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**Food and Feed Compliance**

European Union Reference Laboratory
Feed Additives

JRC F.5/CvH/ZE/AS/Ares

# 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

## Chlorophyllins

***(FAD-2021-0065; CRL/220048)***

European Union Reference Laboratory
Feed Additives

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| Dossier related to: | **FAD-2021-0065 - CRL/220048** |
| Name of Product / Feed Additive: | ***Chlorophyllins*** |
| Active Agent (s): | **Chlorophyllins** |
| Rapporteur Laboratory: | **European Union Reference Laboratory for Feed Additives (EURL-FA)**<br>**JRC Geel, Belgium** |
| Report prepared by: | **Zigmas Ezerskis** |
| Report checked by:<br>Date: | **María José González de la Huebra**<br>**14/11/2022** |
| Report approved by:<br>Date: | **Christoph von Holst**<br>**14/11/2022** |

## EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *chlorophyllins* under the category/ functional group (4d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for the use of the *feed additive* as faecal marker compound for chickens and turkeys for fattening, and minor poultry species.

According to the Applicant, the *feed additive* is a dark green, free flowing powder composed of processed chlorophylls, namely of *chlorophyllins*, considered by the Applicant as active substances. In addition, the Applicant specified several additional criteria for the *feed additive* which are similar to the ones for *chlorophyllins E140(ii)* as food additive specified in Commission Regulation (EU) No 231/2012.

The *feed additive* is intended to be used in *feedingstuffs* or *water* for drinking at a minimum level of 0.045 g *chlorophyllins* / animal / day for a period not longer than 24 h before slaughtering.

For the determination of *chlorophyllins* in the *feed additive* the Applicant submitted a spectrophotometric method, which is based on the internationally recognised method for the determination of total colouring matters content described in the FAO JECFA monographs No. 1 (Vol. 4).

Based on all available information, the EURL recommends for official control the above mentioned spectrophotometric method based on the internationally recognised method for the determination of total colouring matters content described in the FAO JECFA monographs No. 1 (Vol. 4) for the determination of *chlorophyllins* in the *feed additive*.

For the determination of *chlorophyllins* in *feedingstuffs* and *water* for drinking the Applicant did not prove the suitability of the corresponding methods for official control and did not express the dose of *chlorophyllins* in *feedingstuffs* or *water* for drinking as the mass fraction of the active substances in the mentioned matrices.

Therefore, the EURL is not able to recommend any method for the determination of *chlorophyllins* in *feedingstuffs* or *water* for drinking.

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**

*Chlorophyllins*, zootechnical additives, chickens for fattening, turkeys for fattening, minor poultry species.

## 1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new *feed additive*) for *chlorophyllins* under the category/ functional group (4d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. Specifically, the authorisation is sought for the use of the *feed additive* as faecal marker compound for chickens and turkeys for fattening, and minor poultry species [2].

According to the Applicant, the *feed additive* is a dark green, free flowing powder composed of processed chlorophylls, namely of *chlorophyllins*, which consist of water soluble compounds such as chlorin e6 and rhodin g7 as major components with minor amounts of magnesium containing derivatives (chlorophyllin a and b) and pheophorbide derivatives (carboxypyropheophorbide a and b) in the sodium salt form [3], considered by the Applicant as active substances [4].

In addition, the Applicant specified the following criteria for the *feed additive* [5]:

- two absorbance bands at 405 and 630 nm in aqueous buffer at pH 7.5;
- a specific absorbance (E 1%, 1 cm) *ca.* > 1100 at 405 nm (maximum absorbance wavelength) in aqueous buffer at pH 7.5;
- an absorbance ratio at 405 and 630 nm at pH 7.5 ranging between 4.75 and 4.95;
- a pH (1 %, w/v, aqueous solution) ranging from 9.5 to 10.2; and
- a maximum moisture content of 6.0 % (w/w).

These criteria are similar for *chlorophyllins E140(ii)* as food additive specified in Commission Regulation (EU) No 231/2012 [6].

The *feed additive* is intended to be used in *feedingstuffs* or *water* for drinking at a minimum level of 0.045 g *chlorophyllins* / animal / day for a period not longer than 24 h before slaughtering [7].

## 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 *chlorophyllins* 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 *chlorophyllins* in the *feed additive* the Applicant proposed [8] and submitted a spectrophotometric method [9], which is based on the internationally recognised method for the determination of total colouring matters content described in the FAO JECFA monographs No. 1 (Vol. 4) [10].

According to the Applicant, the dried sample (10 mg) is dissolved in 100 ml of a phosphate buffer (pH 7.5) solution. An aliquot (0.5 ml) of the latter solution is diluted with 10 ml of the buffer solution for further analysis by a spectrophotometry. The spectrophotometric measurements of the prepared samples are performed at maximum absorbance wavelength of 405 nm. The *chlorophyllins* expressed as total colouring matters content is determined by using a referential specific absorbance value at the mentioned wavelength for *chlorophyllins E140(ii)* specified in Commission Regulation (EU) No 231/2012 [6,9].

The Applicant analysed several batches of the *feed additive* and a relative standard deviation for *repeatability* ($RSD_r$) of 2.4 % was derived for the average value for *chlorophyllins* as total colouring matters content of 193.7 % (w/w) [11]. According to the Applicant, this value for total colouring matters content is a relative value obtained when using the specific absorbance value of *chlorophyllins E140(ii)* as a reference, which is rather outdated as the Applicant states [9].

In addition, during the analysis of the *feed additive* batches the Applicant demonstrated the presence of two absorbance bands at 405 and 630 nm by a spectrophotometry in aqueous buffer solution at pH 7.5 and presented the data for: i) a specific absorbance (E 1%, 1 cm) at 405 nm at pH 7.5; ii) an absorbance ratio at 405 and 630 nm at pH 7.5; iii) a pH of 1 % (w / v) aqueous solution and iv) a maximum moisture content. The obtained results were complying with the criteria specified for the *feed additive* [11].

Based on all available information, the EURL recommends for official control the above mentioned spectrophotometric method based on the internationally recognised method for the determination of total colouring matters content described in the FAO JECFA monographs No. 1 (Vol. 4) for the determination of *chlorophyllins* in the *feed additive*.

For the determination of *chlorophyllins* in *feedingstuffs* the Applicant submitted another spectrophotometric method similar to the one submitted for the *feed additive* [12,13].

According the method, an aliquot of the feedingstuffs sample (5 g) is mixed with 50 ml of acetone and water mixture (1:1, v / v) and shaken. An aliquot (2 ml) of the extract is centrifuged and the supernatant is diluted with the acetone / water mixture. The diluted solution is then analysed by a spectrophotometry in the range between 300 and 700 nm. The quantification of *chlorophyllins* is performed using a calibration curve prepared from the standard solutions of the product [12,13].

The Applicant performed recovery and stability studies of the method when analysing feed samples spiked with *chlorophyllins* at the levels of 100 and 1000 mg / kg *feedingstuffs* using the above-mentioned method for feed. Too low recoveries of *chlorophyllins* in feed, ranging from 32 to 74 % were obtained [12,13]. Moreover, no limit of quantification (LOQ) and no verification data were provided.

For the determination *chlorophyllins* in *water* the Applicant suggested using the same method than for the determination of *chlorophyllins* in the *feed additive* without providing the proofs of the method's applicability at the levels corresponding to the conditions of use.

Furthermore, in the conditions of use the Applicant did not express the dose of *chlorophyllins* in *feedingstuffs* or *water* for drinking as the mass fraction of the active substances in the mentioned matrices [7]. Finally, according to the Applicant the *feed additive* would be added to *feedingstuffs* or *water* for drinking for a maximum period of 24 h before slaughtering and therefore, more profound performance studies of the methods for the determination of *chlorophyllins* in *feedingstuffs* and *water* for drinking were not conducted [8].

Based on overall available data, the EURL is not able to recommend any method for the determination of *chlorophyllins* in *feedingstuffs* or *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) – (Include this section ONLY when relevant for the dossier evaluation e.g. probiotics)***

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 spectrophotometric method based on the internationally recognised method for the determination of total colouring matters content described in the FAO JECFA monographs No. 1 (Vol. 4) for the determination of *chlorophyllins* in the *feed additive*.

For the determination of *chlorophyllins* in *feedingstuffs* and *water* for drinking the Applicant did not prove the suitability of the corresponding methods proposed for official control and did not express the dose of *chlorophyllins* in *feedingstuffs* or *water* for drinking as the mass fraction of the active substance (or the *feed additive*) in the mentioned matrices. Therefore, the EURL is not able to recommend any method for the determination of *chlorophyllins* in *feedingstuffs* or *water* for drinking.

***Recommended text for the register entry (analytical method)***

For the determination of *chlorophyllins* as total colouring matters content in the *feed additive*:

- spectrophotometry based on FAO JECFA monographs No. 1 (Vol. 4)

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *chlorophyllins* 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_FWD. APPL. 1831-0055-2021

[2] *Application, Annex 1 – submission number 1616773753395-2986

[3] *Technical dossier, Section II: 2.1.3. Qualitative and Quantitative Composition

[4] *Technical dossier, Section II: 2.2.1. Description of the Active Substances

[5] *Technical dossier, Section II: 2.1.3.3. Compositional Product Specifications

[6] Commission Regulation (EU) No 231/2012 of 9 March 2012, laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council, OJ L 83, 22.3.2012

[7] *Technical dossier, Section II: 2.5.1. Proposed Mode of Use in Animal Nutrition

[8] *Technical dossier, Section II: 2.6. Methods of analysis and reference samples

[9] *Technical dossier, Section II – Annex_II-12B

[10] FAO JECFA monographs No. 1 (Vol. 4), Combined Compendium for Food Additive Specifications

[11] *Technical dossier, Section II: 2.1.3.4. Analytical Batch Data

[12] *Technical dossier, Section II – Annex_II-13

[13] *Technical dossier, Section II – Annex_II-10

*Refers to Dossier no: FAD-2021-0065

## 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)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Wageningen Food Safety Research (WFSR)[1] (NL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)

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