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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 (ATTCC SD 5340)

(FAD-2010-0060; CRL/100042)

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-2010-0060 - CRL/100042**
Name of Feed Additive:	***Astaxanthin-rich Phaffia rhodozyma (ATCC SD 5340)***
Active Agent (s):	**Astaxanthin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini & Ursula Vincent** **09/11/2018**
Report approved by: Date:	**Christoph von Holst** **12/11/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 10(2) of Regulation (EC) No 1831/2003 for *Astaxanthin-rich Phaffia rhodozyma.* The authorisation as *feed additive* is sought under the 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 10(2) of Regulation (EC) No 1831/2003, the authorisation for salmon and trout is requested.

The active substance in the *feed additive* is *astaxanthin*, produced by the red yeast *Phaffia rhodozyma (ATCC SD 5340).* The *feed additive* is a dark purple powder containing as active substance at least 5000 mg/kg of total *astaxanthin,* i.e. the sum of the all-E, 9Z-, 13Z and 15Z- isomers. The *feed additive* is intended to be incorporated directly into *feedingstuffs* with a proposed maximum content (expressed as total *astaxanthin*) of 100 mg/kg complete *feedingstuffs*.

For the quantification of *astaxanthin* in the *feed additive* and in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on Normal Phase High Performance Liquid Chromatography with UV/VIS detection (NP-HPLC-UV/VIS).

In addition, the EURL has developed and single-laboratory validated a multi-analyte method based on reversed phase HPLC coupled to spectrophotometric detection (RP-HPLC-UV/VIS) for the determination of all carotenoids currently authorised as *feed additive* within the EU, including thus the one of the current application. This method has also been subjected to ring-trial validation.

Based on the performance characteristics presented, the EURL recommends for official control the validated and further verified NP-HPLC-UV/VIS method for the quantification of *astaxanthin* in the *feed additive* and in *feedingstuffs* and the ring-trial validated RP-HPLC-UV/VIS for the quantification of *astaxanthin* 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Astaxanthin , sensory, colourants, *feed additives*, salmon, trout

1. BACKGROUND

Astaxanthin-rich Phaffia rhodozyma (ATCC SD 5340) is a *feed additive* currently authorised by Commission Regulation (EC) No 828/2007 for salmon and trout [1] belonging to the "colourants, including pigments" group listed in Directive 70/524/EEC. In the current application 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 [2]. In the current application submitted according to Article 10(2) of Regulation (EC) No 1831/2003, the authorisation for salmon and trout is requested [2,3].

The active substance in the *feed additive* is *astaxanthin*, produced by the red yeast *Phaffia rhodozyma (ATCC SD 5340).* The *feed additive* is a dark purple powder consisting of dried, inactivated *astaxanthin* rich yeast *Phaffia rhodozyma* containing as active substance at least 5000 mg/kg of total *astaxanthin* i.e. the sum of the all-E, 9Z-, 13Z and 15Z- isomers and as antioxidant ascorbic acid at a level of 0.5 % w/w [3-5].

The *feed additive* is intended to be incorporated directly into *feedingstuffs* with a proposed maximum content (expressed as total *astaxanthin*) of 100 mg/kg complete *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 *Astaxanthin-rich Phaffia rhodozyma (ATCC SD 5340)* 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 quantification of *astaxanthin* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on normal phase High Performance Liquid Chromatography with UV/VIS detection (NP-HPLC-UV/VIS) [5].

The proposed method [5] involves a multi-extraction procedure using mixtures of organic solvents. The first step is wetting the sample with deionised water; then extraction of the sample with a mixture of butylated hydroxytoluene (BHT) and warm dimethylsulfoxide (DMSO) follows. Afterwards, sodium sulfate, methylene chloride and water are consecutively added to the extract and the mixture is then further vortex mixed and centrifuged to separate layers. The upper water layer is removed and the organic bottom layer is washed three more times with deionised water, followed by the removal of the upper water layer. The organic layer is then dried under a nitrogen stream, re-suspended in the mobile phase, mixed, filtered through a 0.45 µm syringe filter and injected into the HPLC system. The HPLC analysis is performed on a silica column using a mixture of n-hexane and acetone as mobile phase and the absorbance is measured at a wavelength of 475 nm. The *feed additive* is quantified against an *astaxanthin* standard.

The NP-HPLC-UV/VIS method was single-laboratory validated and further verified for the *feed additive* [6].

Upon request of the EURL the Applicant provided a slightly modified protocol for the application of the method described above to *feedingstuffs* [7] as well as additional results obtained by an external laboratory for establishing the performance characteristics of the proposed method for the determination of *astaxanthin* in *feedingstuffs* [8]. Furthermore, in the frame of the homogeneity study the Applicant applied this method to *feedingstuffs* obtaining similar precision values (relative standard deviation for repeatability - RSD_r) [9].

Table 1. Performance characteristics of analytical methods for the determination of *astaxanthin* in the *feed additive* (FA), and fish *feedingstuffs* (FS) reported in the frame of validation (Val) and verification (Ver) and the candidate CEN standard method (c-EN)

Matrix	Method	Mass fraction (mg/kg)	Ref.	RSD_r, %	RSD_{ip}, %	R_{Rec} (%)	LOD (mg/kg)	LOQ (mg/kg)
FA	NP-HPLC-UV/VIS	8292-13295	Val	0.6-1.9[(a)]	0.8-2.5[(a)]	98-101	-	-
			Ver	2.2-6.1[(a)]	2.2-7.0[(a)]	94-101	0.01	0.1
FS	NP-HPLC-UV/VIS	16.3	Val	3.0[(a)]	6.7[(a)]	92	0.01	0.04
	RP-HPLC-UV/VIS	34-200	Val	1.0-12.8	4.5-13	90-98	4.5	15
		26-100	c-EN	4.5-6.9	21-30	76-87	-	-
					RSD_R, %			

RSD_r, RSD_{ip} & RSD_R: relative standard deviation for *repeatability, intermediate precision & reproducibility,* respectively; R_{Rec}: *recovery* rate (%); [a] Recalculated by EURL

In addition, the EURL has developed and single-laboratory validated a multi-analyte method based on reversed phase HPLC coupled with a UV/VIS detector (RP-HPLC-UV/VIS) for the determination of all carotenoids currently authorised as *feed additives* within the EU, including thus the one of the current application [14].

Table 1 presents the performance characteristics for *astaxanthin* [10-12] based on experimental data obtained in the frame of the validation and verification studies using the NP-HPLC-UV/VIS method [10-12] and the RP-HPLC-UV/VIS method [14].

This multi-analyte method involves four steps namely i) enzymatic digestion; ii) pressurised liquid extraction or conventional liquid/solid extraction, iii) centrifugation and iv) quantification of the analytes by reversed-phase HPLC-UV. The HPLC analysis is performed on a C18 column using gradient elution. The measured signal is quantified against an external calibration curve by applying the isosbestic concept, i.e. selection of specific wavelengths at which the absorbance coefficients are identical for all geometrical isomers of a specific analyte [13, 14].

This method has also been ring-trial validated recently for different *feed materials*, i.e. *feedingstuffs* and *premixtures* containing different carotenoids combinations, and is currently under evaluation to become a CEN standard (c-EN).

Based on the documentation available, the EURL recommends for official control the validated and further verified NP-HPLC-UV/VIS method for the quantification of *astaxanthin* in the *feed additive* and in *feedingstuffs* and the ring-trial validated RP-HPLC-UV/VIS for the quantification of *astaxanthin* 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.

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)

An 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 the single laboratory validated and further verified NP-HPLC-UV/VIS method submitted by the Applicant for the quantification of *astaxanthin* in the *feed additive* and *feedingstuffs* and the ring-trial validated RP-HPLC-UV/VIS for the quantification of *astaxanthin* in *feedingstuffs.*

Recommended text for the register entry (analytical method)

For the quantification of *astaxanthin* in the *feed additive*

- Normal Phase High Performance Liquid Chromatography with UV/VIS detection (NP-HPLC-UV/VIS)

For the quantification of *astaxanthin* in *feedingstuffs*

- Normal Phase High Performance Liquid Chromatography with UV/VIS detection (NP-HPLC-UV/VIS) or
- Reversed Phase High Performance Liquid Chromatography with UV/VIS detection (RP-HPLC-UV/VIS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Astaxanthin-rich Phaffia rhodozyma (ATCC SD 5340)* 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] Commission Regulation (EC) No 828/2007 of 13 July 2007 concerning the permanent and provisional authorisation of certain additives in feedingstuffs
[2] *Application, Reference SANCO/G1: Forw. Appl. 1831/0033-2015 (FAD 2010-0060)
[3] *Application, Proposal for Registry Entry – Annex A
[4] *Technical dossier, Section II, 2. Characterisation of the active substance(s)/agent(s)
[5] *Technical dossier, Section II- Annex_II_6.01
[6] * Supplementary information - Annex_II_6.02_part1 & Annex_II_6.02_part2
[7] *Supplementary information, Annex_II_6_18 - HPLC AN-130-7 (Feeds)
[8] *Supplementary information, 17-IGE-001 Report QC
[9] *Technical dossier, Section II, 4. Physico-chemical and technological properties of the additives
[10] *Supplementary Information, eurl-anova-calculation-fa-val.pdf

[11] *Supplementary Information, eurl-anova-calculation-fa-ver.pdf

[12] *Supplementary Information, eurl-anova-calculation-fs-val.pdf

[13] Mitrowska K, Vincent U. and von Holst C. Separation and quantification of 15 carotenoids by reversed phase high performance liquid chromatography coupled to diode array detection with isosbestic wavelength approach. J. Chromatogr A. (2012) Vol. 1233, 44-53

[14] Vincent U., Serano F. and von Holst C. Development and validation of a multi-analyte method for the regulatory control of carotenoids used as feed additives in fish and poultry feed. Food Additives & Contaminants: Part A, (2017) Vol. 34, 8, 1285 – 1297

*Refers to Dossier no: FAD-2010-0060

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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