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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

Macleaya cordata extract

(FAD-2010-0071; CRL/100111)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2010-0071 - CRL/100111**
Name of Feed Additive:	***Macleaya cordata extract***
Phytochemical marker(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 (EURL-FA)** **30/04/2018**
Report approved by: Date:	**Christoph von Holst** **03/05/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 10(2) for the botanically defined *macleaya cordata extract* under the category / functional group (2 b) "sensory additives"/"flavouring compounds", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the *feed additive* is sought to be used for all animal species and categories.

According to the Applicant the *feed additive* contains 40 to 60 % (w/w) of the phytochemical marker *sanguinarine*. The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* with a minimum content of Sangrovit® (the *feed additive* preparation) of 15 mg/kg up to a recommended maximum content of 100 mg/kg *feedingstuffs*.

For the determination of *sanguinarine* (phytochemical marker) in the *feed additive* the Applicant submitted the single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) or fluorescence detection (FD).

The following performance characteristics were reported in the frame of the validation and verification studies for the quantification of the phytochemical marker in the *feed additive*: a relative standard deviation for *repeatability* (RSD_r) and for *intermediate precision* (RSD_{ip}) ranging between 1.2 and 4.6 %; and a *recovery* rate ranging from 95 to 112 %.

Based on the acceptable performance characteristics presented the EURL recommends for official control the single-laboratory validated and further verified method based on HPLC coupled to UV or FD for the determination of *sanguinarine* (phytochemical marker) in the *feed additive*.

As the accurate quantification of the *macleaya cordata extract* content added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine the *macleaya cordata extract* 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

Sanguinarine, *macleaya cordata extract*, sensory additives, all animal species

1. BACKGROUND

In the current application authorisation is sought under Article 10(2) (re-evaluation of additives already authorised under the provisions of the Council Directive 70/524/EEC) for the botanically defined *macleaya cordata extract* under the category / functional group (2 b) "sensory additives"/"flavouring compounds", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, the *feed additive* is sought to be used for all animal species and categories [2].

The *feed additive* is a red-orange powder containing 40 % to 60 % (w/w) of *sanguinarine*; 15 to 25 % (w/w) of *chelerythrine*; 1 to 10 % (w/w) of *protopine*; and traces of *allocryptopine*, *dihydrosanguinarine* and *dihydrochelerythrine*. According to the Applicant, the phytochemical marker of the *feed additive* is *sanguinarine* [2,3]. The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* with a minimum content of Sangrovit® (the *feed additive* preparation) of 15 mg/kg up to a recommended maximum content of 100 mg/kg *feedingstuffs* [3]; according to the Applicant this is equivalent to a *sanguinarine* content ranging from 0.23 to 1.9 mg/kg *feedingstuffs* [3].

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 *macleaya cordata extract* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [4].

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures and feedingstuffs

For the determination of *sanguinarine* (phytochemical marker) and the other major component *chelerythrine* in the *feed additive* (including its preparation), the Applicant submitted the single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) or fluorescence detection (FD) [5,6].

The sample (0.05 to 1 g) is shaken with 100 ml of a mixture containing 3 % of hydrochloric acid in methanol (v/v) for 1 h and filtered. The filtrate, after an appropriate dilution with the extraction mixture, is further analysed by HPLC. The analytes are detected by UV at 270 nm wavelength or by FD at 330 nm (excitation) and 570 nm (emission). The quantification is performed by external standard calibration [5,6].

The performance characteristics reported for the two analytes in the frame of the validation [5,6] and verification [7,8] studies 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-UV/FD for the determination of *sanguinarine* (phytochemical marker) in the *feed additive*.

The HPLC-UV/FD method is also suitable for the determination of *chelerythrine* in the *macleaya cordata extract* and its preparation (Sangrovit®).

Table 1 The performance characteristics of the single laboratory validated and verified HPLC-UV/FD method for the determination of *sanguinarine* and *chelerythrine* in the *feed additive*

	sanguinarine		**chelerythrine**	
	Validation	**Verification**	**Validation**	**Verification**
	[5,6]	[7,8]	[5,6]	[7,8]
Mass fraction, %	1.7 – 49.0		0.9 – 23.2	
RSD_r, %	1.2 – 3.9	3.0 – 3.7	0.8 – 2.6	2.0 – 3.6
RSD_{ip}, %	-	3.2 – 4.6	-	3.2 – 6.9
R_{rec}, %	95 – 98	101 – 112	95	95 – 101

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery* rate.

As the accurate quantification of the *macleaya cordata extract* content added to *premixtures*, *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine the *macleaya cordata extract* in *premixtures* and *feedingstuffs*.

However, for the *qualitative identification* of *sanguinarine* and *chelerythrine* in *premixtures* and *feedingstuffs* the Applicant applied the above mentioned HPLC-FD method by using 10 g of the sample for the extraction of the target analytes [9,10]. The identification was based on comparing the retention times of *sanguinarine* and *chelerythrine* in the samples with the ones of the standard solutions. In addition, for the confirmation of the identity of the analytes in *premixtures* and *feedingstuffs*, the samples were re-analysed by using another method based on HPLC coupled to tandem mass spectrometry (MS/MS) [9,10]. When the HPLC-MS/MS method was applied, the identity of the analytes was confirmed by analysing their retention times, parent and daughter ions [9,10].

Based on the acceptable data presented the EURL considers the above mentioned methods based on HPLC-FD together with the HPLC-MS/MS fit-for-purpose for the *qualitative identification* of *sanguinarine* and *chelerythrine* in *premixtures* and *feedingstuffs*.

Furthermore, the Applicant has revised and consolidated the above mentioned methods by submitting another single-laboratory validated and further verified HPLC-MS/MS method for the *quantitative determination* of *sanguinarine* and *chelerythrine* in *premixtures* and *feedingstuffs* [11].

The sample of *premixtures* (1 g) or *feedingstuffs* (20 g) is shaken with 100 ml of a mixture containing 3 % of hydrochloric acid in methanol (v/v) for 1 h and filtered. The extract, after an appropriate dilution with a mixture of 40 % acetonitrile and 60 % water (v/v), is spiked with the internal standard (palmatine chloride) solution for further analysis. The identification of the analytes is based on the comparison of their retention times, parent and daughter ions in the samples with the ones of the standard solutions. The quantification is performed by using external standard calibration with the presence of the internal standard in the samples and the standard solutions or by the standard additions method. The internal standard is not used when the standard additions method is applied for the quantification [11].

While acceptable performance characteristics were reported in the frame of the verification study for *premixtures* [12] and *feedingstuffs* [13], the validation data presented were not sufficient enough [11] to consider the reliable transferability of the method to another laboratory. Hence, the EURL cannot consider this LC-MS/MS method as fit-for-purpose to determine *sanguinarine* and *chelerythrine* 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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography coupled to ultraviolet or fluorescence detection (HPLC-UV/FD) for the determination of *sanguinarine* (phytochemical marker) in the *feed additive*.

As the accurate quantification of the *macleaya cordata extract* content added to *premixtures*, *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine the *macleaya cordata extract* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *sanguinarine* (phytochemical marker) in the *feed additive*:

- reversed phase high performance liquid chromatography coupled to ultraviolet or fluorescence detection (HPLC-UV/FD)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the *macleaya cordata extract* 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 SANCO_D2_FWD. APPL. 1831/0057-2010
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis
[4] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories
[5] *Technical dossier, Section II – Annex_II_54
[6] *Technical dossier, Section II – Annex_II_56
[7] *Technical dossier, Section II – Annex_II_55

[8] *Technical dossier, Section II – Annex_II_57
[9] *Technical dossier, Section II – Annex_II_58
[10] *Technical dossier, Section II – Annex_II_60
[11] *Technical dossier, Section II – Annex_II_62
[12] *Technical dossier, Section II – Annex_II_59
[13] *Technical dossier, Section II – Annex_II_61

*Refers to Dossier no: FAD-2010-0071

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

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- RIKILT Wageningen UR, Wageningen (NL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)