was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

HPLC-MS/MS Analysis parameters:

HPLC system	Applied Biosystems Sciex 3200QTrap coupled with Agilent 1200 Series HPLC System			
Column	TSKgel ® ODS-100V (2.0 cm x 75 mm, 3 µm)			
Injection volume	10 µL			
Mobile phases	Eluent A: Methanol:Water (10/90, v/v) + 0.01M ammonium formate + 0.1 % formic acid Eluent B: Methanol + 0.1 % formic acid			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0	80	20	0.3
	0.5	80	20	0.3
	4.5	0	100	0.3
	5.5	0	100	0.3
	5.51	80	20	0.3
	8.0	80	20	0.3

Analyte	Ionisation mode	Transition	
Flutolanil	ESI+	324 / 262	Quantification
		324 / 242	Confirmation
M4	ESI+	282 / 262	Quantification
		282 / 242	Confirmation

Assessment of method validation

Parameters		Flutolanil and M4	
Linearity	**Concentration Range (ng/mL)**	5 calibration points 0.2 to 20 ng/mL (corresponding to 0.002 – 0.2 mg/kg in sample) covering the concentration range from the 30% of the LOQ to 10xLOQ + at least 20%.	
	Equation	Quantification	Confirmatory
		See table below (B.5.2.2-6)	
Limit of Quantification (LOQ)		0.01 mg/kg	
Precision and Accuracy (% Recovery)		See table below (Table B.5.2.2-7)	
Acceptable Limit % [SANCO]		Mean recoveries within 70-120% with RSD ≤ 20%	
Confirmatory		Additional confirmatory analysis is not required as the primary method is a highly specific method (LC-MS/MS).	
Specificity		No interference above 30% of the LOQ were observed in the control soil samples at the retention time of flutolanil and its metabolites.	

Table B.5.2.2-6 Typical calibration curves and correlation coefficient for standards in matrix

Flutolanil	Milk		Liver	
Quantitation	y = 31268.42 x +3710.66	r = 0.9999	y = 26837.01 x + 944.11	r = 0.9999
Confirmation	y = 30813.40 x + 1496.10	r = 1.0000	y = 26871 x – 358.85	r = 0.9997
	Kidney		Muscle	
Quantitation	y = 32582.91 x + 2083.95	r = 1.0000	y = 38298.85 x + 5418.69	r = 0.9999
Confirmation	y = 31613.37 x + 3067.30	r = 1.0000	y = 37970.67 x + 2408	r = 1.0000
	Fat		Egg	
Quantitation	y = 36793.18 x + 6164.90	r = 0.9995	y = 39520.75 x + 3090.67	r = 1.0000
Confirmation	y = 36766.84 x + 4342.40	r = 1.0000	y = 38872.29 x + 3613.40	r = 1.0000

M4	Milk		Liver	
Quantitation	y = 8496.24x +1076.83	r = 0.9999	y = 8746.37x + 746.31	r = 1.0000
Confirmation	y = 7096.68x + 88.67	r = 1.0000	y = 7136.43x – 993.15	r = 0.9995
	Kidney		Muscle	
Quantitation	y = 9768.1x – 259.88	r = 1.0000	y = 7903.34x – 545.99	r = 0.9996
Confirmation	y = 8101.94x – 676.85	r = 1.0000	y = 6547.93x – 140.51	r = 1.0000
	Fat		Egg	
Quantitation	y = 8343.70x + 707.08	r = 0.9999	y = 9322.97x + 404.57	r = 1.0000
Confirmation	y = 6955.34x + 81.72	r = 1.0000	y = 7909.02x + 8.70	r = 0.9999
	Milk, enzyme hydrolysis		Liver, enzyme hydrolysis	
Quantitation	y = 10511.16x – 157.35	r = 1.0000	y = 9684.31x – 40.51	r = 0.9998
Confirmation	y = 8838.68x – 10.00	r = 0.9998	y = 7987.80x – 33.46	r = 1.0000
	Kidney, enzyme hydrolysis		Muscle, enzyme hydrolysis	
Quantitation	y = 11327.11x + 428.03	r = 1.0000	y = 10103.50x + 402.94	r = 1.0000
Confirmation	y = 9184.60x – 87.38	r = 0.9997	y = 8223.64x + 287.24	r = 0.9999

Table B.5.2.2-7 Precision and Accuracy (% Recovery)
Flutolanil

Matrix	Ion transition	Spike level (mg/kg)	Mean Recovery (%)	RSD (%)	n
Milk	Quant.	0.01	84	4.2	5
		0.1	92	1.8	5
	Conf.	0.01	91	3.3	5
		0.1	94	2.3	5
Liver	Quant.	0.01	71	3.5	5
		0.1	70	2.4	5
	Conf.	0.01	75	3.0	5
		0.1	71	3.0	5
Kidney	Quant.	0.01	91	4.1	5
		0.1	91	2.7	5
	Conf.	0.01	92	2.3	5
		0.1	92	3.3	5
Muscle	Quant.	0.01	78	3.9	5

		0.1	85	2.4	5
	Conf.	0.01	84	3.2	5
		0.1	85	1.9	5
fat	Quant.	0.01	72	9.3	5
		0.1	72	7.7	5
	Conf.	0.01	74	8.3	5
		0.1	71	7.2	5
eggs	Quant.	0.01	85	3.5	5
		0.1	87	2.2	5
	Conf.	0.01	83	3.0	5
		0.1	87	1.9	5

M4

Matrix	Ion transition	Spike level (mg/kg)	Mean Recovery (%)	RSD (%)	n
Milk	Quant.	0.01	84	2.3	5
		0.1	97	3.3	5
	Conf.	0.01	97	5.1	5
		0.1	97	1.1	5
Liver	Quant.	0.01	78	5.6	5
		0.1	81	2.5	5
	Conf.	0.01	71	2.4	5
		0.1	82	2.9	5
Kidney	Quant.	0.01	91	3.1	5
		0.1	83	2.7	5
	Conf.	0.01	94	5.4	5
		0.1	83	2.4	5
Muscle	Quant.	0.01	109	5.5	5
		0.1	100	3.3	5
	Conf.	0.01	106	3.9	5
		0.1	99	3.7	5
fat	Quant.	0.01	102	7.4	5
		0.1	103	2.3	5
	Conf.	0.01	108	8.4	5
		0.1	103	3.3	5
eggs	Quant.	0.01	92	3.0	5
		0.1	92	1.9	5
	Conf.	0.01	93	2.8	5
		0.1	91	1.8	5

M4 conjugate

Matrix	Ion transition	Spike level (mg/kg)	Mean Recovery (%)	RSD (%)	n
Milk	Quant.	0.01	99	4.5	5
		0.1	93	3.0	5
	Conf.	0.01	94	5.3	5
		0.1	93	2.6	5
Liver	Quant.	0.01	99	4.1	5
		0.1	97	4.4	5
	Conf.	0.01	98	4.6	5
		0.1	95	5.0	5
Kidney	Quant.	0.01	98	4.3	5
		0.1	99	5.1	5
	Conf.	0.01	104	4.4	5
		0.1	100	6.3	5

Muscle	Quant.	0.01	89	6.6	5
		0.1	86	11.7	5
	Conf.	0.01	86	7.7	5
		0.1	84	10.2	5

Conclusion:
The method was independently validated according to the guidance document SANCO/825/00 rev.8.1 for the determination of Flutolanil, M4 and its conjugate in milk, liver, kidney, muscle, fat and eggs.

The report also includes validation data for metabolites M-2, M-7 and M-101. The RMS has not received a summary for these compounds. Considering these compounds are not included in the residue definition, the data is not considered required.

Extraction efficiency
The applicant has given the following justification that the extraction efficiency should be considered addressed:
In the cow study, the extraction was conducted with acetonitrile and acetonitrile/0.1N HCl (4/1), except for fat, where the extraction solvents were hexane/acetonitrile (1/1) and hexane/ acetonitrile/0.1N HCl (5/4/1) and the acetonitrile layer was analysed. In the hen study, the extraction was conducted with acetonitrile, except for fat, where hexane/ acetonitrile (1/1) was the extraction solvent, and acetonitrile layer was analysed. In the goat metabolism study (B.7.2.3.2, B.7.2.3.3), the majority of extractable residual radio-activity was recovered in acetonitrile, acetonitrile /water (4/1) and acetonitrile /0.1N HCl (4/1) extract fraction. In fat matrix, hexane fraction was further extracted with acetonitrile, and radio-activity was remained only in acetonitrile layer. In milk matrix, the majority of residual radio-activity was recovered in acetonitrile extracts. In the hen metabolism study (B.7.2.2.2), the majority of extractable residual radio-activity was also recovered in acetonitrile.

B.5.2.3 Methods for the determination of residues in soil

Data on analytical monitoring methods for the determination of the active ingredient in soil, was submitted for the first inclusion of flutolanil into Annex I and was reviewed under uniform principles. The method in the DAR addendum is still considered adequate to address this endpoint.

Reference	:	Ihara, T. (2007a) K-CA 4.2/07	GLP statement	:	Yes
Type of study	:	Validation of analytical method for flutolanil in soil	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3048

Principle of the method:
Flutolanil was extracted from soil samples with acetonitrile. The solid phase was extracted again with acetonitrile. Supernatant was filtrated under slight suctioning. Thereafter the supernatant was liquid-liquid partitioned with NaCl phosphate buffer solution (pH 7). Resultant upper organic layer was then

withdrawn and evaporated in vacuo. Obtained residue was dissolved with acetonitrile/water and then loaded to a graphite carbon /NH_2 cartridge (Envi-carb®). Analyte was eluted from the column with acetonitrile/acetic acid (95/5, v/v). The eluate was then diluted with water. Flutolanil was determined by HPLC/MS/MS.

Characteristics of Kochi soil used in this study are presented in the table below.

Items	**Kochi soil**
Sampling location	Kochi, Japan
Sampling date	25 June 2001
Soil order	Alluvial soil
Soil texture Rough sand (%) Fine sand (%) Silt (%) Cray (%)	Clay loam 4.4 45.0 26.8 23.8
pH (H_2O) at 25°C pH (KCl) at 25°C pH ($CaCl_2$) at 25°C	6.6 5.5 5.9
Organic carbon (%)	1.46
Organic matter	2.52
Phosphate adsorbtion coefficient (10 mg/kg)	560
Cation exchange capacity (meq./100 g)	12.3
Maximum water holding capacity (10 g/kg)	52.2
Clay fraction mineralogy	Chlorite, Illite
Microbial biomass (10 mg/kg) at zero day	12

HPLC-MS/MS conditions:

Instrument: Agilent 1200 High Performance Liquid-Liquid Chromatograph

Column: Cadenza CD-C18, 50 mm × 2.0, 3 µm ODS

Solvent system: Solvent A: Distilled water containing 0.1% formic acid (v/v)
Solvent B: Methanol containing 0.1% formic acid (v/v)

Column temperature: 40°C

Time [min]	solvent A [%]	solvent B [%]	Gradient
0	50	50	linear
0.5	30	70	linear
5.5	0	100	hold
7.5	0	100	

Injection volume: 10 µL

Flow: 0.20 mL/min

Mass spectrometer: 3200Q trap triple quadrupole mass spectrometer

Ionization mode: ESI, positive

Analyte	Transition	CE (eV)	Dwell Time (ms)	Method
Flutolanil	324.1 / 262.1	23	100	Primary
	324.1 / 242.0	35	100	Confirmatory
	324.1 / 282.1	19	100	Confirmatory

Assessment of method validation in soil

Validation data are presented in the table below.

Parameters		Flutolanil
Linearity	Concentration Range (ng/mL)	6 calibration points - 0.2 to 50 µg/L (corresponding to 0.002 – 0.5 mg/kg in sample) covering the concentration range from 20% of LOQ to at least 10xLOQ + 20%.
	Intercept (a)	-
	Slope of the line (b)	24270.3
	Correlation Coefficient (r)	0.9999
Limit of Detection (LOD)		0.2 µg/L (corresponding to 0.002 mg/kg in sample)
Limit of Quantification (LOQ)		0.01 mg/kg
Precision and Accuracy (% Recovery)	Transition used	m/z 324 → 262
	Level-I	0.01 mg/kg
	Mean Recovery (n=5)	92.0%
	% RSD	7.3%
	Level-II	0.1 mg/kg
	Mean Recovery (n=5)	86.6%
	% RSD	7.3%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-120% with RSD ≤ 20%
Confirmatory	The confirmatory method was demonstrated by using the ratio method. Peak area ratio was calculated as the quotient of peak for one qualifier by the quantifier (m/z 324). Peak area ratios have been calculated for each calibration standards, given an average of c.a. 1 for the qualifier (m/z 242) and c.a. 0.63 for the qualifier (m/z 282). Peak area ratios have also been calculated for fortification samples, given similar value for the two qualifier. Therefore any results (linearity, precision, accuracy and specificity) demonstrated on the first m/z mass would be also demonstrated on the 2 other m/z masses The confirmatory method is considered acceptable.	
Specificity	No interference above 30% of the LOQ were observed in the control soil samples at the retention time of flutolanil.	

Conclusion:

As determined in addendum 5 to the original DAR, method Ihara (2007) is validated in compliance with SANCO/825/00 rev. 7 for the determination of flutolanil in soil with an LOQ of 0.01 mg/kg. Compliance with SANCO/825/00 rev. 8.1 is demonstrated.

B.5.2.4 Methods for the determination of residues in water

A new monitoring method has been developed and validated for the determination of flutolanil in water (drinking and surface water), in order to fulfil the requirement of the SANCO/825/00 rev. 8.1.

Reference	:	Nishimura, Y. (2015) K-CA 4.2/08	GLP statement	:	Yes
Type of study	:	Validation of analytical method for flutolanil in water	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3057

Principle of the method:

An aliquot (50 mL) of aqueous samples was combined with 5 mL of acetonitrile. Samples were loaded to SPE cartridges that were sequentially washed with 6 mL of acetonitrile and equilibrated with 6 mL of 10% aqueous acetonitrile (v/v) prior to use. After loading of sample water, the SPE cartridges were then sequentially washed with 6 mL of 10% aqueous acetonitrile (v/v). Finally flutolanil was eluted from the SPE cartridge by 9 mL of acetonitrile. Flutolanil in a large volume of aqueous matrices was concentrated by solid phase extraction (SPE) and analysed by reverse-phase HPLC-MS/MS. Surface water samples were collected from Ishikawa River (pH 7.4, clear, no suspended material).

LC-MS/MS conditions:

Instrument: Agilent 1200 High Performance Liquid Chromatograph (Agilent technologies Inc.)

Solvent system: 0.1% formic acid (v/v) in water
0.1% formic acid (v/v) in methanol

Time [min]	solvent A [%]	solvent B [%]
0	50	50
0.5	30	70
5.5	0	100
7.5	0	100
7.6	50	50
12.0	50	50

Column: Cadenza CD-C18, 3 µm, 2.0 x 50 mm

Flow: 0.2 mL/min

Mass spectrometer: 3200Q trap triple quadrupole mass spectrometer
Ionization mode: ESI
Scan Mode: Multiple Reaction Monitoring (MRM)

Analyte	Transition	CE (eV)	Dwell Time (msec)	Method
Flutolanil	324 / 262	23	100	Primary
	324 / 242	35	100	Confirmatory
	324 / 282	19	100	-

Assessment of method validation in water

Validation data are presented in the table below.

Parameters		**Flutolanil**	
Linearity	**Concentration Range (ng/mL)**	6 calibration points – 0.2 - 50 µg/L (corresponding to 0.02 – 5 µg/L in sample) covering the concentration range from the 20% of the LOQ to 10xLOQ + at least 20%.	
		Quantification	Confirmatory
	Intercept (a)	4.7756	4.7648
	Slope of the line (b)	0.9353	0.9400

	Correlation Coefficient (r)	0.9998	0.9998
Limit of Quantification (LOQ)		0.1 µg/L (corresponding to 1 µg/L in the extract sample)	
Precision and Accuracy (% Recovery)	**Matrix**	Distilled water	River water
	Level-I	0.1 µg/L	0.1 µg/L
	Mean Recovery (n=5)	93.0%	94.9%
	% RSD	4.1%	2.1%
	Level-II	1.0 µg/L	1.0 µg/L
	Mean Recovery (n=5)	98.4%	101.3%
	% RSD	0.7%	2.0%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%	
CONFIRMATORY Precision and Accuracy (% Recovery)	**Matrix**	Distilled water	River water
	Level-I	0.1 µg/L	0.1 µg/L
	Mean Recovery (n=5)	93.8%	93.9%
	% RSD	5.1%	1.9%
	Level-II	1.0 µg/L	1.0 µg/L
	Mean Recovery (n=5)	97.8%	101.0%
	% RSD	1.5%	2.3%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-110% with RSD ≤ 20%	
Specificity		No interference above 30% of the LOQ were observed in the control samples at the retention time of flutolanil.	
Stability		Stable up to 6 days, when stored below 0°C in the dark.	

Conclusion:

The method is sufficiently validated for surface water, in compliance with SANCO/825/00 rev. 8.1. For the validation of drinking water, distilled water has been used, which is not acceptable. In addition, insufficient information is available on the characteristics of the tested water. The ILV does include adequate data, however. The RMS considers the data requirements to be fulfilled. The ILV can be used as primary validation for surface water, for which no ILV is required. The sampled water can be considered to address the drinking water requirement as it is expected to be worst-case. The LOQ of 0.1µg/L is acceptable for drinking water.

An independent laboratory validation (ILV) of the analytical method for the determination of flutolanil in water was conducted.

Reference	:	Clark, S. (2016) K-CA 4.2/09	GLP statement	:	Yes
Type of study	:	Independent Laboratory Validation of the Flutolanil Analytical Method Described in Nihon Nohyaku Co., Ltd. Final Report No. LSRC-A11-010A, Study Protocol No. GE-04, 11-0001, entitled "Validation of Analytical Method for Flutolanil in Water"	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3066 (+amendment)

Principle of the method:

Flutolanil in a large volume of aqueous matrices was concentrated by solid phase extraction (SPE) and analysed by reverse-phase HPLC-MS/MS. The ILV was conducted on surface water (river) at the LOQ of 0.1 µg/L, which is covering a worst case scenario than the validation requirement of the SANCO/825/00 rev 8.1.

Surface water samples were collected from the American River near Howe Avenue.

Water characteristics: pH 7.2, 5.4ppm Ca, 1.7ppm Mg, 2.1ppm Na, hardness 21 mg/L $CaCO_3$, conductivity 0.05 mmhos/cm, SAR 0.20, total dissolved solids 58 ppm, turbidity 1.13 NTU

LC-MS/MS conditions:

Instrument: Agilent Biosystems/Sciex API 4000 LC/MS/MS System with ACQUITY UPLC system

Solvent system: 0.1% formic acid (v/v) in water
0.1% formic acid (v/v) in methanol

Time [min]	solvent A [%]	solvent B [%]
0	50	50
0.5	30	70
5.5	0	100
7.5	0	100
7.6	50	50
12.0	50	50

Column: Cadenza CD-C18, 3 µm, 2.0 x 50 mm

Flow: 0.2 mL/min

Ionization mode: Positive

Scan Mode: Multiple Reaction Monitoring (MRM)

Analyte	Transition	CE (eV)	Dwell Time (msec)	Method
Flutolanil	324 / 262	23	100	Primary
	324 / 282	35	100	Confirmatory
	324 / 242	19	100	-

Assessment of method validation in water

Validation data are presented in the table below.

Parameters		**Flutolanil**
Linearity	**Concentration Range (ng/mL)**	6 calibration points – 0.2 - 50 µg/L (corresponding to 0.02 – 5 µg/L in sample) covering the concentration range from the 20% of the LOQ to 10xLOQ + at least 20%.

	Transition	324 / 262	324 / 242
	Intercept (a)	66.7	172
	Slope of the line (b)	6670	5760
	Correlation Coefficient (r)	0.9997	0.9995
Limit of Quantification (LOQ)		0.1 µg/L (corresponding to 1 µg/L in the extract sample)	
Precision and Accuracy (% Recovery)	**Transition**	324 / 262	324 / 242
	Level-I	0.1 µg/L	0.1 µg/L
	Mean Recovery (n=5)	84%	87%
	% RSD	8.0%	12%
	Level-II	1.0 µg/L	1.0 µg/L
	Mean Recovery (n=5)	89%	88%
	% RSD	4.2%	3.1%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-120% with RSD ≤ 20%	
Specificity		No interference above 30% of the LOQ were observed in the control samples at the retention time of flutolanil.	

Conclusion:

The analytical method described in Clark (2016) is suitable as independent laboratory validation to the method described in Nishimura (2015). The analytical method for the determination of residues of flutolanil in drinking and surface water is therefore sufficiently validated in compliance with SANCO/825/00 rev. 8.1.

B.5.2.5 Methods for the determination of residues in air

Data on monitoring analytical methods for the determination of the active ingredient in air was submitted for the first inclusion of flutolanil into Annex I and was reviewed under uniform principles. The method described in the DAR is still considered adequate to address this endpoint.

Reference	:	Dorn, U. (1999) K-CA 4.2/10	GLP statement	:	Yes
Type of study	:	Development and validation of an analytical method for the determination of flutolanil in air	Guideline	:	SANCO/825/00 rev. 7
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3042

Principle of the method:

During a 6-hour period with a flow rate of 1-2 l/min measured volume of air (500 l) is drawn through two-bed XAD-2 air sampling adsorption tubes to trap residues of flutolanil. The residues are extracted

from the adsorbent with ethyl acetate. The extracts are filtered and the extract volume adjusted to 10 ml. An aliquot of 9 ml extract is evaporated to dryness and the residue redissolved in 1 ml acetonitrile/water (1/1, v/v). The final extract is analysed by reversed phase HPLC with UV-detection at 240 nm and external standardisation.
For confirmation purposes 1 ml of the ethyl acetate extract (above) is analysed by fused silica capillary gas chromatography with MSD and external standardisation. Monitoring of characteristic ion m/z 173 was used for quantitation.
The residues are extracted separately from the adsorbent in the front and back segments with ethyl acetate.

HPLC/UV conditions:

Instrument:	Varian 9050 variable wavelength UV/VIS detector
Column:	Latek Nucleosil RP C-18; 150 mm x 4 mm, 5 µm
Eluent:	Acetonitrile/water 50/50 v/v isocratic
Flow rate:	1.0 mL/min
Detection:	UV at 240 nm

GC-MS conditions (confirmatory):

GC instrument:	Varian 3400 GC and temperature programmed SPI injector, Varian Saturn III Ion Trap MS
Injector:	temperature programmable SPI injector
MS instrument:	Saturn 3 ion trap
Ionisation:	EI
Column:	J&W DB-5ms (30m x 0.3 mm i.d. x 0.25 µm)
Column temp.:	Hold 70°C for 1 min., rate 20°C/min to 310°C, hold for 1 min at 310°C
Injector temp.:	Hold 120°C for 0.1 min., rate 200°C/min to 260°C, hold for 1 min at 260°C
Injection volume:	1 µL, splitless
Detection:	Ion for quantification 173 m/z

Assessment of method validation in air

Validation data are presented in the table below.

Parameters		**Flutolanil**	
Linearity	**Concentration Range**	>5 calibration points 0.1 to 20 µg/mL (corresponding to 0.2 – 40 µg/m^3 in sample), covering the concentration range from less than 10% of the LOQ to 10xLOQ + at least 20%.	
	Detector	HPLC/UV	GC-MS

	Intercept (a)	-	-
	Slope of the line (b)	28424.59	1251908.5839
	Correlation Coefficient (r)	0.999947	0.9981
Limit of Quantification (LOQ)		2.7 µg/m^3 (which is well in conformity with the acceptable minimum concentration "C" of 270 µg/m^3)	
Precision and Accuracy (% Recovery)		See table below (**Table B.5.2.5-1**)	
Acceptable Limit % [SANCO]		Mean recoveries within 70-110% with RSD ≤ 20%	
Breakthrough		There was no breakthrough to the rear segment in XAD-2 tube (< 1 % of fortifications at higher fortification level)	
Stability		The stability of fortified XAD-2 tubes stored at room temperature and in freezer overnight and for 7 days was analysed.	
Confirmatory		GC/MS with full scan mass spectra was demonstrated as confirmatory method.	

Table B.5.2.5-1: Precision and Accuracy (% Recovery)

Type of method Developed by	Substrate	Fortification level (µg/m^3)	LOQ (µg/m^3)	Recovery (%) mean range	RSD (%)	N
HPLC/UV Dorn, U. 1999 (A-3042)	Ambient air (24 °C and 30 % humidity)	2.7 27 overall	2.7	97 93-100 104 101-107 100	3 2 2	5 5 10
	Warm, humid air (36 °C and 88 % humidity)	2.7 27 overall	2.7	103 96-107 110 108-111 106	4 1 4	5 5 10
GC/MSD (confirmation)	Ambient air (24 °C and 30 % humidity)	2.7 27 overall	2.7	105 88, 122 102 103-100 103	- - 14	2 2 4
	Warm, humid air (36 °C and 88 % humidity)	2.7 27 overall	2.7	92 88, 96 102 101, 102 97	- - 7	2 2 4

Limit of Quantification: The determined LOQ was 2.7 µg/m^3; the lowest fortification level undertaken demonstrating successful recoveries.

This LOQ is well below the concentration C calculated from the acceptable daily intake (ADI) (in [mg/kg bw]) according to the following equation:

$$C = (\text{ADI value} \times \text{safety factor} \times \text{body weight}) / \text{air intake}$$

$$C = (0.09 \times 0.1 \times 60) / 20 = 0.27 \text{ mg/m}^3$$

$$\mathbf{C = 270 \text{ µg/m}^3}$$

where ADI value = 0.09 mg/kg body weight

safety factor = 0.1

body weight = 60 kg (body weight)

air intake = 20 m^3/day

Conclusion:

The method was successfully validated compliant with SANCO/825/00 rev 8.1., with an LOQ of 2.7 µg/m^3, and is suitable for the determination of residues of flutolanil in air as enforcement method.

B.5.2.6 Methods for the determination of residues in body fluids and tissues

Reference	:	Airs, D. (2015) K-CA 4.2/11	GLP statement	:	Yes
Type of study	:	Flutolanil: validation of methodology for the determination of residues of flutolanil and metabolites in bovine liver, kidney, muscle, fat, whole milk, skimmed milk and cream	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	A-3073

This method validation is used to cover the requirement for body tissue, and is also described in the section for animal matrices. The method is sufficiently sensitive, and therefore no additional method or validation study is required. The methodology and summary of results of this report are described in section B.5.2.2.

This method has been validated with an LOQ of 0.01 mg/kg in liver, kidney, muscle and fat.

The following method and validation has been submitted to cover the requirement for body fluid.

Reference	:	Taoudi, M. (2016) K-CA 4.2/12	GLP statement	:	Yes
Type of study	:	Development and method validation – determination of residues of flutolanil in body fluid by LC-MS/MS	Guideline	:	SANCO/825/00 rev. 8.1
			Acceptability	:	Acceptable
Test substance	:	Flutolanil	Method reference	:	XG/16/003

Principle of the method

Residues of flutolanil were extracted from dog plasma by protein precipitation in acetonitrile, followed by vortex mixing and centrifugation for 5 minutes. A portion of the final extract was then taken for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two ion mass transitions.

LC-MS/MS conditions:

	HPLC Conditions			
Column:	Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 x 50 mm			
Injection Volume:	10 µL			
Flow Rate:	500 µL/min			
Mobile Phase A:	0.1% Formic acid in water			
Mobile Phase B:	0.1% Formic acid in acetonitrile			
Gradient:	Time (min)	%A	%B	

	0.0	95		5			
	4.0	5		95			
	5.0	5		95			
	5.1	95		5			
	6.0	95		5			
	Mass Spectrometer Conditions						
Mass Spec:	API5500						
Polarity:	Positive						
Analyte	Transition	Ion Mass Transitions (*m/z*)	Dwell Time (msec)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)	Declustering Potential (DP)	Approx. Retention Time (mins)
Flutolanil	1	324.1/242.0	100	35 V	16 V	80 V	3.4
	2	324.1/262.0	100	25 V	12 V	80 V	3.4

Validation data are presented in the table below:

Parameters		**Flutolanil**	
Linearity	**Concentration Range (ng/mL)**	8 calibration points - 0.5 to 4 ng/mL (corresponding to 0.01 – 0.08 mg/kg in sample) covering the concentration range from 20% of LOQ to 120% above the concentration level	
	Transition used	324.1/242.0 (Quantitation)	324.1/262.0 (Confirmation)
	Equation	y = 45400 x + 483	y = 43800 x + 1660
	Correlation Coefficient (r)	0.9995	0.9998
Limit of Quantification (LOQ)		0.05 mg/L	
Precision and Accuracy (% Recovery)	**Transition used**	324.1/242.0 (Quantitation)	324.1/262.0 (Confirmation)
	Level-I	0.05 mg/L	0.05 mg/L
	Mean Recovery (n=5)	97.0%	97.9%
	% RSD	1.9%	1.3%
	Acceptable Limit % [SANCO]	Mean recoveries within 70-120% with RSD ≤ 20%	
Confirmatory		Additional confirmatory analysis is not required as the primary method is a highly specific method (LC-MS), with an analysis using 2 transitions.	
Specificity		No interference above 30% of the LOQ were observed in the control samples at the retention time of flutolanil	

Conclusion

The analytical method described in Taoudi 2015 has been sufficiently validated in compliance with SANCO/825/00 rev. 8.1 in body fluids with an LOQ of 0.05 mg/L.

B.5.3 References relied on

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.1.2-01	Van de Ruit A. N. R.	1998	Method Validation Study for the Analysis of Flutolanil in Soil and in Soil/Potato Mixture by Gas Chromatography/Mass Spectrometry BCO Analytical Services B.V. Report No.: 12395 - CRLD No. 97-83, Analytico (BCO) project number 4497040003 (A-3039) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-02	Ihara T.	2007a	Validation of analytical method for flutolanil in soil Research Center, Nihon Nohyaku Co., Ltd Report No.: LSRC-A07-161A (A-3048) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-03	Castro L.	1994	Dissipation of flutolanil on bare soil following application of Flutolanil 50WP, USA, 1989 NOR-AM Chemical Company Report No.: R642.07.89 (E-3018) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-04	Castro L.	1993	Long-term field dissipation of flutolanil under conditions of peanut cultivation initiated 1989, USA NOR-AM Chemical Company Report No.: R642.08.89 (E-3023) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.1.2-05	Bourgade C., Yslan F.	1998a	Flutolanil: Analytical method for the determination of residues in drinking water and surface water Rhone-Poulenc AGRO Report No.: R&D/CRLD/AN/9816454 (A-3040) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-06	Bourgade C., Yslan F.	1998b	Flutolanil: Confirmatory chromatographic method for the determination of residues in water Rhone-Poulenc AGRO Report No.: R&D/CRLD/ANH/9816748 (A-3041) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-07	Dorn U.	1999	Development and validation of an analytical method for the determination of flutolanil in air PTRL Europe Report No.: B 361 G (A-3042) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-08	Wouters G.A.J.M.	2000	Method Validation Study for the Analysis of Flutolanil in Potato by GC/MS Analytico Medinet B.V. Report No.: CRLD No. 97-82, 4497040002 (A-3037) + Final Report amendment-1 GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.1.2-09	Fuchsbichler G	2001	Determination of flutolanil in potatoes: Independent laboratory validation of the method described in report CRLD 97-82 Bayerische Hauptversuchsanstalt fur Landwirtschaft Report No.: HVA 30/00, 00-154, (A-3038) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-10	Ihara T.	2007b	Validation of Analytical Method for Flutolanil and its Metabolites in Potato Toxicological & Pharmaceutical Research Center, Nihon Nohyaku Co., Ltd Report No.: LSRC-A07-133A (A-3050) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-12	Burton D.	2011	Validation of methodology for the determination of residues of flutolanil and metabolites M-2 and M-4 in potato Huntingdon Life Sciences Ltd Report No.: LMS0047 (A-3056) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-13	Bernal J.	2016	Flutolanil - Validation of the Analytical Method for the Determination of Flutolanil and its Metabolites M-2, M-4, M-101 and M-102 in Potatoes Tubers Eurofins Agroscience services Report No.: S14-04022 (A-3070) + Final Report Amendment No.1 GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.1.2-14	Merdian H.	2016	Validation of the Analytical Method for the Determination of Flutolanil and its Metabolites M-2, M-4, M-101 and M-102 in Potato after Hydrolysis Eurofins Agroscience services Report No.: S16-00710 (A-3081) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-15	Robinson J. D.	1999	Validation of an analytical method for the determination of residues in products of animal origin Huntingdon Life Sciences Ltd Report No.: RNP 628/993392 (A-3025) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-16	Wouter G.A.J.M.	1999	Independent laboratory validation of Rhone-Poulenc analytical method AR 192-99 for the determination of flutolanil in products of animal origin RHONE-POULENC Report No.: 99-173 (A-3027) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.1.2-17	Airs D.	2015	Flutolanil: Validation of methodology for the determination of residues of flutolanil and metabolites in bovine liver, kidney, muscle, fat, whole milk, skimmed milk and cream Envigo CRS Ltd Report No.: LMS0125 (A-3073) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.1.2-18	Dias N. A.	2016	Flutolanil: Residues of flutolanil and its metabolites in eggs and tissues of laying hens Envigo CRS Ltd Report No.: LMS0104 (A-3075) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-19	Brekelmans M.J.C.	2003a	Development and validation of an analytical method for the analysis of flutolanil in sediment samples from the sediment water chironomid toxicity test NOTOX B.V. Report No.: 355488 (A-3035) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-20	Brekelmans M.J.C.	2003b	Development and validation of an analytical method for the analysis of flutolail in iso-medium samples from the sediment water chironomid toxicity test NOTOX B.V. Report No.: 355477 (A-3036) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-21	Kendall T.Z., Nixon W.B.	2011	Analytical method verification for the determination of flutolanil in freshwater Wildlife International Ltd. Report No.: 397C-111 (A-3059) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.1.2-22	Bowman J.H.	1987a	Acute toxicity of flutolanil technical to rainbow trout (salmo gairdneri) Analytical Bio-Chemistry Laboratories Inc. Report No.: ABC LABS 35378 (W-3008) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-23	Bowman J.H.	1987b	Acute toxicity of flutolanil technical to Bluegill Sunfish (Lepomis macrochirus) Analytical Bio-Chemistry Laboratories Inc. Report No.: ABC LABS 35377 (W-3009) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-24	Bowman J.H., Bussard J.	1990	Acute toxicity of flutolanil technical to Fathead Minnow (Pimephales promelas) Analytical Bio-Chemistry Laboratories Inc. Report No.: ABC LABS 38101 (W-3010) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-25	Forbis A.D.	1991	Acute Toxicity of Flutolanil to *Mysidopsis bahia* Analytical Bio-Chemistry Laboratories Inc. Report No.: 38720 (W-3015) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.1.2-26	Scheller K.	2016	Repeated exposure of Flutolanil 40SC to honey bee (*Apis mellifera*) larvae under laboratory conditions (*in vitro*) BioChem agrar GmbH Report No.: 16 10 48 035 B (N-3079) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.1.2-27	Ruhland S.	2016	Chronic toxicity of Flutolanil SC to the honey bee *Apis mellifera* L. under laboratory conditions BioChem agrar GmbH Report No.: 16 10 48 034 B (N-3078) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-01	Fuchsbicher G.	2002	Development and validation of the multi-residue method DFG S19 modified, for the determination of the residue of flutolanil in potatoes Bayerische Hauptversuchsanstalt fur Landwirtschaft Report No.: HVA 19/02 (A-3034) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd
CA 4.2-02	Taoudi M.	2016a	Method Validation – Determination of Residues of Flutolanil in Crops by LC-MS/MS Battelle UK Ltd Report No.: XG/16/002 GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-03	Thorn J.	2016	Independent Laboratory Validation for the Determination of Residues of Flutolanil in Crops by GC/MS and LC-MS/MS Battelle Report No.: 100075555 GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.2-04	Airs D.	2015	Flutolanil: Validation of methodology for the determination of residues of flutolanil and metabolites in bovine liver, kidney, muscle, fat, whole milk, skimmed milk and cream) Envigo CRS Ltd Report No.: LMS0125 (A-3073) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-05	Dias N. A.	2016	Flutolanil: Residues of flutolanil and its metabolites in eggs and tissues of laying hens Envigo CRS Ltd Report No.: LMS0104 (A-3075) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-06	Ihara T.	2016	Flutolanil: ILV (Independent Laboratory Validation) Study of Analytical Method for Flutolanil and its Metabolites in Foodstuffs of Animal Origin (Bovine and Hen) Research Center, Nihon Nohyaku Co., Ltd Report No.: LSRC-R15-141A (A-3074) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-07	Ihara T.	2007a	Validation of analytical method for flutolanil in soil Research Center, Nihon Nohyaku Co., Ltd Report No.: LSRC-A07-161A (A-3048) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.2-08	Nishimura Y.	2015	Validation of Analytical Method for Flutolanil in Water Research Center, Nihon Nohyaku Co., Ltd Report Report No.: LSRC-A11-010A (A-3057) + amendment to Final Report GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-09	Clark S.	2016	Independent Laboratory Validation of the Flutolanil Analytical Method Described in Nihon Nohyaku Co., Ltd. Final Report No. LSRC-A11-010A, Study Protocol No. GE-04, 11-0001, entitled “Validation of Analytical Method for Flutolanil in Water” Morse Laboratories, LLC Report No.: 66925 Final amended report (A-3066-1) + Original report (A-3066) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-10	Dorn U.	1999	Development and validation of an analytical method for the determination of flutolanil in air PTRL Europe Report No.: B 361 G (A-3042) GLP: yes Published: no	N	N	Evaluated in DAR	Nihon Nohyaku Co. Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 4.2-11	Airs D.	2015	Flutolanil: Validation of methodology for the determination of residues of flutolanil and metabolites in bovine liver, kidney, muscle, fat, whole milk, skimmed milk and cream) Envigo CRS Ltd Report No.: LMS0125 (A-3073) GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd
CA 4.2-12	Taoudi M.	2016b	Development and Method Validation - Determination of Residues of Flutolanil in Body Fluid by LC-MS/MS Battelle UK Ltd Report No.: XG/16/003 + Report Amendment 1 GLP: yes Published: no	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co. Ltd