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Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Preparation of macleaya cordata extract and leaves

(FAD-2021-0054; CRL/210023)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0054 - CRL/210023**
Name of Product:	***Preparation of macleaya cordata extract and leaves***
Active Agent (s):	***Sanguinarine***
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **14/02/2022**
Report approved by: Date:	**Christoph von Holst** **14/02/2022**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4 for a *preparation of macleaya cordata extract and leaves* under the category / functional group (4 d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the *feed additive* preparation is sought to be used for all avian species (excluding laying and breeding birds).

The *feed additive* (Sangrovit® extra) is a *preparation of macleaya cordata extract and leaves* containing a minimum of 0.4 % (w/w) of *sanguinarine*, which according to the Applicant is the phytochemical marker of Sangrovit® extra. The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* with a minimum Sangrovit® extra content of 45 mg / kg up to a recommended maximum content of 150 mg / kg *feedingstuffs*, which would lead to *sanguinarine* contents ranging from 0.225 to 0.750 mg / kg *feedingstuffs*.

For the determination of *sanguinarine* in Sangrovit® extra the Applicant submitted a single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography (HPLC) coupled to fluorescence detection (FLD).

For the determination of *sanguinarine* in *premixtures* and *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified method based on HPLC coupled to tandem mass spectrometer (MS/MS).

Based on the acceptable performance characteristics presented the EURL recommends for official control (i) the single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) for the determination of *sanguinarine* in Sangrovit® extra and (ii) the single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography coupled to tandem mass spectrometer (HPLC-MS/MS) for the determination of *sanguinarine* in *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Preparation of macleaya cordata extract and leaves, Sangrovit® extra, *sanguinarine*, zootechnical additives, all avian species (excluding laying and breeding birds).

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for a *preparation of macleaya cordata extract and leaves* under the category / functional group (4 d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, the *feed additive* preparation is sought to be used for all avian species (excluding laying and breeding birds) [2].

The *feed additive* (Sangrovit® extra) is a reddish-orange dry granular *preparation* of *macleaya cordata extract and leaves* containing a minimum of 0.4 % (w/w) of *sanguinarine*, (phytochemical marker) combined with some other excipients acting in the preparation as carrier, binder or preservative [3].

According to the Applicant the *feed additive* preparation is intended to be incorporated directly into *feedingstuffs* or through *premixtures* with a minimum content of Sangrovit® extra (the *feed additive* preparation) of 45 mg / kg up to a recommended maximum content of 150 mg / kg *feedingstuffs* which is equivalent to a *sanguinarine* content ranging from 0.225 to 0.750 mg / kg *feedingstuffs* [2,4].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with the *preparation of macleaya cordata extract and leaves* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the determination of *sanguinarine* in Sangrovit® extra (the *feed additive* preparation), the Applicant submitted a single-laboratory validated [5] and further verified [6] method based on reversed phase high performance liquid chromatography (HPLC) coupled to fluorescence detection (FLD) [7].

The Sangrovit® extra sample (1 to 2 g) is shaken with 50 ml of a mixture of hydrochloric acid and methanol for 1 h and filtered. The filtrate, is then further diluted with methanol and analysed by HPLC. The analytes are detected by FLD at wavelengths of 330 nm (excitation) and 570 nm (emission). The quantification is performed by external standard calibration [7].

The performance characteristics reported for *sanguinarine* in the frame of the validation [5] and verification [6] studies for Sangrovit® extra (the *feed additive* preparation) are presented in Table 1.

Based on the acceptable performance characteristics presented the EURL recommends for official control the single-laboratory validated and further verified method based on reversed phase HPLC-FLD for the determination of *sanguinarine* in Sangrovit® extra.

For the determination of *sanguinarine* in *feedingstuffs* the Applicant submitted another single-laboratory validated [9] and further verified method [10] based on HPLC coupled to tandem mass spectrometer (HPLC-MS/MS) [11].

The sample of *feedingstuffs* (10 g) is spiked with the appropriate volume of the internal standard (palmatine chloride hydrate solution), then is shaken during 1 h with 250 ml of a mixture of hydrochloric acid and methanol and filtered. The clear extract obtained, after an appropriate dilution with a mixture of acetonitrile, water and formic acid is directly injected in the chromatographic system. The identification of the analyte is based on the comparison of the retention times, ion ratio and signal-to-noise ratio between the standards and the sample. The quantification of *sanguinarine* is performed by using the standard addition method or by using a recovery rate correction (using a blank feed sample spiked with a *sanguinarine* standard solution) [11].

Table 1 The performance characteristics of the single laboratory validated and verified HPLC-FLD method for the determination of *sanguinarine* in Sangrovit® extra

	Sanguinarine	
	Validation	**Verification**
	[5]	[6]
Mass fraction, %	0.48	
RSD_r %	1.83*	0.44*
RSD_{ip} %	2.06*	0.44*
R_{rec} %	95-100	97

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery* rate. * Recalculated by EURL after removal of outliers [8]

Table 2 The performance characteristics of the single laboratory validated and verified HPLC-MS/MS method for the determination of *sanguinarine* in *feedingstuffs.*

	Sanguinarine	
	Validation	**Verification**
	[9]	**[10]**
Mass fraction, mg/kg	0.23-0.87	0.38
RSD_r, %	6.7-9.3*	5.9*
RSD_{ip}, %	6.9-10.2*	5.9*
R_{rec}, %	95-108	109

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery* rate. * Recalculated by EURL [12]

Furthermore, the Applicant reported for the method described above a limit of detection (LOD) of 0.01 mg *sanguinarine* / kg *feedingstuffs* and a limit of quantification (LOQ) of 0.02 mg *sanguinarine* / kg *feedingstuffs* [11].

The performance characteristics reported in the frame of the validation [9] and verification [10] studies for the determination of *sanguinarine* in *feedingstuffs* are presented in Table 2.

Regarding *premixtures*, the Applicant has reported low recovery rates and justified by strong matrix interferences [13]. However, these interferences are not observed when analysing *sanguinarine* in *feedinfstuffs* in which Sangrovit® Extra is introduced via the vitamin-mineral *premixture* [14]. Consequently, the Applicant proposed the determination of *sanguinarine* in *premixtures* according to the method proposed for *feedingstuffs* (prior solid dilution with blank feed). The EURL considers it a suitable approach.

Based on the acceptable performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified method based on reversed phase HPLC-MS/MS for the determination of *sanguinarine* in *premixtures* and *feedingstuffs*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The Applicant provided a method based in ultra-high performance liquid chromatography coupled to tandem mass spectrometer (UPLC-MS/MS) [15] for the determination of *sanguinarine* in tissues and verified it in poultry tissues (muscle, kidney, skin/fat and liver) [16].

However taking into account that i) the Applicant did not propose any MRLs and ii) no MRLs have been set for *sanguinarine*, the EURL considers that the evaluation of the corresponding methods of analysis is not relevant for the present application.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005. as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control (i) the single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) for the determination of *sanguinarine* in Sangrovit® extra (the *feed additive* preparation) and (ii) the single-laboratory validated and further verified method based on reversed phase HPLC-MS/MS for the determination of *sanguinarine* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *sanguinarine* in the *feed additive*:

- reversed phase high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD)

For the determination of *sanguinarine* in *premixtures* and *feedingstuffs*:

- reversed phase high performance liquid chromatography coupled mass spectrometry detection (HPLC-MS/MS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the *preparation of macleaya cordata extract and leaves* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANTE/E5: FORW. APPL. 1831-0068-2021
[2] *Application, Annex I - Submission number 1614332887371-2836
[3] *Technical dossier, Section II: 2.1 Identity of the additive
[4] *Technical dossier, Section II: 2.5 Conditions of use
[5] *Technical dossier, Section II – Annex_II.6.2
[6] *Technical dossier, Section II – Annex_II.6.12
[7] *Technical dossier, Section II – Annex_II.6.1
[8] Supplementary information: eurl-anova-fa.pdf

[9] *Technical dossier, Section II – Annex_II.6.4

[10] *Technical dossier, Section II – Annex_II.6.11

[11] *Technical dossier, Section II – Annex_II.6.3

[12] Supplementary information: eurl-anova-fs.pdf

[13] *Technical dossier, Section II – Annex_II.6.5

[14] *Technical dossier, Section II – Annex_II.4.2

[15] *Technical dossier, Section II – Annex_II.6.6

[16] *Technical dossier, Section II – Annexes_II.6.7 to II.6.10

*Refers to Dossier no: FAD-2021-0054

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Wageningen Food Safety Research (WFSR)[1] (NL)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)

[1] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.