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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

Red carotenoid rich *Paracoccus carotinifaciens* (2a(ii)167)

(FAD-2017-0048; CRL/170039)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2017-0048 - CRL/170039**
Name of Feed Additive:	**Red carotenoid rich *Paracoccus carotinifaciens* (2a(ii)167)**
Active Agent (s):	**Astaxanthin, canthaxanthin and adonirubin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis 11/07/2018**
Report approved by: Date:	**Christoph von Holst 12/07/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 14 for the *feed additive* (Red carotenoid rich *Paracoccus carotinifaciens*) under the category/functional group/subgroup '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. The *feed additive* is currently authorised for salmon and trout by Commission Regulation (EC) No 721/2008, further amended by Commission Regulation (EU) No 334/2010.

The *feed additive* marketed as *Panaferd AX* is a granular powder composed of dried cells of astaxanthin-rich *Paracoccus carotinifaciens*, containing 20 to 23 g of *astaxanthin*, 7 to 15 g of *adonirubin* and 1 to 5 g of *canthaxanthin* (as active substances) per kilogram of the *feed additive*. The *feed additive* is intended to be incorporated directly into *feedingstuffs* with a proposed total maximum content (expressed as the sum of *astaxanthin*, *adonirubin* and *canthaxanthin*) of 100 mg/kg complete *feedingstuffs*.

Furthermore, the Applicant proposed Maximum Residue Limits (MRLs) for the sum of *adonirubin* and *canthaxanthin* in muscle *tissues* of 5 mg/kg for salmon and 8 mg/kg for trout.

For the quantification of *astaxanthin*, *adonirubin* and *canthaxanthin* in the *feed additive* and *feedingstuffs*, and for the quantification of the sum of *adonirubin* and *canthaxanthin* in salmon and trout muscle *tissues* the Applicant submitted a ring-trial validated method based on normal phase high performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV/VIS). Based on acceptable performance characteristics available the EURL recommends for official control this HPLC-UV/VIS method for the quantification of *astaxanthin*, *adonirubin* and *canthaxanthin* in the *feed additive* and *feedingstuffs*; and the above mentioned HPLC-UV/VIS method or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC, to enforce the proposed MRLs in the relevant *tissues*.

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

Red carotenoid rich *Paracoccus carotinifaciens*, *2 a (ii)167*, *Panaferd AX*, *astaxanthin*, *adonirubin*, *cantaxanthin*, sensory additives, colourants, salmon, trout

1. BACKGROUND

In the current application authorisation is sought under Article 14 (renewal of existing authorisation) for the *feed additive* (Red carotenoid rich *Paracoccus carotinifaciens*) under the category/functional group/subgroup '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][2]. The *feed additive* is currently authorised for salmon and trout by Commission Regulation (EC) No 721/2008, further amended by Commission Regulation (EU) No 334/2010.

The *feed additive* marketed as *Panaferd AX* is a granular powder composed of dried cells of astaxanthin-rich *Paracoccus carotinifaciens*, containing 20 to 23 g of *astaxanthin*, 7 to 15 g of *adonirubin* and 1 to 5 g of *canthaxanthin* (as the active substances) per kilogram of the *feed additive* [3][4]. The *feed additive* is intended to be incorporated directly into *feedingstuffs* with a proposed total maximum content (expressed as the sum of *astaxanthin*, *adonirubin* and *canthaxanthin*) of 100 mg/kg complete *feedingstuffs* [3][4].

Furthermore, the Applicant proposed a reduction of the Maximum Residue Limits (MRLs) already established by Commission Regulation (EU) No 721/2008 for the sum of *adonirubin* and *canthaxanthin* in salmon muscle from 10 to 5 mg/kg. Therefore, in the frame of this application the Applicant suggested MRLs for the sum of *adonirubin* and *canthaxanthin* in muscle *tissues* of 5 mg/kg for salmon and 8 mg/kg for trout [3].

Note: The EURL previously evaluated and recommended the analytical methods for the quantification of *astaxanthin*, *adonirubin* and/or *canthaxanthin* in the frame of several dossiers [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 Red carotenoid rich *Paracoccus carotinifaciens 2 a (ii)167* 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*, *adonirubin* and *canthaxanthin* in the *feed additive* and *feedingstuffs* the Applicant submitted a ring-trial validated method based on normal phase high performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV/VIS) [6]. The method allows the quantification of eight carotenoids, namely *beta-carotene*, *echinenone*, *3-hydroxyechinenone*, *canthaxanthin*, *adonirubin*, *astaxanthin*, *asteroidenone* and *adonixanthin*.

The proposed method [6] 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 tetrahydrofuran (THF) and methanol is followed. Afterwards, n-hexane is added to the extract, further mixed and centrifuged. An aliquot of the supernatant containing the target analytes is transferred to a volumetric flask. This procedure is repeated twice, and both aliquots of the obtained supernatants are combined, further diluted with the mobile phase, and injected into the HPLC system. The HPLC analysis is performed on two serially connected silica columns using a mixture of n-hexane, THF and methanol as mobile phase and measured at a wavelength of 470 nm. The target carotenoids are quantified against an *astaxanthin* standard using specific conversion factors for *canthaxanthin* and *adonirubin*, respectively.

Table 1 Performance characteristics of the analytical method for the quantification of *astaxanthin, adonirubin* and *canthaxanthin* in the *feed additive* (FA) and *feedingstuffs* (FS) reported in the frame of the ring-trial validation [11]

Matrix	Carotenoid	Mean content (mg/kg)	*RSD_r %	*RSD_R %
FA [15]	Astaxanthin	21700	0.6	4.0
	Canthaxanthin	2300	1.7	2.8
	Adonirubin	8900	1.3	3.9
FS [16]	Astaxanthin	24.0 - 50.5	0.9 - 1.8	7.8 - 9.4
	Canthaxanthin	3.1 - 5.6	1.7 - 2.6	5.6 - 8.4
	Adonirubin	10 - 19.3	0.9 - 2.1	7.2 - 8.1

RSD_r & RSD_R: relative standard deviation for *repeatability* & *reproducibility* ; * recalculated by the EURL

The HPLC-UV/VIS method was single-laboratory validated [7], verified [8][9][10] and ring-trial validated [11] for 4 different materials, i.e. one *feed additive* and three *feedingstuffs* materials. Table 1 presents the performance characteristics recalculated by the EURL [15][16] based on experimental data obtained by the Applicant in the frame of the ring-trial validation studies [11]. Similar performance characteristics were obtained by the Applicant in the frame of the single-laboratory validation and verification studies for the quantification of the analytes in the *feed additive* and *feedingstuffs*.

Based on the performance characteristics presented the EURL recommends for official control the normal phase HPLC-UV/VIS method submitted by the Applicant for the quantification of *astaxanthin*, *adonirubin* and *canthaxanthin* in the *feed additive* and *feedingstuffs*.

In addition, the EURL has developed a multi-analyte method based on reversed phase HPLC coupled to spectrophotometric detection for the determination of all carotenoids currently authorised as feed additives within the EU, including thus the *feed additive* of the current application. The method is currently under evaluation to become a CEN standard [12].

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)

For the quantification of the sum of *adonirubin* and *canthaxanthin* in salmon and trout muscles the Applicant proposed the same normal phase HPLC-UV/VIS method as described above [6].

The method was single-laboratory validated [7], verified [8][9][10] and ring-trial validated with four laboratories [11] for the quantification of *adonirubin* and *canthaxanthin* in *tissues* (salmon muscle). Table 2 summarises the performance characteristics recalculated by the EURL [17] based on experimental data obtained by the Applicant in the frame of the ring-trial validation studies [11] for the target carotenoids.

Table 2 Performance characteristics of the analytical method for the quantification of *adonirubin* and *canthaxanthin* in *tissues* (salmon muscle) reported in the frame of the ring-trial validation [11]

Matrix	Carotenoid	Mean content (mg/kg)	*RSD_r, %	*RSD_R, %
Salmon muscle [17]	Canthaxanthin	0.4	2.7	8.9
	Adonirubin	2.1	2.3	9.4

RSD_r & RSD_R: relative standard deviation for *repeatability* & *reproducibility*; * recalculated by the EURL

Even if outside the scope of this application the data provided by Applicant in the frame of the ring-trial validation indicated the suitability of the proposed method for the quantification of *astaxanthin* in fish muscle at a content of 6.0 mg/kg.

In order to comply with the identification requirements of Commission Decision 2002/657/EC [13] and upon EURL request, the Applicant carried out the analyses of a relevant set of samples, i.e. carotenoid standards, blank fish tissue and fish tissue spiked with the target carotenoids using two different chromatographic systems [14].

Based on the performance characteristics available the EURL recommends for official control the ring-trial validated method based on HPLC-UV/VIS method or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC to enforce the proposed MRLs for the sum of *adonirubin* and *canthaxanthin* in salmon and trout *tissues*.

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

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 ring-trial validated normal phase high performance liquid chromatography coupled to UV/VIS detection (HPLC-UV/VIS) for the quantification of *astaxanthin, adonirubin* and *canthaxanthin* in the *feed additive* and *feedingstuffs*; and the above mentioned HPLC-UV/VIS method or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs for the sum of *adonirubin* and *canthaxanthin* in the relevant *tissues*.

Recommended text for the register entry (analytical method)

For the quantification of *astaxanthin, adonirubin* and *canthaxanthin* in the *feed additive* and *feedingstuffs*:

- normal phase high performance liquid chromatography coupled to UV/VIS detection (HPLC-UV/VIS)

For the quantification of the sum of *adonirubin* and *canthaxanthin* in salmon and trout *tissues*:

- normal phase high performance liquid chromatography coupled to UV/VIS detection (HPLC-UV/VIS) or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

The dossier related to Red carotenoid rich *Paracoccus carotinifaciens, 2 a (ii)167* has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANTE_E5_FWD. APPL. 1831-0040-2017

[2] *Application, Reference Annex 1 (Submission No. 1502439309182-2136)

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

[4] *Technical dossier, Section II: Identity, characterisation and conditions of use of the feed additive; methods of analysis

[5] EURL Evaluation Reports FAD 2006-0021, FAD 2013-0032 and FAD 2008-0048
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2006-0021.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/revisedfinrep-fad-2013-0032-phaffia.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2008-0048.pdf

[6] *Technical dossier, Section II: Annex II.20

[7] *Technical dossier, Section II: Annex II.23

[8] *Technical dossier, Section II: Annex II.24

[9] *Technical dossier, Section II: Annex II.25

[10] *Technical dossier, Section II: Annex II.26

[11] *Technical dossier, Section II: Annex II.22

[12] 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

[13] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

[14] *Technical dossier, Supplementary information, Request for supplementary information - FAD-2017-0048_SIn_EURL_010218.pdf

[15] *Technical dossier, Supplementary Information, eurl-anova-calculation-panaferd-fa.pdf

[16] *Technical dossier, Supplementary Information, eurl-anova-calculation-panaferd-fs.pdf

[17] *Technical dossier, Supplementary Information, eurl-anova-calculation-panaferd-tissue.pdf

*Refers to Dossier no: FAD-2017-0048

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)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
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