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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 dimethyldisuccinate
(FAD-2017-0029; CRL/170025)

**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-2017-0029 - CRL/170025**

Name of Feed Additive: **Astaxanthin dimethyldisuccinate**

Active Agent (s): **Astaxanthin dimethyldisuccinate**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

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Date: **12/01/2018**

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Date: **15/02/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1), 13 and 14 for *astaxanthin dimethyldisuccinate* under the category/ functional group (2 a) "sensory additives"/"colourants", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the *feed additive* is sought to be used for salmon, trout, crustaceans and other fish.

The *feed additive*, which is already authorised by Commission Regulation (EC) No 393/2008, is to be marketed as Carophyll® Stay-Pink 10%-CWS. This is a brown to violet-red free flowing powder formulation, consisting of a minimum of 13.8 % *astaxanthin dimethyldisuccinate* (equivalent to a minimum of 10 % astaxanthin) and formulated in an organic matrix. In addition, maximum limits for triphenylphosphine oxide (100 mg/kg) and dichloromethane (600 mg/kg) are specified for the *feed additive*.

The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* with a proposed maximum *astaxanthin dimethyldisuccinate* content of 138 mg/kg *feedingstuffs*. If the *feed additive* is mixed with other sources of astaxanthin, the total maximum dose of astaxanthin equivalent is set to 100 mg/kg *feedingstuffs*.

For the quantification of the total *astaxanthin dimethyldisuccinate* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on spectrophotometry at 486 nm wavelength. The following performance characteristics were reported: a *precision* (relative standard deviations for *repeatability* – RSD_r and *intermediate precision* – RSD_{ip}) ranging from 0.3 to 1.2 %; and a *recovery* rate (R_{rec}) ranging from 98 to 103 %. Based on the acceptable performance characteristics available, the EURL recommends this method for official control.

For the quantification of the total *astaxanthin dimethyldisuccinate* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified method based on normal phase high performance liquid chromatography coupled with UV/VIS detection (HPLC-UV/VIS). The following performance characteristics were reported: a *precision* (RSD_r and RSD_{ip}) ranging from 0.7 to 3.9 %; R_{rec} ranging from 98 to 105 %; and a limit of quantification (LOQ) ranging from 0.2 to 2 mg *astaxanthin dimethyldisuccinate* (expressed as astaxanthin equivalent) /kg *feedingstuffs*. Based on the acceptable performance characteristics presented, the EURL recommends this method for official control.

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 dimethylsuccinate, *astaxanthin*, sensory additives, colourants, salmon, trout, crustaceans and other fish

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use of an already authorised *feed additive*), article 13 (modification of the existing authorisation) and article 14 (renewal of existing authorisation) for *astaxanthin dimethylsuccinate* under the category / functional group (2 a) "sensory additives"/"colourants", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the *feed additive* is sought to be used for salmon, trout, crustaceans and other fish [1].

The *feed additive*, which is already authorised by Commission Regulation (EC) No 393/2008, is to be marketed as Carophyll® Stay-Pink 10%-CWS. It is a brown to violet-red free flowing powder formulation, consisting of a minimum of 13.8 % *astaxanthin dimethylsuccinate* (equivalent to minimum of 10 % astaxanthin) and formulated in an organic matrix [2, 3]. In addition, maximum limits for triphenylphosphine oxide (100 mg/kg) and dichloromethane (600 mg/kg) are specified for the *feed additive* [2].

The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* with a proposed maximum *astaxanthin dimethylsuccinate* content of 138 mg (equivalent to 100 mg astaxanthin) /kg *feedingstuffs* [2, 3]. If the *feed additive* is mixed with other sources of astaxanthin, the total maximum dose of astaxanthin equivalent is set to 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 (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 dimethylsuccinate* 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 [4].

The Applicant suggested using gas chromatography coupled to flame photometric detection (GC-FPD) [5] and head space gas chromatography coupled to flame ionisation detection (GC-FID) [6] for the quantification of triphenylphosphine oxide and dichloromethane in the *feed additive*, respectively. The EURL considers these two methods fit-for-purpose for the quantification of the two impurities of interest in the *feed additive*.

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

For the quantification of the total *astaxanthin dimethyldisuccinate* (sum of *all-E*-isomer and other geometrical isomers of astaxanthin dimethyldisuccinate) in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on spectrophotometry at 486 nm [7].

The sample (90 to 110 mg) is treated by sonication with water at 60 °C and subsequently with ethanol and chloroform at room temperature. The final volume is adjusted with chloroform, shaken and centrifuged. The aliquot of the supernatant is evaporated to dryness and the residue is dissolved in a mixture of ethanol and chloroform for further analysis by spectrophotometry at 486 nm [7]. The quantification of total *astaxanthin dimethyldisuccinate* (expressed as astaxanthin equivalent) is performed using the specific absorbance value ($E_{1\%, 1\text{cm}} = 1830$) of an isomerised mixture of astaxanthin in chloroform at 486 nm. If the concentration of total *astaxanthin dimethyldisuccinate* should be reported as such, then the astaxanthin equivalent resulting from the quantification is multiplied by a factor of 1.384 [7].

Two formulations of the *feed additive* (Carophyl[®] Stay Pink, see FAD-2006-0032 [8] and Carophyll[®] Stay-Pink 10%-CWS, current dossier) were used in the frame of the validation and verification studies [9,10]. The reported performance characteristics are presented in Table 1.

Based on the acceptable performance characteristics available, the EURL recommends for official control this spectrophotometric method for the quantification of total *astaxanthin dimethyldisuccinate* in the *feed additive*.

Table 1 Performance characteristics of the validated and verified spectrophotometry method for the quantification of total *astaxanthin dimethylsuccinate* (expressed as astaxanthin equivalent) in the *feed additive*

	Feed additive	
	Validation	Verification
Mass fraction, g/kg	79 – 108	108
RSD _r , %	0.3 – 1.2	0.5 – 0.7
RSD _{ip} , %	1.2	0.9
R _{rec} , %	98 ^(*)	103
Reference	[9, 10]	[10]

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery rate*;
 (*) blank feed additive samples spiked with *astaxanthin dimethylsuccinate* at concentration ranging from 5 to 15% (w/w).

For the quantification of the total *astaxanthin dimethylsuccinate* (sum of *all-E*-isomer and other geometrical isomers of *astaxanthin dimethylsuccinate*) in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified method based on normal phase high performance liquid chromatography coupled with UV/VIS detection (HPLC-UV/VIS) [11, 12].

The sample (0.1 to 10 g) is mixed with an appropriate amount of bacterial alkaline protease and placed in an ultrasonic bath at 50 °C for 30 min. The enzymatic reaction is stopped by the addition of ethanol and cooled down to ambient temperature. For the *feed additive* or *premixtures* samples, the mixture is adjusted with dichloromethane, shaken and allowed to rest for 2 h. The aliquot of the supernatant is then diluted with a mixture of n-hexane:acetone. For *feedingstuffs* samples, the aliquot of the dichloromethane sample extract is cleaned-up by solid phase extraction (containing silica). All extracted samples are centrifuged and aliquots are analysed by HPLC-UV/VIS at 470 nm [11, 12]. The quantification of the total *astaxanthin dimethylsuccinate* is performed spectrophotometrically by external matrix-free calibration using the standard solutions [11, 12].

In the previous dossier (FAD-2006-0032 [8]) the Applicant submitted this method together with a method validation study. In the current dossier, the Applicant provided in addition the verification data [13-15]. Two formulations of the *feed additive* were used (Carophyll® Stay Pink [8] and Carophyll® Stay-Pink 10%-CWS, current dossier) as such, or included in *premixtures* and *feedingstuffs*.

Table 2 The performance characteristics of the single laboratory validated (Val) and verified (Ver) normal phase HPLC-UV/VIS method for the quantification of total *astaxanthin dimethyldisuccinate* (expressed as astaxanthin equivalent) in the *feed additive, premixtures* and *feedingstuffs*

	Feed additive		Premixtures		Feedingstuffs	
	Val.	Ver.	Val.	Ver.	Val.	Ver.
Mass fraction, mg/kg	111000		1074 – 1445	1074	36 – 41	41
RSD _r , %	0.7	0.9 – 1.4	2.6 – 2.8	1.4 – 3.3	2.0 – 3.8	3.0 – 3.9
RSD _{ip} , %	0.7	1.4	2.8	3.3	3.8	3.9
R _{rec} , %	-	99	99 ^(*)	102	98 ^(*)	105
Reference	[11, 13]	[13]	[11, 14]	[14]	[11, 15]	[15]

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec} - a *recovery rate*; (*) recoveries are based on blank premixture or blank feed samples spiked with astaxanthin dimethyldisuccinate.

The performance characteristics reported for the quantification of total *astaxanthin dimethyldisuccinate* in the *feed additive, premixtures* and *feedingstuffs* are shown in Table 2. Furthermore, a limit of quantification (LOQ) ranging from 0.2 to 2 mg/kg *feedingstuffs* was reported [11, 15].

Based on the acceptable performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified normal phase HPLC-UV/VIS method for the quantification of total *astaxanthin dimethyldisuccinate* in the *feed additive, premixtures* and *feedingstuffs*.

While this HPLC-UV/VIS method allows determining other sources of astaxanthin (e.g. canthaxanthin, adonirubin, astacene, lutein and zeaxanthin) in *feedingstuffs*, the Applicant did not provide any performance characteristics for the determination of these substances with this method. However, similar methods of analysis for the determination of astaxanthin, canthaxanthin and adonirubin in *feedingstuffs* were previously evaluated by the EURL and are currently recommended in the Commission Implementing Regulations: (EC) 721/2008, (EU) 2015/1415, and (EU) 2015/1486. In addition, the EURL has developed a multi-analyte method [16] for the determination of all carotenoids currently authorised as feed additive within the EU including the feed additive of this application and this method is currently under evaluation to become a CEN standard.

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 two single-laboratory validated and further verified methods based on (i) spectrophotometry at 486 nm for the quantification of total *astaxanthin dimethylsuccinate* in the *feed additive*; and (ii) normal phase HPLC-UV/VIS for the quantification of total *astaxanthin dimethylsuccinate* in the *feed additive, premixtures* and *feedingstuffs*.

Furthermore, the HPLC-UV/VIS method – allowing the determination of other sources of astaxanthin (e.g. canthaxanthin, adonirubin, astacene, lutein and zeaxanthin) in *feedingstuffs* – is already recommended in the Commission Implementing Regulations (EC) 721/2008, (EU) 2015/1415, and (EU) 2015/1486.

Recommended text for the register entry (analytical method)

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

- spectrophotometry at 486 nm

For the quantification of *astaxanthin dimethylsuccinate* in the *feed additive, premixtures* and *feedingstuffs*:

- normal phase high performance liquid chromatography coupled to UV/VIS detection (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 dimethylsuccinate* 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. 1831-0024-2017
- [2] *Application, Proposal for Register Entry – Annex A
- [3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the feed additive; methods of analysis
- [4] 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
- [5] *Technical dossier, Section II – Annex II_33
- [6] *Technical dossier, Section II – Annex II_34

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- [7] *Technical dossier, Section II – Annex II_24
- [8] #FAD-2006-0032 – D08/FSQ/CVH/GS/D(2007)18791
- [9] *Technical dossier, Section II – Annex II_21
- [10] *Technical dossier, Section II – Annex II_23
- [11] *Technical dossier, Section II – Annex II_16
- [12] *Technical dossier, Section II – Annex II_17
- [13] *Technical dossier, Section II – Annex II_18
- [14] *Technical dossier, Section II – Annex II_19
- [15] *Technical dossier, Section II – Annex II_20
- [16] 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-2017-0029

#<https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports>

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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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- RIKILT Wageningen UR, Wageningen (NL)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Elintarviketurvallisuusvirasto / Livsmedelssäkerhetsverket (Evira), Helsinki / Helsingfors (FI)