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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

Botanically Defined Flavourings Group BDG 14 - Malvales

(FAD-2010-0324; CRL/100208)

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-2010-0324 - CRL/100208**
Name of Feed Additive:	***althaea tincture, cocoa distillate (withdrawn), cocoa extract and cocoa absolute from botanically defined flavourings group (BDG 14) - Malvales***
Phytochemical marker (s):	**tiliroside, theobromine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**S. Bellorini & Z. Ezerskis** **13/07/2020**
Report approved by: Date:	**Christoph von Holst** **14/07/2020**

EXECUTIVE SUMMARY

In the current grouped application, an authorisation is sought under Articles 4(1) and 10(2) for *althaea tincture*, *cocoa distillate*, *cocoa extract* and *cocoa absolute* from botanically defined flavourings group 14 (BDG 14) as *feed additives* under the category/functional group (2b) "sensory additives"/"flavouring compounds", according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additives* for all animal species and categories. Following the withdrawal of *cocoa distillate*, in this report the EURL will focus exclusively on the evaluation of the suitability of analytical methods for official control of the remaining *feed additives* namely *althaea tincture*, *cocoa extract* and *cocoa absolute*.

According to the Applicant, *althaea tincture* contains between 0.0005 to 0.0015 % (w/v) of *tiliroside* as phytochemical marker; *cocoa extract* has *theobromine* as phytochemical marker ranging from 3 to 5 % (w/w) and *cocoa absolute* contains between 0.01 to 0.05 % (w/w) of *theobromine* as phytochemical marker. The *feed additives* are intended to be incorporated into *feedingstuffs* or *water* for drinking alone or through flavouring *premixtures* with no proposed minimum or maximum levels in *feedingstuffs* or *water* for drinking. However, the Applicant suggested the typical maximum inclusion level of the *feed additive* of 25 mg/kg *feedingstuffs*.

For the determination of *tiliroside* in *althaea tincture*, the Applicant submitted a single-laboratory validated and further verified method based on high performance liquid chromatography coupled to photometric detection (HPLC-UV) based on a general method for polar phenolics from the Institut für Angewandte Botanik und Pharmakognosie Veterinärmedizinische Universität Wien (IAB, AT). For the quantification of the *theobromine* content in *cocoa absolute* and in *cocoa extract* the Applicant applied a ring-trial validated method based on HPLC-UV developed by the National Institute of Standards and Technology (NIST, USA).

Based on the experimental evidences provided the EURL recommends for official control the single-laboratory validated and further verified HPLC-UV method from IAB for polar phenolics for the determination of *tiliroside* in *althaea tincture* and the ring-trial validated HPLC-UV method developed by NIST for the determination of *theobromine* in *cocoa absolute* and *cocoa extract*.

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

Tiliroside, theobromine, althaea tincture, cocoa extract and *cocoa absolute*, sensory additives, flavouring compounds, all animal species

1. BACKGROUND

In the current grouped application, an authorisation is sought under Articles 4(1) (authorisation of a *feed additive* or a new use) and 10(2) (authorisation of an existing product) for *althaea tincture, cocoa distillate, cocoa extract* and *cocoa absolute* from botanically defined flavourings group 14 (BDG 14) as *feed additives* under the category/functional group (2b) "sensory additives"/"flavouring compounds", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. However as the European Commission has acknowledged the request from the Applicant to withdraw the application for one of the *feed additives* of this grouped application, namely *cocoa distillate* [2] the EURL will focus in this report exclusively on the evaluation of the suitability of analytical methods for official control of the remaining *feed additives* namely *althaea tincture, cocoa extract* and *cocoa absolute*. The authorisation is sought for the use of the *feed additives* for all animal species and categories [3].

According to the Applicant, *althaea tincture* is a colourless to pale yellow-brownish liquid with a characteristic odour containing between 0.0005 to 0.0015 % (w/v) of *tiliroside* as phytochemical marker; *cocoa extract* is a brown liquid with a cocoa odour with *theobromine* as phytochemical marker ranging from 3 to 5 % (w/w) and *cocoa absolute* is a reddish amber liquid with a characteristic odour containing between 0.01 to 0.05 % (w/w) of *theobromine* as phytochemical marker [4].

The *feed additives* are intended to be incorporated into *feedingstuffs* or *water* for drinking alone or through flavouring *premixtures* with no proposed minimum or maximum levels in *feedingstuffs* or *water* for drinking [5]. However, the Applicant suggested the typical maximum inclusion level of the *feed additive* of 25 mg/kg *feedingstuffs* [5].

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 *botanically defined flavourings group 14*

(BDG 14) - *Malvales* 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 *tiliroside* in *althaea tincture* the Applicant submitted a single-laboratory validated [6][7] and further verified method [8][9] based on high performance liquid chromatography coupled to photometric detection (HPLC-UV) [10][11]. This method is based on general method for polar phenolics from the Institut für Angewandte Botanik und Pharmakognosie Veterinärmedizinische Universität Wien (IAB, AT) [10].

The *althaea tincture* is directly injected into the HPLC system and *tiliroside* is determined by reversed-phase HPLC using a gradient elution [12]. *Tiliroside* is eluted at 53.4 min and quantified at 320 nm. This method was single-laboratory validated and further verified. Based on the verification data the EURL calculated a relative standard deviation for *repeatability* (RSD_r) of 5.1 % and a relative standard deviation for *intermediate precision* (RSD_{ip}) of 7.3 % [8][13]. Additionally, the Applicant reported a recovery rate of 96 %, a limit of quantification (LOQ) of 0.15 µg *tiliroside* /ml of the tincture [7] and provided typical chromatograms for *althaea tincture* that demonstrate the lack of potential interferences for the determination of *tiliroside* in *althaea tincture* [7].

Furthermore, the Applicant provided information on three different batches of *althaea tincture* reporting *tiliroside* contents ranging from 9.1 to 12.9 µg/ml [14].

The Applicant slightly modified the method described above and successfully applied it to a solid *premixture* prepared by applying *althaea tincture* to a mixture of silicic acid and calcium carbonate [12].

For the quantification of the *theobromine* content in *cocoa absolute* and in *cocoa extract* the Applicant applied a high performance liquid chromatography coupled to photometric detection (HPLC-UV) [15]. This method has been developed by the National Institute of Standards and Technology (NIST, USA) for the value assignment of caffeine, *theobromine* and theophylline in the standard reference material (SRM) 2388 Baking chocolate [15].

In this method an aliquot of the SRM 2388 material melted and homogenised is extracted with hexane to remove the lipid components. Then, the hexane layer is removed and the extraction procedure is consecutively repeated for another three times. After the fourth extraction the defatted chocolate is dried under a nitrogen stream, reconstituted in water,

sonicated and filtered. After centrifugation the supernatant is further filtered through nylon filter before being injected in the high performance liquid chromatography (HPLC) system for further analysis [15].

The *theobromine* content is determined using a photometric detector (UV) set at a wavelength of 274 nm. The *theobromine* content is quantified against an external standard calibration curve using beta-hydroxyethyltheophyline as internal standard. This method has been ring-trial validated in the frame of the value assignment for the reference material SRM 2388 Baking chocolate leading to a relative standard deviation for *repeatability* (RSD_r) of 2.3 % and a relative expanded uncertainty (*U*) of 10 %. Furthermore a limit of quantification (LOQ) of 30 ng/ml was reported for *theobromine* [15].

The Applicant provided experimental evidences on the applicability of the method in data for the analysis of *theobromine* in *cocoa absolute* and in *cocoa extract* [16]. Additionally the Applicant provided information on three different batches of *cocoa extract* and two different batches of *cocoa absolute* reporting average *theobromine* contents of 4.0 % (w/w) and of 0.01 % (w/w), respectively [14].

Furthermore, upon request of the EURL the Applicant provided the modified standard operating procedure (SOP) for applying the method to the *feed additives* i.e. *cocoa absolute* and *cocoa extract* [17].

The Applicant proved the applicability of this method [15] to a solid *premixture* composed by a mixture of flavouring substances containing *cocoa extract*, calcium carbonate and silicic acid, and to a liquid *premixture* composed by a mixture of flavouring substances containing *cocoa absolute* and 1,2-propanediol [18].

Based on the experimental evidence available, the EURL recommends for official control (i) the single-validated and further verified HPLC-UV method from IAB for polar phenolics method for the determination of *tiliroside* in *althaea tincture* and (ii) the ring-trial validated HPLC-UV method developed by NIST for the determination of *theobromine* in *cocoa absolute* and in *cocoa extract*.

The Applicant did not provide experimental data or an analytical method for the determination of *althaea tincture*, *cocoa absolute* and *cocoa extract* in *feedingstuffs* and in *water* for drinking. As the unambiguous determination of the *feed additive* added to these matrices is not achievable experimentally, the EURL cannot evaluate or recommend any method for official control for the determination of *althaea tincture*, *cocoa absolute* and *cocoa extract* in *feedingstuffs* and *water* for drinking.

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)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not considered necessary by the EURL.

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 high performance liquid chromatography coupled to photometric detection (HPLC-UV) for the determination of *tiliroside* (phytochemical marker) in the *feed additive (althaea tincture)* and (ii) the ring-trial validated method on based on HPLC-UV for the determination of *theobromine* (phytochemical marker) in the *feed additives (cocoa absolute* and *cocoa extract).*

Recommended text for the register entry (analytical method)

For the determination of *tiliroside* in the *feed additive (althaea tincture)*:

- High performance liquid chromatography coupled to photometric detection (HPLC-UV)

For the determination of *theobromine* in the *feed additives (cocoa absolute* and *cocoa extract)*:

- High performance liquid chromatography coupled to photometric detection (HPLC-UV)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *botanically defined flavourings group 14 (BDG 14) - Malvales* 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, Ref. SANCO/D/2: FORW. APPL. 00198 (10189)/1831/-2010

[2] *Supplementary information – Partial withdrawal of applications for various Botanically Defined Groups from FEFANA ASBL – Ares(2019)129932

[3] *Application, Proposal for Register Entry - Annex A

[4] *Technical dossier, Section II, 2.1 Identity of the additive

[5] *Technical dossier, Section II, 2.5 Conditions of use of the additive

[6] *Technical dossier, Annex_II_10_HPLC-B14-2

[7] *Technical dossier, Annex_III_IAB

[8] *Technical dossier, Annex_IVa_IAB

[9] *Technical dossier, Annex_IVb_IAB

[10] *Technical dossier, Annex_I_IAB

[11] *Technical dossier, Annex_II_IAB

[12] *Technical dossier, Annex_V_IAB_Method_Premixture_Reports

[13] *Supplementary information, EURL_ANOVA_tiliroside_ver.pdf

[14] *Technical dossier, Annex_II_1

[15] *Technical dossier, Annex_II_9_HPLC_B14-1

[16] *Technical dossier, Annex_II-HPLC_B14-1_Matrices

[17] *Supplementary information, Annex_SOP determination of theobromine by hplc.pdf

[18] *Technical dossier, Annex_II-HPLC_B14-1_Premixtures

*Refers to Dossier no: FAD-2010-0324

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:

- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)

- Wageningen Food Safety Research[1] (WFSR) (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.