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

Dossier related to:	FAD-2009-0054 CRL/090010
Name of Additive:	Astaxanthin (E161j)
Active Substance(s):	Astaxanthin
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
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EXECUTIVE SUMMARY

Astaxanthin is a *feed additive* for which authorisation is sought under the category "sensory additives", functional group 2(a) "colourants", sub-classification (ii) "substances which, when fed to animals, add colours to food of animal origin", (iii) "substances which favourably affect the colour of ornamental fish or birds" according to Annex I of Regulation (EC) No 1831/2003. In the current application submitted according to Article 4(1) (new use in water) and Article 10(2) (re-evaluation of additives already authorised under Directive 70/524/EC) of Regulation (EC) No 1831/2003, authorisation is requested for salmon and trout, ornamental fish and birds, crustaceans and other fish.

The active ingredient and the additive for registration is *Astaxanthin*, produced by a synthetic process and marketed in a stabilised form, e.g. with a spray-dried coating material (i.e. carbohydrates, protein).

The applicant proposed a maximum *Astaxanthin* concentration of 100 mg/kg *feedingstuffs* for salmon and trout and for minor species (crustaceans and other fish). However, no maximum content was proposed for pets (ornamental fish and birds). No minimum contents were proposed by the applicant.

The *Astaxanthin* concentration in *premixtures* and in *feedingstuffs* corresponds to the sum of geometrical *Astaxanthin* isomers detected, namely (1) all-E *Astaxanthin*, (2) 9Z *Astaxanthin*, (3) 13Z *Astaxanthin* and (4) other non-identified Z isomer(s). Here the E/Z-isomers notation is used instead of the terms trans/cis.

Furthermore, the presence of *Canthaxanthin* with *Astaxanthin* in completed *feedingstuffs* is allowed for salmon and trout with a maximum concentration of the sum of both substances of 100 mg/kg.

For the determination of the purity of the *crystalline Astaxanthin (feed additive)*, the applicant proposed a spectrophotometric method measuring at 486-487 nm, and a Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) using a visible detector with a wavelength measuring at 474 nm. The CRL considers the two methods submitted by the applicant suitable for intended purposes.

For the determination of *Astaxanthin* in the *premixtures* and *feedingstuffs*, the applicant proposed a ring-trial validated chromatographic method, based on Normal-Phase High-Performance Liquid Chromatography (NP-HPLC) using a visible detector at 470 nm. The following performance characteristics were reported:

For *premixtures* containing Astaxanthin at 4500 mg/kg:

- a relative standard deviation for *reproducibility* (RSD_R) of 10.2 %.

For *feedingstuffs*, in the concentration ranging of Astaxanthin from 20 to 80 mg/kg:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 1.5 to 3.2 %,
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 3.5 to 12.6 %
- a *recovery* rate (R_{Rec}) ranging from 98 to 102 %
- a limit of detection (LOD) and quantification (LOQ) of 0.005 and 0.01 mg/kg *feedingstuffs, respectively.*.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Astaxanthin* in *premixtures* and *feedingstuffs.*

Upon request of CRL, the applicant submitted experimental data proving that the ring-trial validated spectrophotometry method for the determination of *Astaxanthin* in the powdery or water dispersible formulations is also applicable for the determination of *Astaxanthin* in *water*. The target values were ranging from 30 to 100 mg/kg of *Astaxanthin* in drinking water. The following performance characteristics were reported: - RSD_r ranging from 0.45 to 1.10 %; - RSD_R ranging from 1.0 to 3.3 %; - R_{Rec} = 99.9 %.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Astaxanthin* in *water*.

For the determination of *Canthaxanthin* in *feedingstuffs,* the applicant submitted, upon request from the CRL, a single laboratory validated method, similar to the chromatographic method for *Astaxanthin* in *premixtures* and *feedingstuffs*, with slight modification of chromatographic conditions. The following performance characteristics for *feedingstuffs,* in the concentration ranging from 5 to 1000 mg/kg, were reported: - RSD_r ranging from 1 to 7 %; - R_{Rec} ranging from 101 to 103 %; and – LOD = 0.003 mg/kg.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Canthaxanthin* in *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) is not considered necessary.

KEYWORDS

Astaxanthin, sensory additive, colourant, salmon and trout, pets (ornamental fish and birds), minor species (crustaceans and other fish)

1. BACKGROUND

Astaxanthin is a *feed additive* for which authorisation is sought under the category "sensory additives", functional group 2(a) "colourants", sub-classification (ii) "substances which, when fed to animals, add colours to food of animal origin", (iii) "substances which favourably affect the colour of ornamental fish or birds" according to Annex I of Regulation (EC) No 1831/2003 [1].

In the current application submitted according to Article 4(1) (new use in water) and Article 10(2) (re-evaluation of additives already authorised under Directive 70/524/EC) of Regulation (EC) No 1831/2003, authorisation for salmon and trout, pets (ornamental fish and birds), minor species (crustaceans and other fish) is requested [2].

The active ingredient and the additive for registration is *Astaxanthin*, produced by a synthetic process [3].

When referring to the purity of the crystalline *Astaxanthin*, the applicant proposed the following technical specification [2]:

- a minimum *Astaxanthin* colour contribution of 96 %; and
- a maximum contribution from other carotenoids colouring agents of 5 %.

Crystalline Astaxanthin is only stable in absence of light and oxygen and is sensitive to heat; therefore, it is stabilized with a spray-dried coating material (i.e. carbohydrates, protein), before being incorporated in *premixtures* and *feedingstuffs* and placed on the market [3].

The *Astaxanthin* concentration in *premixtures* and in *feedingstuffs* corresponds to the sum of *Astaxanthin* isomers detected, namely (1) all-E *Astaxanthin*, (2) 9Z *Astaxanthin*, (3) 13Z *Astaxanthin* and (4) other non-identified Z isomer(s) [4]; the E/Z-isomers notation is used instead of the terms trans/cis.

The applicant proposed a maximum *Astaxanthin* concentration of 100 mg/kg *feedingstuffs*, for salmon and trout, and for minor species (crustaceans and other fish). However, no maximum content was proposed for pets (ornamental fish and birds) [2]. No minimum contents were proposed by the applicant [2].

Furthermore, the presence of *Canthaxanthin* with *Astaxanthin* in complete *feedingstuffs* is allowed for salmon and trout provided that the total concentration of the substances does not exceed 100 mg/kg *feedingstuffs* [2].

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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application or for each group of application. For this particular dossier, the methods of analysis submitted in connection with *Astaxanthin* were evaluated for their suitability for official controls.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and qualitative composition of impurities in the additive

Several impurities can be generated during the synthetic production process from various manufacturing company. When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins and solvents are available from the respective Community Reference Laboratories [5].

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

The applicant provided single-laboratory validated methods to monitor the purity specifications of the *crystalline Astaxanthin*.

For the determination of *Astaxanthin* in *crystalline Astaxanthin (feed additive)*, the applicant submitted a single-laboratory validated method based on VIS-spectrophotometry [6]. The sample is dissolved in dichloromethane and diluted. The content is determined at 486-487 nm with a relative standard deviation of *repeatability* (RSD_r) of 0.5 %. The validation report "VB 970197", referred in the standard operating procedure [6], was requested by the CRL, but not provided by the applicant.

For the determination of *Carotenoids other than Astaxanthin* in *crystalline Astaxanthin (feed additive)*, the applicant suggested a single-laboratory validated method based on Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) [7]. The peak area at 474 nm is normalised by the sum of areas of all detected peaks, resulting in RSD_r of 1.0 % [7]. The validation report "VD 080012", referred in the standard operating procedure [7], was requested by the CRL, but not provided by the applicant.

The CRL considers the two methods submitted by the applicant suitable for intended purposes.

For the determination of *Astaxanthin* in the powdery or water dispersible formulations, the applicant proposed a ring-trial validated spectrophotometry method by 9 laboratories [8]. The powdery or water dispersible formulations are digested in water with protease. After dilution with acetone the mass fraction of *Astaxanthin* is determined photometrically at the wavelength of 431 nm. The following performance characteristics determined in the concentration range from 8 to 20 g/100 g were reported [8]:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.45 to 1.10 %;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 1.0 to 3.3 %;
- a *recovery* rate (R_{Rec}) of 99.9 %.

Upon request of CRL, the applicant submitted experimental data [10] to prove that the ring-trial validated spectrophotometry method [8], requiring only minor modification in the sample preparation, is also applicable for the determination of Astaxanthin in water; therefore, we can assume to have the same performance characteristics. The target values were ranging from 30 to 100 mg/kg of *Astaxanthin* in drinking water.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Astaxanthin* in *water*.

For the determination of *Astaxanthin* in the *premixtures* and *feedingstuffs*, the applicant proposed a ring-trial validated chromatographic method (by 7 laboratories in 1993 and by 8 laboratories in 2007) based on Normal-Phase High-Performance Liquid Chromatography (NP-HPLC) using a visible detector at 470 nm [9]. The assay comprises an enzymatic (protease) digestion of the samples followed by extraction with ethanol and dichloromethane. Prior to the HPLC analysis the extract is purified by the means of silica gel chromatography. Two alternative procedures can be used, namely large and small scale procedure. In the large scale procedure the extract is purified and also concentrated (2.5-5 times). The NP-HPLC is able to resolve the all-E isomer and the main Z isomers of *Astaxanthin*. Furthermore, the

system is able to separate *Astaxanthin* from its oxidation products astacene and semiastacene as well as from carotenes and other xanthophylls present in feed (i.e. canthaxanthin, lutein and zeaxanthin) [9]. *Astaxanthin* is comprised of the sum of all-E isomer, 9Z *Astaxanthin*, 13Z *Astaxanthin* and other non-identified Z isomer(s). For the determination of Astaxanthin, the peaks corresponding to these isomers are identified via their specific retention times. Then, the measured peak areas of each isomer are corrected by using specific relative response factors, experimentally determined compared to the all-E isomer. Finally these corrected areas are summed up and the sum is used to calculate the total *Astaxanthin* content in the sample [9], by calibrating against the measured peak area obtained from an all-E Astaxanthin standard. The following method performance characteristics were obtained.

For *premixtures*, with a concentration of 4500 mg/kg:

- RSD_R = 10.2 %;

For *feedingstuffs*, for concentration ranging from 20 to 80 mg/kg:

- RSD_r ranging from 1.5 to 3.2 %;
- RSD_R ranging from 3.5 to 12.6 %;
- R_{Rec} ranging from 98 to 102 %;
- a limit of detection (LOD) and quantification (LOQ), according to the purification scale, are respectively in a range from 0.005 to 0.02 and from 0.01 to 0.05 mg/kg *feedingstuffs*.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Astaxanthin* in *premixtures* and *feedingstuffs.*

Furthermore, for salmon and trout the presence of *Canthaxanthin* with *Astaxanthin* in complete *feedingstuffs* is allowed provided that the total concentration of the substances does not exceed 100 mg/kg *feedingstuffs* [2]. The applicant submitted, upon request from the CRL, a single laboratory validated method for the determination of *Canthaxanthin* [11]. This method is similar to the NP-HPLC method for *Astaxanthin* in *premixtures* and *feedingstuffs*, with slight modification of chromatographic conditions (i.e. mobile phase and detection at 466 nm), to resolve the all-trans isomer and the main cis isomers of *Canthaxanthin*. The following performance characteristics for *feedingstuffs,* in the concentration ranging from 5 to 1000 mg/kg, were reported [11]:

- RSD_r ranging from 1 to 7 %;
- R_{Rec} ranging from 101 to 103 %; and,
- a LOD, according to the purification scale, is ranging from 0.003 to 0.1 mg/kg *feedingstuffs*.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Canthaxanthin* in *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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

The CRL considers single-laboratory validated methods, submitted by the applicant to monitor the purity specifications of the *crystalline Astaxanthin*, suitable for intended purposes.

The CRL recommends for official control - in the frame of this authorisation - the ring-trial validated spectrophotometry method submitted by the applicant for the determination of *Astaxanthin* in drinking *water*.

The CRL recommends for official control - in the frame of this authorisation - the ring-trial validated chromatographic methods submitted by the applicant for the determination of *Astaxanthin* in *premixtures* and *feedingstuffs*.

The CRL recommends for official control - in the frame of this authorisation - the single laboratory validated method submitted by the applicant for the determination of *Canthaxanthin* in *feedingstuffs*.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

For the determination of purity of the *crystalline Astaxanthin* (*feed additive*):

- a spectrophotometry method at 486-487 nm, and a Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) using a visible detector at 474 nm.

For the determination of *Astaxanthin* in *water*:

- a spectrophotometry method at the wavelength of 431 nm.

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

- a Normal-Phase High-Performance Liquid Chromatography (NP-HPLC) using a visible detector with a wavelength at 470 nm.

For the determination of *Canthaxanthin* in *feedingstuffs*:

- Normal-Phase High-Performance Liquid Chromatography (NP-HPLC) using a visible detector with a wavelength at 466 nm.

The *Astaxanthin* concentration in *premixtures* and in *feedingstuffs* corresponds to the sum of geometrical *Astaxanthin* isomers detected: all-E *Astaxanthin*, 9Z *Astaxanthin*, 13Z *Astaxanthin* and other non-identified Z isomer(s).

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Astaxanthin* have been sent to the Community Reference Laboratory for Feed Additives.

The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Reference SANCO/D/2 Forw. Appl. 1831/0044-2009
[2] *Annex A: Proposal for Registry Entry
[3] *Section II, chapter 2.3. - Manufacturing process
[4] *Supplementary information: "clarification Astaxanthin mail 15-7-2010.pdf"
[5] 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
[6] *Annex Section II - 2.6.1.a. "Photometrische Gehaltsbestimmung von Astaxanthin krist"
[7] *Annex Section II - 2.6.1.b. "Bestimmung der Flächenprozentverteilung von Astaxanthin und Nebenfarbstoffen mittels Reversed-Phase HPLC"
[8] *Annex Section II - 2.6.1.c. "Determination of Astaxanthin tel quel by Photometry at the Isobestic Wavelength of 431 nm"
[9] *Annex Section II - 2.6.1.d. "Determination of Stabilised Astaxanthin in Premixes and Feedstuffs"
[10] *Supplementary information. "BASF astaxanthin in drinking water 2010.pdf"
[11] *Supplementary information. "Determination of Stabilised Canthaxanthin in Premixes and Feedstuffs"

* Refers to Dossier No. FAD-2009-0054

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community 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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- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer (DE)
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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (I)
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