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ERM
European Reference Materials

CERTIFICATION REPORT

The certification of Fipronil sulfone and the Sum of fipronil and fipronil sulfone expressed as fipronil in egg powder ERM®-BB125

EUR 30157 EN

European Commission
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JRC120285
EUR 30157 EN

ISBN 978-92-76-17604-6 (PDF)
ISSN 1831-9424 (online)
doi:10.2760/822406

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Abstract

This report describes the production of the ERM-BB125, which is an egg material certified for the mass fraction of fipronil sulfone and the sum of fipronil and fipronil sulfone expressed as fipronil. This material was produced following ISO 17034 and is certified in accordance with ISO Guide 35.
Eggs from a farm embargoed by the Belgian food safety authorities were freeze-dried, cryo-milled and homogenised.
Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2017.
The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity, instability and characterisation.
The material is intended for the quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or during validation studies. The CRM is available in sealed glass vials containing at least 5 g of dried egg powder. The minimum amount of sample to be used is 1 g of the dry material.

How to cite this report: P. Shegunova, S. Harbeck, M. Dabrio, J. Seghers, H. Emteborg, *The certification of fipronil sulfone and the sum of fipronil and fipronil sulfone expressed as fipronil in egg powder ERM®-BB125*, EUR 30157 EN, Publications Office of the European Union, Luxembourg, 2020, ISBN 978-92-76-17604-6, doi:10.2760/822406, JRC120285.

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Summary

This report describes the production of the ERM-BB125, which is an egg material certified for the mass fraction of Fipronil sulfone and the Sum of fipronil and fipronil sulfone expressed as fipronil. This material was produced following ISO 17034 [1] and is certified in accordance with ISO Guide 35 [2].

Eggs from a farm embargoed by the Belgian food safety authorities were freeze-dried, cryo-milled and homogenised.

Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2017 [2].

The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025 [3]. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The material is intended for the quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or during validation studies. The CRM is available in sealed glass vials containing at least 5 g of dried egg powder. The minimum amount of sample to be used is 1 g of the dry material.

The following values were assigned:

	Mass Fraction	
	Certified value [5] [mg/kg]	Uncertainty [6] [mg/kg]
Fipronil sulfone[1,2)]	0.060	0.005
Sum of fipronil[3)] and fipronil sulfone[2)] expressed as fipronil[1,4)]	0.058	0.005

[1)] As obtained by chromatography and mass spectrometry. Based on dry mass basis and corrected for recovery

[2)] 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfonyl]-1H-pyrazole-3-carbonitrile, CAS No 120068-36-2

[3)] 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile, CAS No 120068-37-3

[4)] Corresponds to the parameter “Fipronil (sum of fipronil + sulfone metabolite expressed as fipronil)” as listed in Commission Regulation (EU) 2019/1792.

[5)] Certified values are values that fulfil the highest standards of accuracy and represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value and its uncertainty are traceable to the International System of units (SI).

[6)] The uncertainty is the expanded uncertainty of the certified value with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

Table of contents

Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
BEH	Ethylene Bridged Hybrid
c	Mass concentration $c = m / V$ (mass / volume)
CLSI	Clinical and Laboratory Standards Institute
CRM	Certified reference material
dSPE	dispersive Solid Phase Extraction
EC	European Commission
ECD	Electron capture detection
EI	Electron ionisation
EN	European norm (standard)
ERM®	Trademark of European Reference Materials
EU	European Union
GC	Gas chromatography
GC-ECD	Gas chromatography-electron capture detection
GC-MS	Gas chromatography-mass spectrometry
GUM	Guide to the Expression of Uncertainty in Measurements *[ISO/IEC Guide 98-3:2008]*
HPLC	High performance liquid chromatography
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MRL	Maximum residue level
MS	Mass spectrometry
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
MQC	Method quality control
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
n.c.	Not calculated

n.d.	Not detectable
PGC	Porous Graphitic Carbon
PSA	Primary-secondary amine sorbent
QuEChERS	“quick, easy, cheap, effective, rugged, and safe”
RASFF	Rapid Alert System for Food and Feed
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
r^2	Coefficient of determination of the linear regression
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
se	Standard error
SweEt	Swedish ethyl acetate method
SI	International System of Units
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
s_{ns}	Standard deviation of results of normal stock samples
SPE	Solid phase extraction
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation
T	Temperature
t	Time
t_i	Time point for each replicate
$t_{\alpha, df}$	Critical t-value for a t-test, with a level of confidence of 1-α and df degrees of freedom
t_{sl}	Proposed shelf life
u	Standard uncertainty
U	Expanded uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_c	Combined standard uncertainty; an additional index "rel" is added as appropriate
u_{cal}	Standard uncertainty of calibration

u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{Δ}	Combined standard uncertainty of measurement result and certified value
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
UPLC	Ultra Performance Liquid Chromatography
u_{rec}	Standard uncertainty related to possible between-unit inhomogeneity modelled as rectangular distribution; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
u_{t}	Standard uncertainty of trueness
$\bar{x}$	Arithmetic mean
$\bar{x}_{ns}$	Arithmetic mean of all results of normal stock samples
$\bar{x}_{ref}$	Arithmetic mean of results of reference samples
α	Significance level
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{s,meas}$	Degrees of freedom for the determination of the standard deviation s_{meas}
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

In August 2017 millions of eggs were destroyed and egg products removed from the shelves of supermarkets and stores in Europe. According to the RASFF (Rapid Alert System for Food and Feed) triggered by the Belgian authorities, chicken eggs were found to contain from 0.0031 to 1.2 mg/kg fipronil [5], far above the maximum level of 0.005 mg/kg in fresh egg allowed by the EU legislation [6,7]. Eggs contaminated with high levels of fipronil were discovered in Belgium, the Netherlands, France and a dozen of other European countries.

Fipronil is used as an insecticide to protect crops as well as in veterinary medicine to kill off fleas, lice, ticks, roaches and mites. If a pest infestation at a farm is treated with fipronil, the animal's skin – or feathers in case of chickens spaces– could absorb the insecticide. Traces can then also be found in animal products like eggs. In Europe, fipronil is exclusively authorised for use as a plant protector in products for seed treatment.

To ensure safe food for the citizens, all products within the EU need to comply with the maximum residue levels (MRLs) of pesticide residues established by the European legislation [6,7] prior their commercialisation. The MRL for fipronil in bird eggs is set as 0.005 mg/kg with a residue definition of the sum of fipronil and sulfone metabolite expressed as fipronil.

Laboratories in charge of control and monitoring of fipronil in food stuff need to offer reliable and comparable results. The use of certified reference materials and the participation in proficiency testing schemes are essential tools for assuring and controlling the quality of analytical data and to provide evidence of analytical method performance [3,4]

To contribute to the harmonisation of reliable analytical results, and thus to the proper implementation of EU legislation, the Joint Research Centre of the European Commission launched a proficiency test for determination of fipronil in eggs. Furthermore a survey to identify the need of Certified Reference Materials (CRMs) for analysis of fipronil in egg or egg products was set up. As a follow up of the survey and as a response of the needs the JRC of the European Commission initiated the production of a CRM for determination of fipronil in eggs as defined in the legislation [6,7].

This report describes in detail the steps for the production of ERM-BB125, an egg powder material certified for the fipronil sulfone and the sum of fipronil and fipronil sulfone expressed as fipronil.

1.2 Choice of the material

Contaminated eggs were collected from a farm embargoed by the Belgian food safety authorities and used as base material for production of the CRM. The target mass fraction of fipronil in the processed egg powder was chosen as to be above the MRL (0.005 mg/kg fresh egg and 0.02 mg/kg dry egg powder) established by the EU legislation [6,7].

1.3 Design of the CRM project

The project was designed, managed and developed at the JRC European Commission, Joint Research Centre, Directorate F– Health, Consumers and Reference Materials.

Dedicated analytical methodology was developed and validated in-house to support the different steps of the CRM production. A method based on tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) was applied during the production to optimise

the material processing conditions as well as for the assessment of homogeneity and stability of the fipronil in the CRM.

Characterisation was based on an interlaboratory comparison involving a number of expert laboratories in the field of pesticides residue analysis. Selected laboratories taking part in the material certification campaign were ISO/IEC 17025 accredited for the particular applications. The participants in the characterisation study were instructed to apply their own validated analytical methodology for the determination of fipronil in egg.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.3 Homogeneity and stability studies

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(BELAC No. 268-RM; measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 268-TEST)

2.4 Characterisation

ANALYTEC® Labor für Lebensmitteluntersuchung und Umweltanalytik, Salzburg, AU
(measurements under the scope of ISO/IEC 17025 accreditation, Akkreditierung Austria, No. 0182)

Bodemkundige Dienst van België, Haverlee, BE
(measurements under the scope of ISO/IEC 17025 accreditation, BELAC; No. 127-TEST)

DUCARES B.V., Utrecht, NL
(measurements under the scope of ISO/IEC 17025 accreditation, Dutch Accreditation Council RvA; No. L494)

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(BELAC No. 268-RM; measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 268-TEST)

Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, IT
(measurements under the scope of ISO/IEC 17025 accreditation, Accredia, No. 0217)

Labor Friedle GmbH, Tegernheim, DE
(measurements under the scope of ISO/IEC 17025 accreditation, Deutsche Akkreditierungsstelle; No. D-P-14646-03-00)

Laboratório Regional de Veterinária e Segurança Alimentar, Funchal, PT
(measurements under the scope of ISO/IEC 17025 accreditation, Instituto Português de Acreditação; No.L0509-1)

Laboratoire du SCL de Montpellier, Montpellier, FR
(measurements under the scope of ISO/IEC 17025 accreditation, Le Comité Français d'Accréditation; No. 1-0154)

Livsmedelsverket, Uppsala, SE
(measurements under the scope of ISO/IEC 17025 accreditation, SWEDAC; No. 1457)

3 Material processing and process control

3.1 Origin of the starting material

Eggs from a farm embargoed by the Belgian food safety authorities were collected. The levels of fipronil compound in the contaminated eggs were lower than expected. Nevertheless the levels of the fipronil sulfone were higher than the MRL. Therefore the target nominal level of the sum of fipronil and fipronil sulfone expressed as firponil in the dry egg powder was set above the MRL. Several egg batches were analysed. Suitable levels of fipronil were found in one of the batches and it was used as the base material for further processing.

3.2 Processing

More than 2000 eggs were cracked, big shell pieces were manually removed and the egg white and egg yolk was pooled into a 100 L stainless steel drum (Figure 1). The egg slurry was stirred for three hours at a low speed to avoid foaming and then stored at +4 °C. The slurry was stirred for an additional two hours at room temperature before being passed it through a 250 µm sieve to remove smaller pieces of shell, lumps and highly viscous parts. Approximately 46 kg of the egg slurry was poured into trays for subsequent freeze drying.

Figure 1. Manual cracking of the contaminated eggs (on the left) and mixing of their content in a stainless steel drum (right).

The egg slurry was freeze dried in the trays in a Martin Christ FD 2-100D freeze drier (Martin Christ, Osterode, DE). The resulting dry egg was stored over liquid nitrogen in metallic drums overnight. The freeze dried egg material was then milled using a Palla VM-KT vibrating cryogenic mill (KHD Humboldt Wedag, Colone, DE). The mill was cooled down with liquid nitrogen to -196 °C prior to use. The cold powder was mixed in a three-dimensional mixer for one hour (Dyna-Mix CM200, Basel, CH) and afterwards stored at +4 °C before filling. The egg material bottling was carried out in a MCPI Vibrating filling machine set up inside a glovebox. Amber glass vials with 50mL capacity were filled with at least 5 g of egg powder. The vials were closed with a lyo-insert capped and labelled according to fill order using a capping and labelling assembly from Bausch & Ströbel (Ilshofen, DE) / BBK (Beerfelden, DE). The vials were stored at +4 °C.

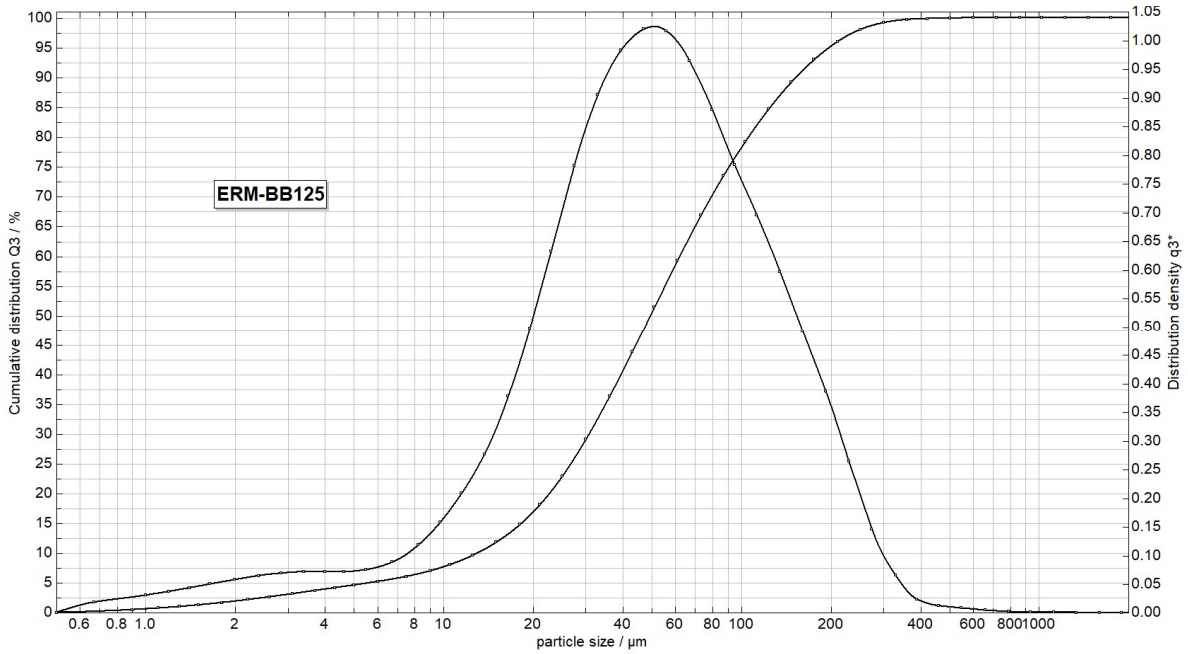
Prior to irradiation of the whole egg material batch, three units were gamma-irradiated at a dose of 10 kGy (Synergyhealth, Ede, NL) in order to check if the analytes (fipronil and fipronil sulfone) could withstand gamma irradiation. These samples were stored at +4 °C before shipment. The irradiated samples were analysed and the results confirmed that irradiation was a suitable preservation method for the material. Afterwards, the whole batch was irradiated with a minimum dose of 10 kGy (minimum calculated dose of 13.9 kGy and maximum dose of 21.1 kGy, Irradiation certificate NL25S12087403-1-1). Thereafter, the vials were placed into pre-labelled aluminium sachets in order to protect the material from light.

3.3 Process control

The water content measurement and particle size analysis was performed in the final material.

The water content in the dried egg powder was measured in duplicate in five samples covering the filling sequence using Volumetric-Karl Fischer titration (Metrohm, Herisau, CH). The average result was 1.78 ± 0.26 % (m/m) (expanded uncertainty).

Figure 2: Results of the particle size analysis of ERM-BB125 using a laser diffraction instrument (Sympatec, Clausthal Zellerfeld, DE)



4 Homogeneity

A key requirement for any reference material aliquoted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO 17034 [1] requires RM producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values in the CRM are valid for all units of the material, within the stated uncertainties.

The number of units selected corresponds to approximately the cube root of the total number of units produced. Fifteen units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into groups (with a similar number of units) and one unit was selected randomly from each group. Three independent sub-samples were taken from each selected CRM unit. The samples were extracted by a modified QuEChERS method and analysed by LC-MS/MS. The measurements were performed under intermediate precision conditions (five different days) due to combining the homogeneity measurements with method validation experiments. Consequently, day-to-day effects can occur and could mask the between bottle variation. Therefore, it had to be first checked if there was a significant difference between the day means using ANOVA for the measurements spread over more than two days. Statistically significant day-to-day effects were identified, since the analytical measurements were spread over 5 days for the two pesticides: fipronil compound and fipronil sulfone. In order to limit day-to-day effects in the between bottle uncertainty evaluation, a correction was applied by dividing every data point by the respective day mean. After normalisation the data were checked for a significant differences between the day means using a t-test at a 95 % confidence interval. No difference was detected. All replicate measurements were done in a randomised manner to be able to separate a potential analytical drift form a trend in the filling sequence. The results are shown as graphs in the Annex B.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends in the filling sequence or the analytical sequence were observed at a 95 % confidence level.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method repeatability if the individual samples were representative for the whole unit.

Evaluation by ANOVA requires mean values per unit, which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per unit was visually tested using histograms and normal probability plots. Too few data are available for the unit means to make a clear statement about the distribution. Therefore, it was checked visually whether all individual data follow a unimodal distribution using histograms and normal probability plots. In general minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. The results of all statistical evaluations are given in Table 1.

Table 1: Results of the statistical evaluation of the homogeneity studies

Pesticide	Trends (before correction)*		Outliers**		Distribution	
	Analytical sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
Fipronil	no	no	none	none	normal	unimodal
Fipronil sulfone	no	no	two (retained)	none	normal	unimodal
Sum of fipronil and fipronil sulfone expressed as fipronil	no	no	two (retained)	none	normal	unimodal

* 95 % confidence level
** 99 % confidence level

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [8]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 1}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{v_{MSwithin}}}}{\bar{y}} \qquad \textbf{Equation 3}$$

MS_{within} mean of squares within-unit from an ANOVA

$MS_{between}$ mean of squares between-unit from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

n mean number of replicates per unit

$v_{MSwithin}$ degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 2. The resulting values from the above equations were converted to relative uncertainties. In most cases, the uncertainty contribution for homogeneity was determined by the method repeatability.

Table 2: Results of the homogeneity studies

Pesticide	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
Fipronil	11.78	4.15	4.85	4.85
Fipronil sulfone	5.20	n.c.	2.14	2.14
Sum of fipronil and fipronil sulfone expressed as fipronil	5.13	n.c.	2.11	2.11

n.c.: cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation can be used as estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. Due to this correlation, individual aliquots of a material will not contain the same amount of analyte if the sample intake is too small. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 1 g sample intake when using LC-MS/MS. This sample intake for LC-MS/MS gives acceptable repeatability, demonstrating that the within-unit inhomogeneity no longer contributes to analytical variation at this sample intake.

The minimum sample intake was determined from the results of the characterisation study, using the method information supplied by the participants. The smallest sample intake that still yielded results with acceptable accuracy to be included in the respective studies was taken as minimum sample intake. Using the data from Annex E, a minimum sample intake of 1 g egg powder reconstituted with 4 g water was established for all methods.

5 Stability

Time, temperature, light and water content were regarded as the most relevant influences on the stability of the materials. The influence of ultraviolet or visible light was minimised by filling the material in amber glass vials which were afterwards placed in aluminium sachets in order to reduce the light exposure. In addition, materials were stored in the dark and dispatched in boxes, thus removing any possibility of degradation by light. The water content was adjusted for optimal stability during processing. Additionally, the material was sterilised by γ-irradiation to eliminate microbial growth. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability).

The stability studies were carried out using an isochronous design [8]. In this approach, samples were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions. Analysis of the

material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

For the short-term stability study, samples were stored at -20 °C, +4 °C and +18 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set at -70 °C. Three units per storage time were selected using a random stratified sampling scheme. From each unit, three subsamples were measured by LC-MS/MS. The measurements were performed under repeatability conditions, and a randomised sequence was used to differentiate any potential analytical drift from a trend over storage time. The results were reported as mass fractions of the compounds in egg powder.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. One outlying individual result was found for the studies at -20 °C and +18 °C (Table 3). As no technical reason for the outliers could be found all data were retained for statistical analysis.

In addition, the data were evaluated against storage time, and regression lines of mass fraction of each compound versus time were calculated, to test for potential increases/decrease of the mass fraction of the pesticides due to shipping conditions. The slopes of the regression lines were tested for statistical significance.

The results of the measurements are shown in Annex C. The results of the statistical evaluation of the short-term stability are summarised in Table 3.

Table 3: Results of the short-term stability tests

Pesticide	Number of individual outlying results*			Significance of the trend **		
	-20 ºC	+4 ºC	+18 ºC	-20 ºC	+4 ºC	+18 ºC
Fipronil	one (retained)	none	one (retained)	no	no	yes
Fipronil sulfone	none	none	none	no	yes	yes
Sum of fipronil and fipronil sulfone expressed as fipronil	none	none	none	no	yes	yes

* 99 % confidence level
** 95 % confidence level

Technical outliers were detected for fipronil and they were retained for the estimation of u_{sts}. For a temperature of -20 °C no statistically significant trends was detected on a 95 % confidence level. At higher temperatures (+4 °C and +18 °C) the trends observed were statistically significant indicating instability of fipronil sulfone.

Standard shipment conditions: The material shall be shipped frozen on dry ice to ensure it is kept frozen upon arrival.

5.2 Long-term stability study

For the long-term stability study, samples were stored at -70 °C and -20 °C for 0, 4, 8 and 11 months (at each temperature). The reference temperature was set to -150 °C. Four units per storage time were selected using a random stratified sampling scheme. From each unit, three subsamples were measured by LC-MS/MS. The measurements were performed under repeatability conditions, in a random sequence to be able to separate any potential analytical drift from a trend over storage time. The results were reported as mass fractions of the compounds in egg powder.

The long-term stability data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No outlying individual results were found (Table 4).

In addition, the data were plotted against storage time and linear regression lines of mass fraction versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected for any of the analytes at a 95 % confidence level with the exception of the fipronil compound at -70 °C.

The results of the long-term stability measurements are shown in Annex D. The results of the statistical evaluation of the long-term stability study are summarised in Table 4.

Table 4: Results of the long-term stability tests

Pesticide	Number of individual outlying results*		Significance of the trend**	
	-70 ºC	-20 ºC	-70 ºC	-20 ºC
Fipronil	none	none	yes	no
Fipronil sulfone	none	none	no	no
Sum of fipronil and fipronil sulfone expressed as fipronil	none	none	no	no

* 99 % confidence level
** 95 % confidence level

No technically unexplained outliers were observed and at -70 °C test temperature and a statistically significant trend was found on a 95 % confidence level. The trend was observed only for the fipronil compound and this trend is a result of the low level of the analyte close to the LOQ of the method and therefore less precise. Since the level of fipronil compound is low the impact to the level of the sum of fipronil and fipronil sulfone expressed as fipronil is negligible. The material is stable at -20°C and can be stored at -20°C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability/intermediate precision, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

The uncertainties of stability during dispatch and storage were estimated, as described in [8] for each analyte. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 4}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 5}$$

s_{rel} relative standard deviation of all results of the stability study

t_i time elapsed at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at -20 ºC)

t_{sl} chosen shelf life (11 months at -20 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the -20 °C studies. The uncertainty describes the possible change during a dispatch at -20 °C lasting for one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -20 °C studies. The uncertainty contribution describes the possible degradation during 11 months storage at -20 °C.

The results of these evaluations are summarised in Table 5.

Table 5: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of -20 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -20 °C and 11 months

Pesticide	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
Fipronil	0.48	1.95
Fipronil sulfone	0.37	1.13
Sum of fipronil and fipronil sulfone expressed as fipronil	0.37	1.13

The material showed no significant degradation for transport below -20 °C. Transport on dry ice is necessary.

After the certification study, the material will be included in the JRC's regular stability monitoring programme, to control its further stability.

6 Characterisation

The material characterisation is the process of determining the property values of a reference material.

This was based on an interlaboratory comparison of expert laboratories, i.e. the analyte mass fraction in the material was determined in different laboratories that applied different measurement procedures to demonstrate the absence of a measurement bias. Due to the nature of the analytes however, all participants used liquid and/or gas chromatographic methods, in most cases followed by mass spectrometric detection, for the measurements.

6.1 Selection of participants

Twelve laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of fipronil measurements in relevant matrices by submitting results for intercomparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. When measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

6.2 Study setup

Each laboratory received two units of ERM-BB125 and was requested to provide six independent results, three per unit expressed as mass fraction on a dry mass basis and corrected for recovery. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over at least two days to ensure intermediate precision conditions. One gram egg powder was reconstituted in 4 g water added prior sample preparation. The water content was determined for dry egg powder in each unit in order to report the results on dry mass basis.

Laboratories were also requested to give estimations of the expanded uncertainties of the results. No approach for the estimation was prescribed, i.e. top-down and bottom-up [4] were regarded as equally valid procedures.

6.3 Methods used

A variety of extraction and clean-up methods with different quantification approaches were used to characterise the material. All methods used during the characterisation study are summarised in Annex E. The laboratory code (e.g. L01) is a random number and does not correspond to the order of laboratories in Section 2. The lab-method code consists of a number assigned to each laboratory (e.g. L01) and abbreviation of the measurement method used, (e.g. LC-MS/MS).

6.3.1 Dry mass determination

For all measurements carried out during certification the following protocol for dry mass determination was applied:

One gram of sample, oven dry at 103 °C ± 2 °C for 1 hour, one replicate per bottle.

The water content determined by the laboratories during the material characterisation was in the range of 0.5–1.8 %. Certified values are expressed as dry mass basis accordingly.

However, results within each laboratory were consistent and in agreement with the results from the processing control (Section 3.3).

6.4 Evaluation of results

The characterisation study resulted in 12 datasets per analyte. All individual results of the participants, grouped per analyte are displayed in tabular and graphical form in Annex F.

6.4.1 Technical evaluation

The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- appropriate validation of the measurement procedure
- compliance with the analysis protocol: sample preparations and measurements performed on two days, and the analytical sequence and water content determination
- absence of values given as below limit of detection or below limit of quantification

Based on the above criteria, all data sets were technically valid with the exception of the L08, which reported results after reconstitution and not based on dry mass. Data form L08 was not used for further evaluation.

6.4.2 Statistical evaluation

The data sets for fipronil sulfone and sum of fipronil and fipronil sulfone expressed as fipronil were plotted in a graph (annex F). From the data plotted a visual difference is observed between the results from methods applying mass spectrometry and the only method using an electron capture detector (L11). After investigation, no technical reason was identified to explain the lower value reported from this laboratory compared to the rest of data sets. This is the only data set obtained using ECD as a detector and it could be speculated that the results might be dependent of the analytical technique used. ECD is less specific than MS. With the available data the influence of the analytical technique cannot be confirmed or discarded. Therefore, it was decided to exclude the technique from the characterisation (i.e. L11) and restrict the identity of the measurand of the certified value to determination by chromatography/mass spectrometry.

Ten technically valid datasets were assessed for normality of dataset means using kurtosis/skewness tests and normal probability plots. Furthermore the presence of outlying means was checked using the Grubbs test and the Cochran test for outlying standard deviations (both at a 99 % confidence level). Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 6.

EU legislation requires the reporting of fipronil (sum of fipronil + sulfone metabolite expressed as fipronil). The calculation to convert the mass fraction of fipronil sulfone to fipronil follows the SANTE/11813/2017 [9] recommendation. The expression of the sum is equal to the sum of mass fraction of fipronil plus the mass fraction of fipronil sulfone multiplied by 0.9647 (the ratio of the molecular weight of fipronil/fipronil sulfone).

Table 6: Statistical evaluation of the technically accepted datasets for the certification of ERM-BB125. *p*: number of technically valid datasets

Pesticide	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [mg/kg]	*s* [mg/kg]	$s_{between}$ [mg/kg]	s_{within} [mg/kg]
Fipronil sulfone	10	0	0	yes	0.060	0.006	0.005	0.005
Sum of fipronil and fipronil sulfone expressed as fipronil	10	0	0	yes	0.058	0.005	0.005	0.005

The laboratory means follow normal distributions. None of the data contain outlying means and variances. The datasets are therefore consistent and the mean of laboratory means is a good estimate of the true value. Standard deviations between laboratories are considerably larger than the standard deviation within laboratories for sum of fipronil and fipronil sulfone expressed as fipronil, showing that confidence intervals of replicate measurements are unsuitable as estimate of measurement uncertainty.

The uncertainty related to the characterisation is estimated as the standard error of the mean of laboratory means (Table 7).

Table 7: Uncertainty of characterisation for ERM-BB125

Pesticide	*p*	Mean [mg/kg]	*s* [mg/kg]	u_{char} [mg/kg]
Fipronil sulfone	10	0.060	0.005	0.002
Sum of fipronil and fipronil sulfone expressed as fipronil	10	0.058	0.005	0.002

7 Value Assignment

Certified values were assigned for fipronil sulfone and the sum of fipronil and fipronil sulfone expressed as fipronil.

Certified values are values that fulfil the highest standards of accuracy. Procedures at the JRC, Directorate F, generally require pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 8 was assigned as certified value for each parameter.

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} (Section 6), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5). The uncertainty related to inhomogeneity/degradation during transport/long-term storage was found to be negligible. These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor *k* given as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2 + u_{char,rel}^2} \qquad \textbf{Equation 6}$$

- u_{char} was estimated as described in Section 6
- u_{bb} was estimated as described in Section 4.1.
- u_{sts} and u_{lts} were estimated as described in section 5.3

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions, a coverage factor *k* of 2 was applied, to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 8.

Table 8: Certified values and their uncertainties for ERM-BB125

Pesticide	Certified value[1)] [mg/kg]	u_{char} [mg/kg]	u_{bb} [mg/kg]	u_{sts} [mg/kg]	$u_{lts,}$ [mg/kg]	U_{CRM} [2)] [mg/kg]
Fipronil sulfone	0.060	0.002	0.001	0.0002	0.0010	0.005
Sum of fipronil and fipronil sulfone expressed as fipronil	0.058	0.002	0.001	0.0002	0.0010	0.005

[1)]: mass fraction refer to dry mass
[2)]: Expanded (k = 2) and rounded uncertainty.

7.2 Additional material information

The data provided in this section should be regarded as informative only on the general composition of the material and cannot be, in any case, used as certified or indicative value.

Fipronil was reported by only four participants. The levels of fipronil in the material were low and below the limit of quantification for most of the participants. Therefore, a certified value could not be assigned. Since most of the information about fipronil was obtained from the homogeneity and stability studies, the results should be regarded as informative only on general composition of the material (Table 9).

Table 9: Mass fraction range for fipronil for ERM-BB125

	Mass fraction range [1)] [mg/kg]
Fipronil	0.0007-0.0027

[1)]: based on dry mass

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

Fipronil and fipronil sufone are chemically clearly defined analytes. Identity was confirmed by mass spectrometry. The participants used different methods for the sample preparation as well as for the final determination, demonstrating to a great extent the absence of measurement bias. Nevertheless, since all participants used a chromatographic separation in combination with mass spectrometry, the measurands are operationally defined chromatography and mass spectrometry.

Quantity value

Only validated methods were used for the determination of the assigned values. Investigation of the method and measurement details of the individual results shows that all the relevant input parameters of each technically accepted dataset have been properly calibrated. Calibrants of known purity, with specified traceability of their assigned values and of different independent commercial origins were used. All values in the technically accepted datasets are therefore traceable to the same reference, namely the SI. The traceability to the SI is also confirmed by the agreement of results within their respective uncertainties through the use of GC and/or LC methods as indicated on the certificate.

8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the CLSI Guideline C53-A [10] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

ERM-BB125 was produced from a naturally contaminated egg material further manipulated by mixing, freeze drying and milling. Once reconstituted, the analytical behaviour of this matrix is expected to be highly similar to routine samples of fresh egg. It should be borne in mind that the methods used in the characterisation are methods routinely applied for measuring fipronil and fipronil sulfone in eggs. The agreement of results from different methods demonstrates that the processing did not affect any properties relevant for these methods and that ERM-BB125 behaves like a real sample.

9 Instructions for use

9.1 Safety information

ERM-BB125 is intended for laboratory use only. The usual laboratory safety measures apply.

9.2 Storage conditions

The materials should be stored at -20 ± 4 °C in the dark.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened vials.

9.3 Reconstitution

The material consists of an amber glass vial containing 5 g of egg powder. The reconstitution should be done as follows:

Leave the content of the vial to thaw at room temperature. The vial shall be shaken by turning upside down by hand for at least 1 min before opening to ensure material re-homogenisation. Remove the cap and weigh 1 g powder and record the weighted amount. Add 4 g water to 1 g egg powder. Record the amount of water. To fully reconstitute the sample use a Vortex shaker for 30 s at maximum speed followed by an ultrasonic bath for 10 min. After reconstitution, the sample should be used within a maximum period of 2 h.

9.4 Minimum sample intake

The minimum sample intake representative for all parameters is 1 g of egg powder.

9.5 Dry mass correction

For all measurements carried out during certification the following protocol for dry mass determination was applied:

Open the bottle and take one gram of sample, oven dry at 103 ºC ± 2 ºC for 1 hour, one replicate per bottle. Weighing of the samples for dry mass determination and weighing for the analysis shall be done at the same time to avoid differences due to possible take up of moisture by the material.

Certified values are expressed as dry mass basis accordingly. Users of the material should perform their own water determination as described above in order to express the result on a dry mass basis.

9.6 Use of the certified value

The main purpose of this material is to assess method performance, i.e. for checking accuracy of analytical results/calibration. As any reference material, it can be used for establishing control charts or during validation studies.

Use as a calibrant

It is not recommended to use this matrix material as a calibrant. If used nevertheless, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1)[11]

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

10 Acknowledgments

The authors would like to acknowledge the extensive support received from the JRC, Directorate F.6 processing team and from M. Contreras concerning the set-up of the required isochronous studies.

Furthermore, the authors would like to thank S. Elordui-Zapatarietxe and S.Boulo (JRC, Directorate F) for reviewing the certification report, as well as the experts of the Reference Material Review Panel, T. Pihlström (National Food Agency, Uppsala, Sweden) and J. de Boer (Vrije Universiteit Amsterdam, The Netherlands) for their constructive comments.

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Annexes

Annex A: List of the analytes for certification in ERM-BB125 with some characteristics

Annex B: Results of the homogeneity measurements

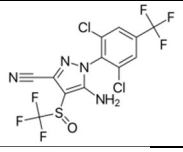
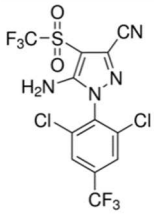
Annex C: Results of the short-term stability measurements at -20°C

Annex D: Results of the long-term stability measurement at -20°C for 11 months

Annex E: Summary of methods used in the characterisation of ERM-BB125

Annex F: Results of the characterisation measurements

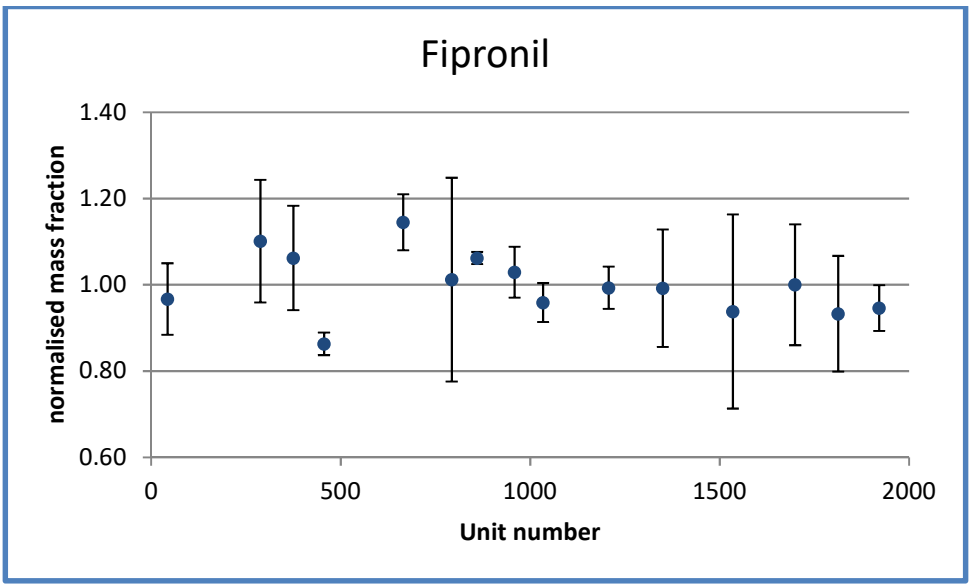
Annex A: List of analytes for the certification in ERM-BB125

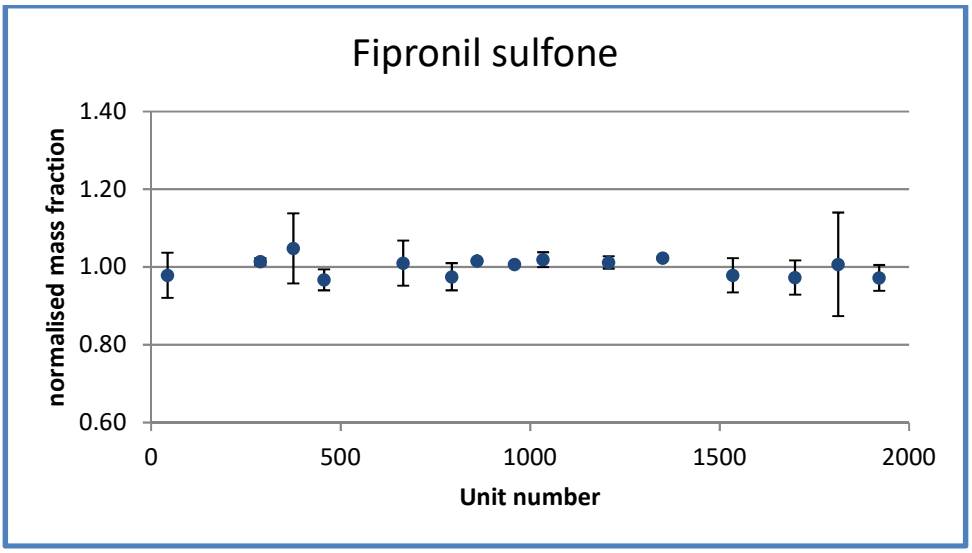
Pesticide	Chemical class	Use type	CAS number	Chemical structure	Molecular weight, [g/mol]	MRL[1] [mg/kg]	Legislation Reg (EU)[2]
Fipronil	pyrazoles	Insecticide	120068-37-3		437.14	0.005	No. 1127/2014
Fipronil sulfone	pyrazoles	Insecticide	120068-36-2		453.14		

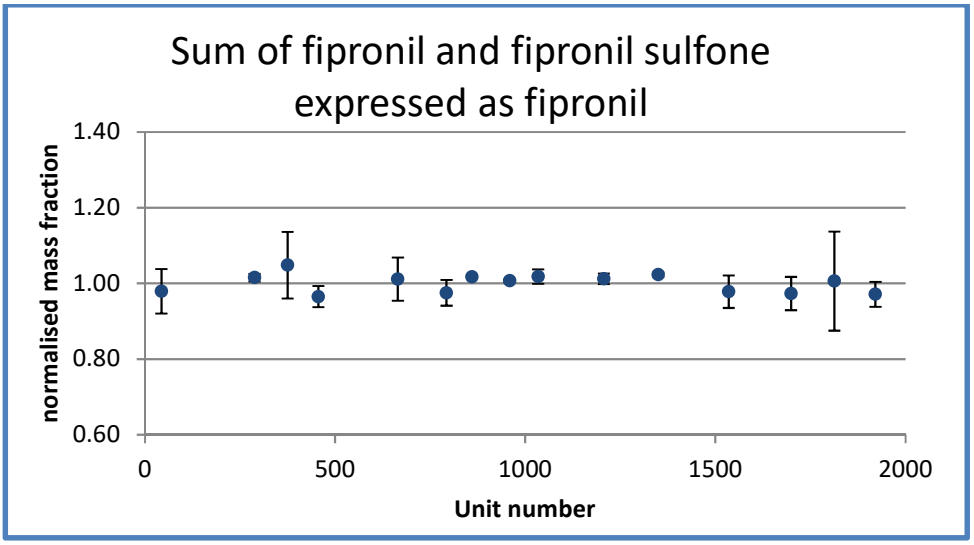
[1] MRL = fipronil (sum of fipronil + sulfone metabolite expressed as fipronil)

[2] Commission regulations (EU) No 1127/2014 of 20 October 2014 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for amitrole, dinocap, fipronil, flufenacet, pendimethalin, propyzamide, and pyridate in or on certain products

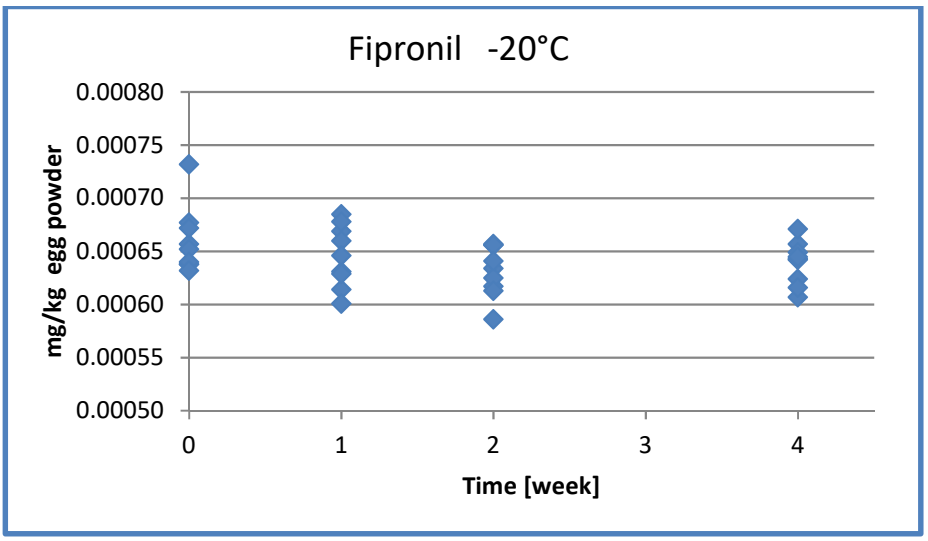
Annex B: Results of the homogeneity measurements - Values are normalised and illustrated as mean mass fractions of pesticide obtained from the analysis of 3 subsamples per unit of ERM-BB125

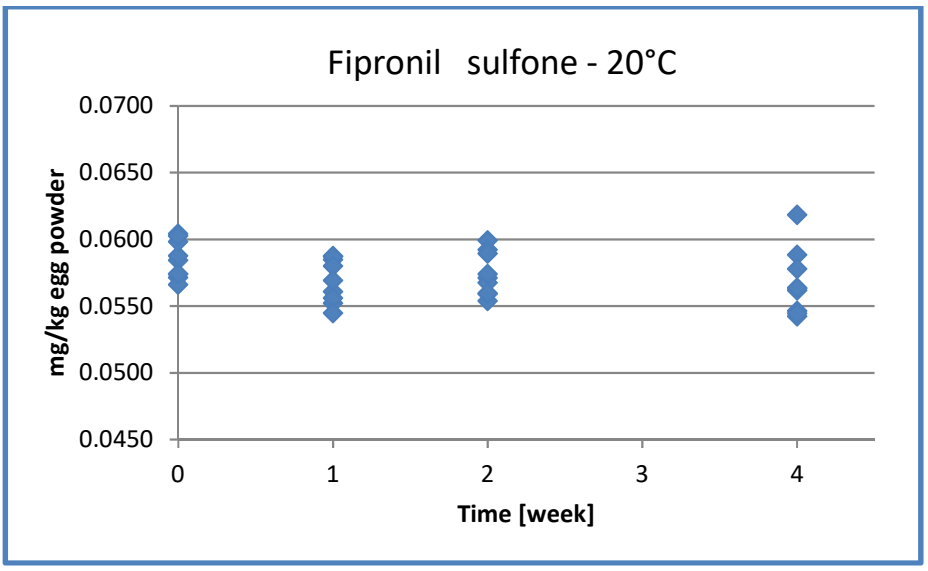


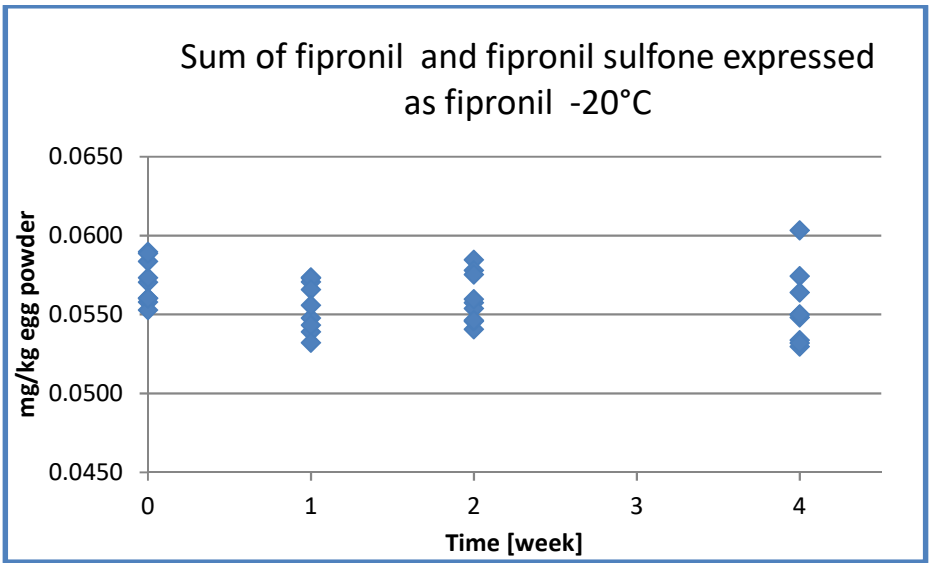




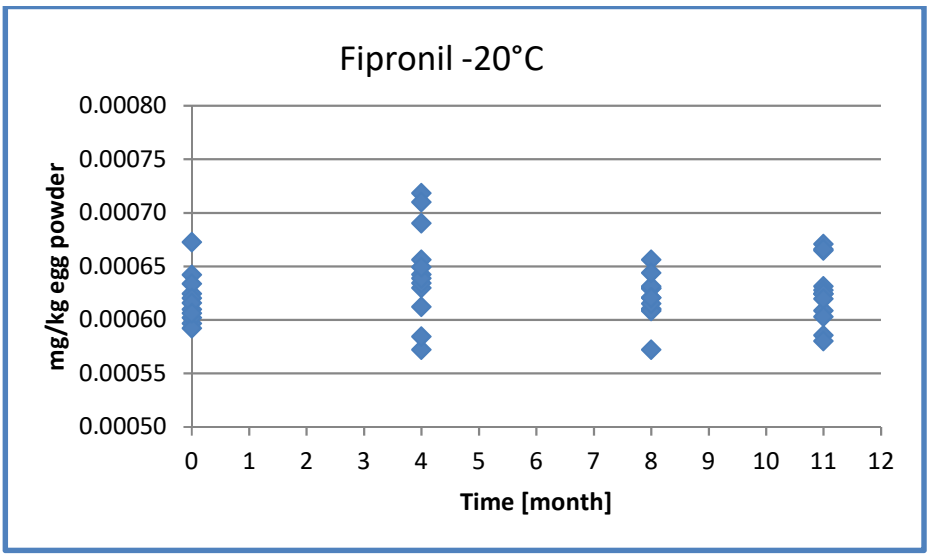
Annex C: Results of the short-term stability measurements at -20°C - Graphs provide individual results (9 replicates per time point) to better illustrate the presence, if any, of outliers

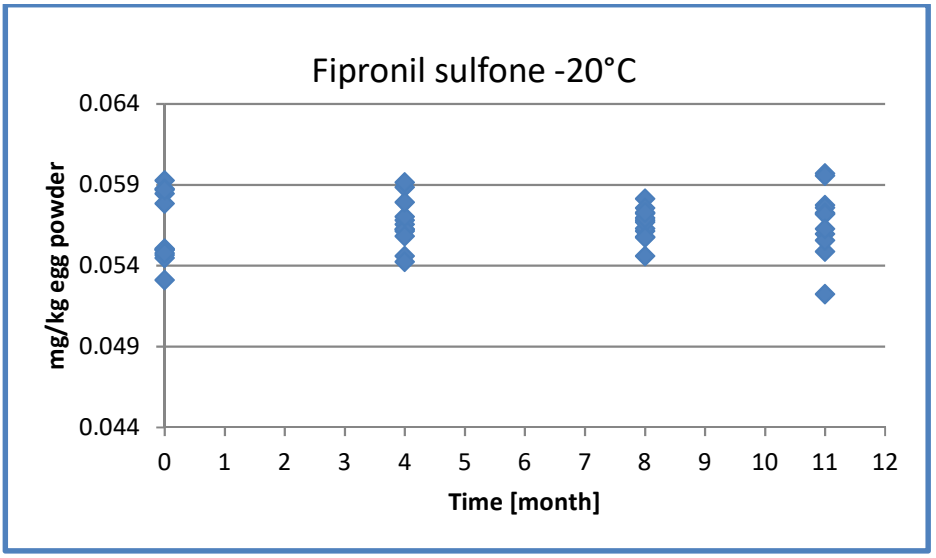


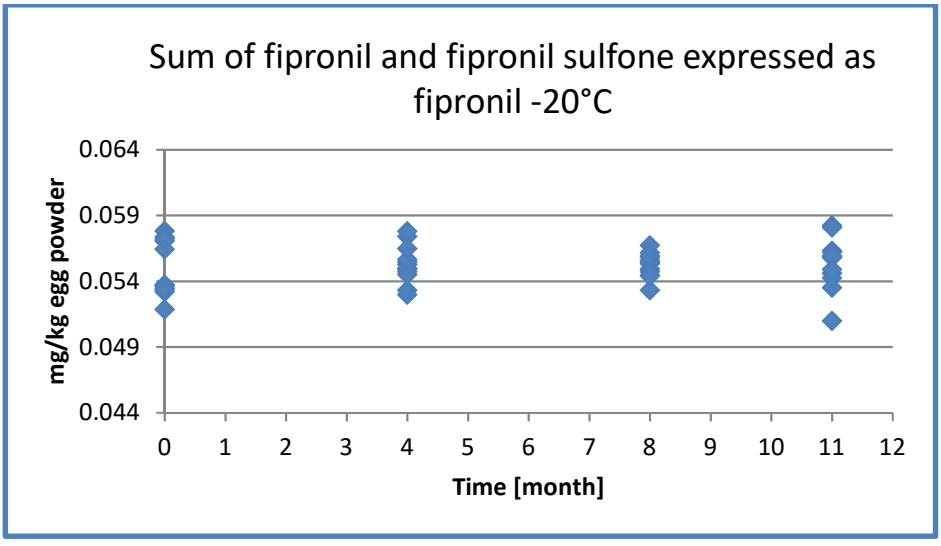




Annex D: Results of the long-term stability measurement at -20°C for 11 months - Graphs provide individual results (12 replicates per time point) to better illustrate the presence of outliers







Annex E: Summary of methods used in the characterisation of ERM-BB125

Table E.1: Details of analytical methods, as given by the laboratories

Laboratory code	Sample intake, [g]	Water added, [g]	Extraction	Clean up
L01 LC-MS/MS	1	4	Modified QuEChERS method with DisQuE extraction salts, water and acetonitrile, 30 min shaker	centrifugation, supernatant filtered over Oasis PRiME HLB syringe filter
L02 LC-MS/MS	3	12	QuEChERS	
L03 LC-MS/MS	0.9819	3.9995	1g sample + 10 mL acetonitrile + (4g anhydrous magnesium sulfate + 1g sodium chloride + 1g trisodium citrate dihydrate + 0.5g disodium hydrogencitrate sesquihydrate)	
L04 GC-MS/MS	1	4	Quechers PSA 150 mg/C18EC 150 mg	
L05 LC-MS/MS	1	4	Ethyl acetate (SWEet method) - 5 mL	PSA/C18 (200+200mg), filtration
L06 LC-TOF/MS	2	8	Extraction with 10 mL acetonitrile	dSPE clean-up with PSA and magnesium sulfate

Laboratory code	Sample intake, [g]	Water added, [g]	Extraction	Clean up
L07 LC-MS/MS	1	4	QuEChERS - 10 mL acetonitrile, shake, add citrate extraction mix and shake	dSPE (PSA)
L08 LC-TOF/MS	1	4	liquid-liquid	n.a.
L09 GC-MS/MS	1	4	QuEChERS - extraction kit (6g magnesium sulfate, 1.5 g sodium acetate) (Perkin Elmer N9306900)	Quechers - clean up kit (1200mg magnesium sulfate, 400 mg PSA, 400 mg C18, 400 mg PGC) (Perkin Elmer N9306914)
L10 GC-MS/MS	3	12	QuEChERS	
L11 GC-ECD	3.0062	12.0021	1g sample + 10 mL acetonitrile + (4g anhydrous magnesium sulfate + 1g sodium chloride + 1g trisodium citrate dihydrate + 0.5g disodium hydrogencitrate sesquihydrate)	
L12 GC-MS/MS	1	4	Ethyl acetate (SweEt method) - 5mL	PSA/C18 (200+200mg), filtration

Table E.2: Details of measurement techniques as given by the laboratories (m/z transitions in bold were employed for quantification purposes and the rest are for identification confirmation)

Laboratory code	Mobile phase	Analytical column	Calibration	Chromatographic technique	Ionisation technique	MS/detector	Fipronil	Fipronil sulfone
L01 LC-MS/MS	1mM C_2H_7NO/Methanol 1mM C_2H_7NO	Acquity UPLC BEH C18 (1.7µm, 2.1x100mm)	Matrix matched	HPLC	ESI	Triple quadrupole	**435>330** 345>250 435>183	**451>415** 451>282 451>244
L02 LC-MS/MS	water (0.1% formic acid), acetonitrile (0.1% formic acid)	Phenomenex - Kinetex 1,7µ XLB, (50 mm, 2.1 µm)	Matrix matched	HPLC	ESI	Triple quadrupole	**435>330,** 435>319, 435>250	**435>330** 435>282 435>244
L03 LC-MS/MS	Methanol/H2O (10/90)+5mmol/L ammonium formate; MeOH/H_2O (90/10)+5mmol/L ammonium formate	Atlantis T3 (5µm, 2.1x150 mm)	Matrix matched	HPLC	ESI	Triple quadrupole	**434.8>329.9** 434.8>249.9 436.8>331.9	**450.8>414.9** 450.8>282.0 452.8>416.9
L04 GC-MS/MS		Restek RTX-CL Pesticides 2 (30 m, 0.25 µm, 0.2 mm)	Matrix matched	GC	EI	Triple quadrupole	**367>213** 367>255	**383>255** 383>213
L05 LC-MS/MS	10mM ammonium formate, pH 4; methanol	Waters HSS T3 (150mm, 2.1.mm, 18.8µm)	Bracketing (matrix matches)	HPLC	ESI	Triple quadrupole	**437>368** 454>368	**452.9>415.0** 452.9>244.0
L06 LC-TOF/MS	90%/10% H_2O / methanol + ammonium formate and formic acid; methanol 100% + ammonium formate and formic acid	Thermo Acclaim RSLC (100 mm, 2.1 mm, 2.2 µm)	Matrix matched	HPLC	ESI	Time of flight		**469.9674**

Laboratory code	Mobile phase	Analytical column	Calibration	Chromatographic technique	Ionisation technique	MS/detector	Fipronil	Fipronil sulfone
L07 LC-MS/MS	H_2O 5mMol/L ammonium-methanol 5mMol/L ammonium, MeOH 5 mMol/L ammonium formate	Zorbax Eclipse Plus (50 mm, 2.1 mm, 1.8 µm)	Calibration curve	HPLC	ESI	Triple quadrupole	**435.0>330.1** 435.0>250.0	**451.1>414.9** 451.1>282.0
L08 LC-TOF/MS	5mM NH_4FA/0.1% FA in water, 5nM NH_4FA/0.1% FA in 95% metahnol	Zorbax Eclipse Plus, (50 mm, 2.1 mm, 1.8 µm)	Matrix matched	HPLC	ESI	Time of flight	**434.9312** 436.9280	**450.9266** 452.9235
L09 GC-MS/MS		VF 5ms (50 m ,0.25 mm, 0.25µm)	Calibration curve	GC	CI	Triple quadrupole	**367.0>212.9** 267.0>227.9	**382.9>255.0** 255.0>228.0
L10 GC-MS/MS		5% Phenyl-95% dimethylpolysiloxane	Matrix matched	GC	EI	Triple quadrupole	**367>213** 367>228	**383>255** 383>228
L11 GC-ECD		Crosslinked 5%PH ME siloxane (30 mm, 320 µm, 0.25 µm)		GC		ECD		
L12 GC-MS/MS		HP-5ms Ultra Inert - 5%-Phenyl-methylpolysiloxane (15 m, 250 µm, 0.25 µm)		GC	EI	Triple quadrupole	**367.0** 213.0 255.0	**383.0** 255.0 385.0

Table E.3: Water content results as given by the laboratories

Laboratory code	Water content [%], unit 1	Water content [%], unit 2
L01	1.58	1.58
L02	0.72	0.44
L03	1.23	1.65
L04	1.84	1.83
L05	1.57	1.11
L06	1.10	0.83
L07	1.30	1.30
L08	n.d.	n.d.
L09	1.76	1.56
L10	0.72	0.44
L11	1.21	1.30
L12	1.08	1.06

Annex F: Results of the characterisation measurements

Figure F1. Laboratory means (six replicates) for fipronil sulfone and their standard deviations represented as error bars for reported data sets applying GC-MS/MS, LC-MS/MS, LC-TOF/MS and GC-ECD

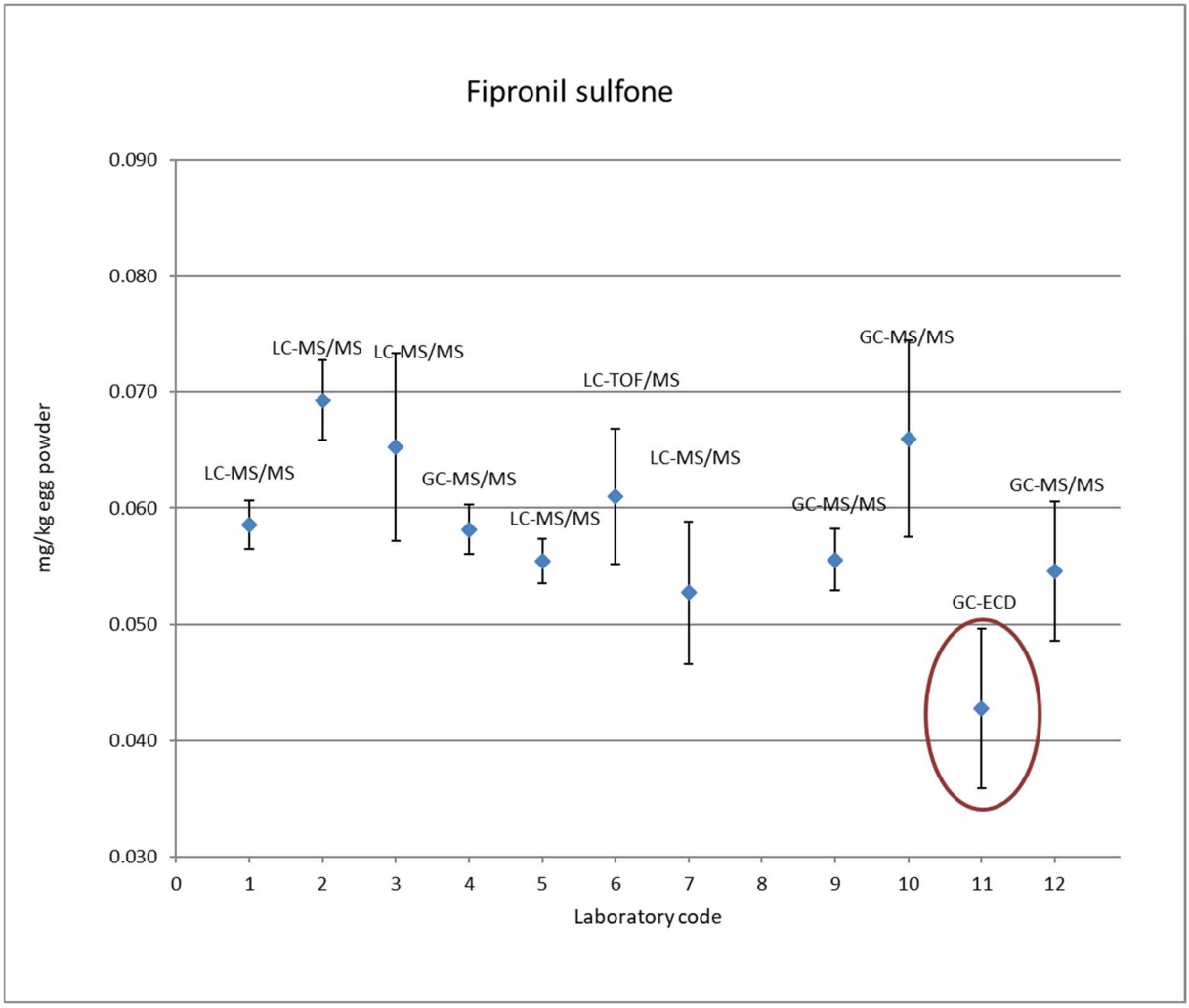


Figure F2. Laboratory means (six replicates) for the sum of fipronil and fipronil sulfone expressed as fipronil and their standard deviations represented as error bars for reported data sets applying GC-MS/MS, LC-MS/MS, LC-TOF/MS and GC-ECD

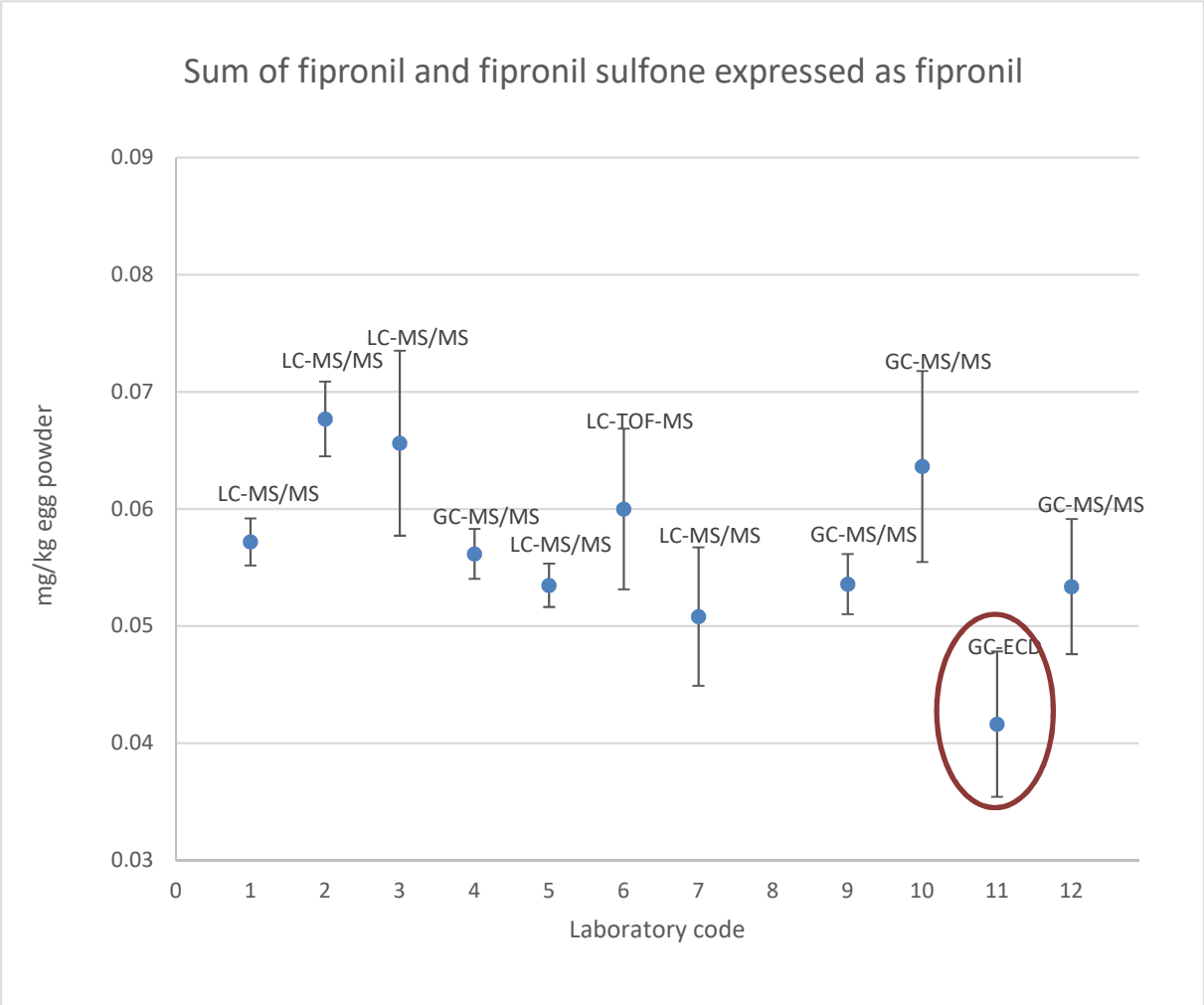


Table F.1. Mass fractions of fipronil sulfone in egg powder (dry mass basis) as reported by participant laboratories

Laboratory code	replicate 1 [mg/kg]	replicate 2 [mg/kg]	replicate 3 [mg/kg]	replicate 4 [mg/kg]	replicate 5 [mg/kg]	replicate 6 [mg/kg]	mean [mg/kg]	s [mg/kg]
L01 -LC-MS/MS	0.057	0.058	0.056	0.062	0.058	0.060	0.059	0.002
L02 -LC-MS/MS	0.068	0.071	0.069	0.065	0.068	0.075	0.069	0.003
L03 -LC-MS/MS	0.074	0.057	0.060	0.060	0.065	0.076	0.065	0.008
L04 -GC-MS/MS	0.056	0.059	0.060	0.055	0.060	0.059	0.058	0.002
L05 -LC-MS/MS	0.057	0.058	0.057	0.054	0.056	0.053	0.055	0.002
L06 -LC-TOF/MS	0.067	0.067	0.064	0.058	0.057	0.053	0.061	0.006
L07 -LC-MS/MS	0.051	0.050	0.044	0.059	0.052	0.061	0.053	0.006
L09 -GC-MS/MS	0.053	0.058	0.055	0.052	0.059	0.056	0.056	0.003
L10 -GC-MS/MS	0.065	0.070	0.074	0.072	0.065	0.050	0.066	0.008
L12 -GC-MS/MS	0.059	0.053	0.061	0.051	0.058	0.045	0.055	0.006
Results not used for certification								
L11 -GC-ECD	*0.052*	*0.041*	*0.050*	*0.036*	*0.036*	*0.042*	*0.043*	*0.007*

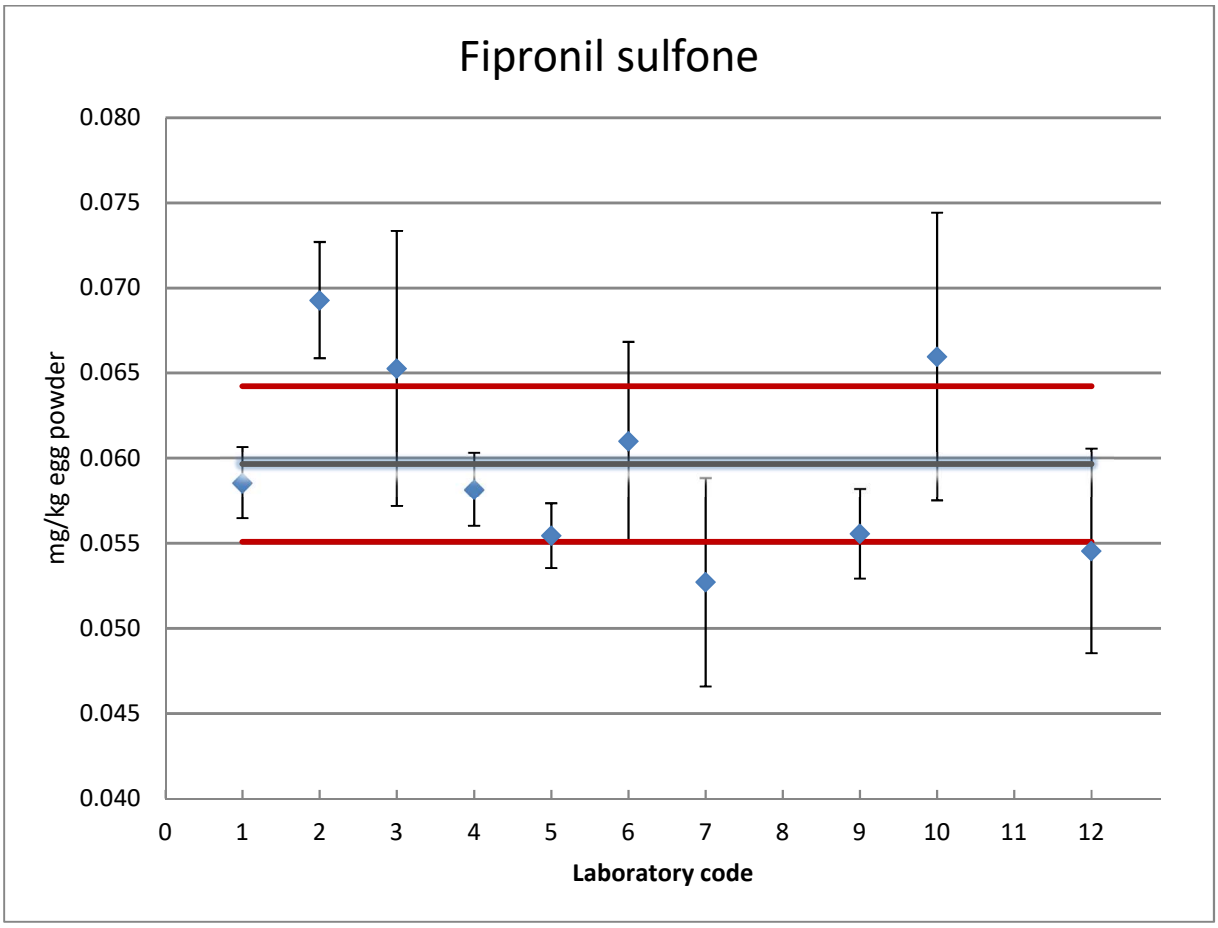


Figure F.3 Laboratory means with their standard deviations represented as error bars for accepted data sets used for certified value assignment of fipronil sulfone in ERM-BB125. Grey line correspond to the certified value and red lines correspond to the U_{CRM}

Table F.2. Mass fractions of the sum of fipronil and fipronil sulfone expressed as fipronil in egg powder (dry mass basis) as reported by participant laboratories

Laboratory code	replicate 1 [mg/kg]	replicate 2 [mg/kg]	replicate 3 [mg/kg]	replicate 4 [mg/kg]	replicate 5 [mg/kg]	replicate 6 [mg/kg]	mean [mg/kg]	s [mg/kg]
L01 -LC-MS/MS	0.056	0.057	0.054	0.060	0.057	0.059	0.057	0.002
L02 -LC-MS/MS	0.066	0.069	0.067	0.063	0.068	0.073	0.068	0.003
L03 -LC-MS/MS	0.074	0.057	0.061	0.060	0.065	0.076	0.066	0.008
L04 -GC-MS/MS	0.054	0.057	0.058	0.053	0.058	0.057	0.056	0.002
L05 -LC-MS/MS	0.055	0.056	0.055	0.052	0.054	0.051	0.053	0.002
L06 -LC-TOF/MS	0.067	0.067	0.064	0.056	0.055	0.051	0.060	0.007
L07 -LC-MS/MS	0.049	0.048	0.043	0.057	0.050	0.059	0.051	0.006
L09 -GC-MS/MS	0.051	0.056	0.053	0.051	0.057	0.054	0.054	0.003
L10 -GC-MS/MS	0.063	0.068	0.071	0.069	0.062	0.048	0.064	0.008
L12 -GC-MS/MS	0.057	0.052	0.060	0.050	0.057	0.044	0.053	0.006
Results not used for certification								
L11 -GC-ECD	*0.050*	*0.039*	*0.049*	*0.035*	*0.037*	*0.041*	*0.042*	*0.006*

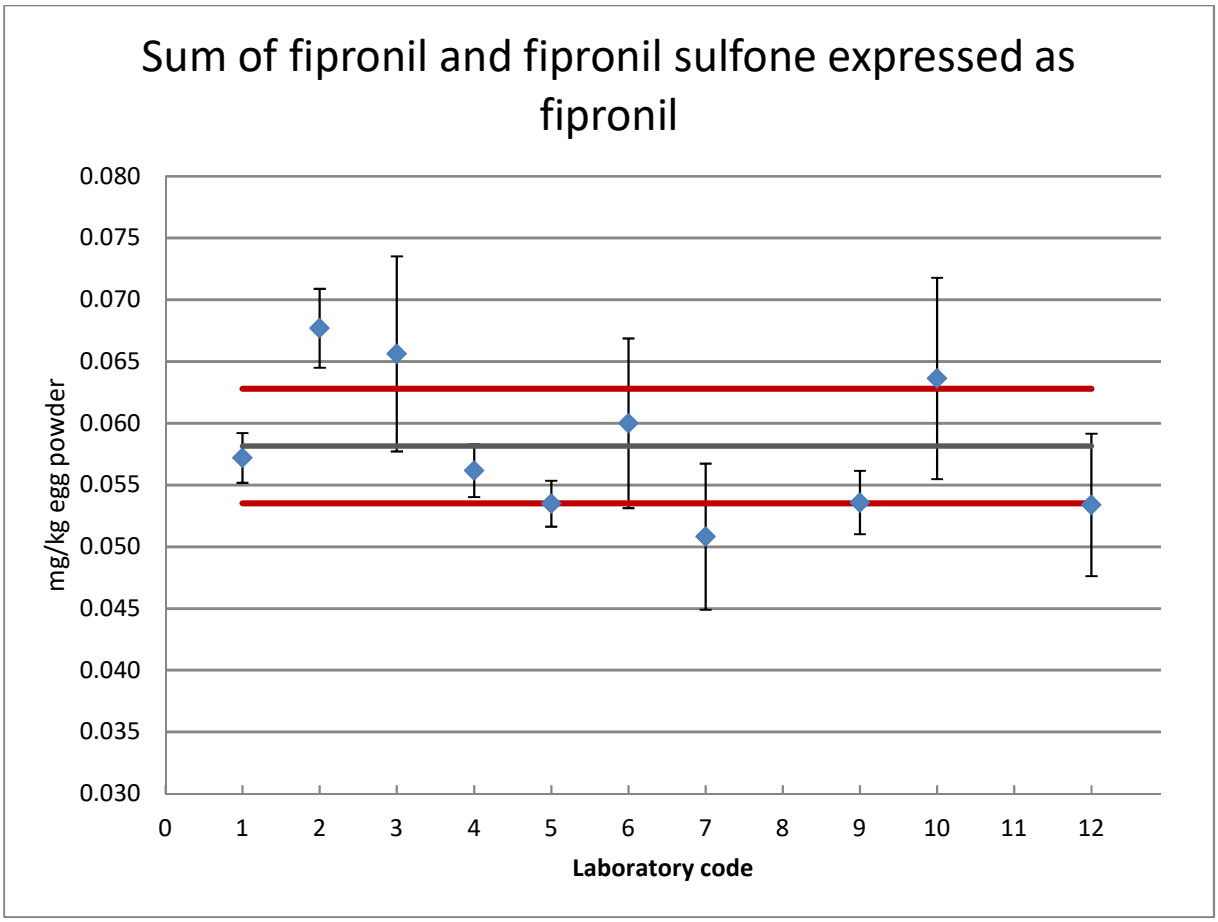


Figure F.4 Laboratory means with their standard deviations represented as error bars. Grey line correspond to the certified value and red lines correspond to the U_{CRM}

Table F.3. Mass fractions of fipronil compound in egg powder (dry mass basis) as reported by participant laboratories

Laboratory code	replicate 1 [mg/kg]	replicate 2 [mg/kg]	replicate 3 [mg/kg]	replicate 4 [mg/kg]	replicate 5 [mg/kg]	replicate 6 [mg/kg]	mean [mg/kg]	s [mg/kg]
L01 -LC-MS/MS	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0000
L02 -LC-MS/MS	0.0006	0.0006	0.0006	0.0005	0.0006	0.0006	0.0006	0.0000
L03 -LC-MS/MS	0.0030	0.0023	0.0032	0.0021	0.0029	0.0024	0.0027	0.0004
L04 -GC-MS/MS	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	n/a	n/a
L05 -LC-MS/MS	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	n/a	n/a
L06 -LC-TOF/MS	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	n/a	n/a
L07 -LC-MS/MS	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	n/a	n/a
L09 -GC-MS/MS	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	n/a	n/a
L10 -GC-MS/MS	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	n/a	n/a
L12 -GC-MS/MS	0.0007	0.0007	0.0007	0.0008	0.0008	0.0008	0.0008	0.0000
Results not used for certification								
L11 -GC-ECD	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	n/a	n/a

European Commission

EUR 30157 EN – Joint Research Centre – Directorate F – Health, Consumers and Reference Materials

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Author(s): P. Shegunova, S. Harbeck, M. Dabrio, J. Seghers, H. Emteborg

Luxembourg: Publications Office of the European Union

2020 –40 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424

ISBN 978-92-76-17604-6

doi:10.2760/822406

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KJ-NA- 30157-EN-N

Publications Office
of the European Union

doi:10.2760/822406

ISBN: 978-92-76-17604-6