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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

Astaxanthin-rich Phaffia rhodozyma KBW 10061 (AJ 14971)

(FAD-2013-0032; CRL/120042)

Revised

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-2013-0032 - CRL/120042**
Name of Product:	***Astaxanthin-rich Phaffia rhodozyma KBW 10061 (AJ 14971)***
Active Agent (s):	**Astaxanthin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Rebeca Fernandez Orozco**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **12/05/2014**
Report approved by: Date:	**Christoph von Holst** **12/05/2014**

EXECUTIVE SUMMARY

Astaxanthin-rich Phaffia rhodozyma is the *feed additive* for which authorisation is sought under category/functional group 2(a) "sensory additives"/"colourants: substances which, when fed to animals, add colours to food of animal origin", according to the classification system of Annex I of Regulation (EC) No 1831/2003. In the current application submitted according to Article 4(1) of Regulation (EC) No 1831/2003, the authorisation for salmon and trout is requested.

The active substance in the product is *Astaxanthin*, produced by the red yeast *Phaffia rhodozyma* (KBW 10061 [(AJ 14971]). *Astaxanthin* occurs in different forms which can be classified according to stereoisomers, geometric isomers and free or esterified forms. The *feed additive* is a dark purple solid consisting of a mixture of *Astaxanthin* (8000-9000 mg/kg), variable amount of antioxidants (ascorbic acid and citric acid) and a calcium carbonate carrier. The *feed additive* is intended to be incorporated in *feedingstuffs*. The recommended maximum concentration of the active substance is 100 mg/kg *feedingstuffs*; this level refers to sum of all-E-*Astaxanthin*, 9z-*Astaxanthin*, 13z-*Astaxanthin* and other z-*Astaxanthin* isomers. The mixture of *Astaxanthin* with *Canthaxanthin* is allowed provided that the total concentration does not exceed 100 mg/kg *feedingstuffs*.

For quantification of *Astaxanthin* in the *feed additive* and in the *feedingstuffs* the Applicant submitted two single-laboratory validated and further verified methods based on High Performance Liquid Chromatography coupled with a UV/VIS detector (HPLC-UV/VIS). The following performance characteristics were reported: - precisions (relative standard deviations for *repeatability* and *intermediate precision*) ranging from 1 to 3.6 %; – recovery rates ranging from 88 to 101 %, and a limit of quantification of 0.26 mg *Astaxanthin* /kg *feedingstuffs*. Based on the performance characteristics presented, the EURL recommends for official control the HPLC-UV/VIS method mentioned above.

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) is not considered necessary.

KEYWORDS

Astaxanthin , sensory, colourants, feed additives, salmon, trouts

1. BACKGROUND

Astaxanthin is the *feed additive* for which authorisation is sought under category/functional group 2(a)(ii) "sensory additives"/"colourants: substances which, when fed to animals, add colours to food of animal origin" according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. In the current application submitted according to Article 4(1) of Regulation (EC) No 1831/2003, the authorisation for salmon and trout is requested [2].

The active substance in the product is *Astaxanthin*, produced by the red yeast *Phaffia rhodozyma* (KBW 10061 [(AJ 14971]). *Astaxanthin* occurs in different forms which can be classified according to stereoisomers, geometric isomers and free or esterified forms. The main stereoisomers present in the feed additive are all-E-*Astaxanthin*, 9z-*Astaxanthin* and 13z-*Astaxanthin*. The *feed additive* is a dark purple solid consisting of a mixture of *Astaxanthin* (8000-9000 mg/kg) with variable amounts of antioxidants (i.e. ascorbic acid, citric acid) and a calcium carbonate carrier [3,4].

The *feed additive* is intended to be incorporated in *feedingstuffs*. The recommended maximum concentration of the active substance is 100 mg/kg of complete *feedingstuffs*. This level corresponds to the sum of all-E-*Astaxanthin* and some minor cis-*Astaxanthin* isomers. Furthermore, the mixture of *Astaxanthin* with *Canthaxanthin* is allowed provided that the total concentration does not exceed 100 mg/kg in the complete *feedingstuffs* [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 *Astaxanthin-rich Phaffia rhodozyma KBW 10061 (AJ 14971)* 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 [6]

Description of the analytical methods for the quantification of the active substance in feed additive and feedingstuffs

For the quantification of *Astaxanthin* in the *feed additive* (*Ajinomoto Phaffia Astaxanthin*) the Applicant submitted a single-laboratory validated and further verified method based on High Performance Liquid Chromatography coupled with a UV/VIS detector (HPLC-UV/VIS) [7].

A sample is enzymatically broken down and extracted with ethanol. The solution is then injected after filtration in the HPLC system to separate the different *Astaxanthin* stereoisomers, which are quantified at 454 nm using external calibration. The *Astaxanthin* trans and cis isomers are quantified and added to calculate the total *Astaxanthin* content in the sample[7].

The performance characteristics obtained in the frame of the validation [8] and verification [9] studies are reported Table 1. Furthermore, the Applicant determined a limit of quantification (LOQ) of 0.63 mg/g [8].

For the quantification of *Astaxanthin* in formulated fish feed (*feedingstuffs*), the Applicant proposed a single laboratory validated method, based on HPLC-UV/VIS [10].

Intracellular *Astaxanthin* is made available for extraction by combined homogenization, sonication, and digestion of yeast cells with lysing enzyme. The digested aqueous sample is further homogenized with methanol and a portion of the resulting preparation is extracted with chloroform. An aliquot of the chloroform extract is evaporated to dryness and reconstituted in mobile phase for subsequent assay by HPLC with a normal phase column. Total *Astaxanthin* (sum of all E-*Astaxanthin* isomers, 9-Z-*Astaxathin*, 13-Z-*Astaxanthin* and other Z-*Astaxanthin* isomers) is quantified by UV/vis detector at 470 nm using external calibration [10].

The performance characteristics obtained in the frame of the validation [11] and verification [12] studies are reported in Table 1. Furthermore, the Applicant determined a limit of quantification (LOQ) of 0.26 mg/kg fish feed [11].

Table 1. Performance characterictics for the quantification of *Astaxanthin* in *feed additive* (FA) and *feedingstuffs* (FS), obtained in the frame of the validation (Val) and verification (Ver) studies.

	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
Matrix	Val	Ver	Val	Ver	Val	Ver
FA	1 [8]	1.3 [9]	1.2 [8]	1.3 [9]	99-101 [8]	99.5 [9]
FS	2.9 [11]	2 [12]	3.6 [11]	2.2 [12]	88 [11]	98 [12]

RSD_r, RSD_R: relative standard deviation for repeatability and reproducibility, respectively. R_{Rec}: recovery rate (%)

Based on the acceptable performance characteristics presented, the EURL recommends for official control, the validated and further verified HPLC-UV/VIS method for the quantification of *Astaxanthin* in the *feed additive* and *feedingstuffs*. However, several NRLs recommended to avoid chloroform and to replace it by another suitable solvent.

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) 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 HPLC-UV/VIS method submitted by the Applicant for the quantification of *Astaxanthin* in *feed additive* and *feedingstuffs.*

Recommended text for the register entry (analytical method)

For the quantification of *Astaxanthin* in *feed additive* and *feedingstuffs*:

- High Performance Liquid Chromatography with UV/VIS detector (HPLC-UV/VIS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ajimoto Phaffia Astaxanthin* 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/G1: Forw. Appl. 1831/0035-2013
[2] *Application, Proposal for Registry Entry – Annex A
[3] *Technical dossier, Section II, 2.2.2. Relevant properties
[4] *Technical dossier, Section II, 2.1.3. Qualitative and quantitative composition (active substance/agent, other components, impurities, batch to batch variation)
[5] *Technical dossier, Section II, 2.5.1. Proposed mode of use in animal nutrition
[6] 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
[7] *Technical dossier, Section II- Annex_II_20
[8] *Technical dossier, Section II- Annex_II_21
[9] *Technical dossier, Section II- Annex_II_23
[10] *Technical dossier, Section II- Annex_II_24
[11] *Technical dossier, Section II- Annex_II_25
[12] *Technical dossier, Section II- Annex_II_26

*Refers to Dossier no: FAD-2013-0032

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Fødevarestyrelsen, Ringsted (DK)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Labore Landwirtschaft, Nossen (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)