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

Dossier related to:	FAD-2008-0048 CRL/080025
Feed Additive Name:	Canthaxanthin
Active Substance(s):	Canthaxanthin
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
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EXECUTIVE SUMMARY

In the current application, authorisation is sought under articles 4(1) and 10(2) for *Canthaxanthin*, under the category/functional group 2(a) "sensory additives"/"colourants" according to the classification system of Annex I of Regulation (EC) No 1831/2003. According to the Applicant, the *active substance* in the formulation is the 10 % of the complete preparation (with a level of at least 96 % of colouring matters expressed as sum of all trans/cis isomers of *Canthaxanthin* and a maximum of 5 % of carotenoids other than *Canthaxanthin*). Specifically, authorisation is sought for the use of the *feed additive* for chickens for fattening, chickens reared for laying, laying hens, other poultry, salmon, trout, other fish, pets and other non-food producing animals. The Applicant proposed the following maximum levels of the *feed additive* in the *feedingstuffs*:

- 8 mg/kg for chickens reared for laying and laying hens;
- 25 mg/kg for chickens for fattening, other poultry, salmon, trout and other fish.

Furthermore, the Applicant proposed the following Maximum Residue Limits (MRLs) for *Canthaxanthin* (sum all trans/cis isomers): 2.5 mg/kg for *poultry fat* and *skin*; 5 mg/kg for *trout flesh*; 10 mg/kg for *salmon flesh*; 15 mg/kg for *poultry liver*; and 30 mg/kg for *egg yolk*.

For the determination of the *active substance, Canthaxanthin,* in the *feed additive,* the Applicant submitted a ring trial validated method based on spectrophotometry. The following performance characteristics were reported:

- a standard deviation for *repeatability* (RSD_r) ranging from 0.2 to 0.8%;
- a standard deviation for *reproducibility* (RSD_R) ranging from 1.3 to 4.0%, and
- a *recovery* rate (R_{Rec}) ranging from 96.2 to 105%.

Based on the performance characteristics presented the EURL recommends for official control the ring trial validated spectrophotometric method, submitted by the Applicant, to determine *Canthaxanthin,* in the *feed additive*.

For the determination of *Canthaxanthin* in *premixtures* and *feedingstuffs* the Applicant submitted a single laboratory validated and further verified method based on Normal Phase High-Performance Liquid Chromatography coupled to VIS detection (NP-HPLC-VIS). The following performance characteristics were reported:

- RSD_r ranging from 1.4 to 15%;
- a standard deviation for *intermediate precision* (RSD_{ip}) ranging from 2.1 to 14.8%;
- R_{Rec} ranging from 85.5 to 107%, and
- a *limit of quantification* (LOQ) of 1 mg/kg in *feedingstuff.*

Based on the performance characteristics presented the EURL recommends for official control the single laboratory validated and further verified NP-HPLC-VIS method, submitted by the Applicant, to determine *Canthaxanthin* in *premixtures* and *feedingstuffs*.

For the determination of *Canthaxanthin* in *water*, the Applicant submitted the above mentioned NP-HPLC-VIS method. However no experimental data have been provided. Therefore the EURL could not evaluate nor recommend a method for official control to determine *Canthaxanthin* in *water.*

For the quantification of *Canthaxanthin* in *poultry tissues (liver, skin, fat)* and *egg yolk* the Applicant proposed a single laboratory validated and further verified method, based on NP-HPLC coupled to VIS detection measuring at 466 nm. For the quantification of *Canthaxanthin* in *fish flesh (salmon* and *trout)* the Applicant proposed the CEN/TS 16233-1:2011 analytical method (further ring trial validated by six laboratories in a collaborative study organised by the University of Trondheim - HIST on behalf of the Norwegian Seafood Federation - FHL), based on NP-HPLC coupled to VIS detection measuring at 470 nm. These methods were validated and verified at the content of *Canthaxanthin* ranging from 0.7 to 114 mg/kg in *poultry tissues*, 2.5 to 40 mg/kg for *egg yolk* and applicable for a rage above 0.02 mg/kg for *fish flesh*. The following performance characteristics were reported:

- for *liver, skin, fat* and *egg yolk*: RSD_r and RSD_{ip} ranging from 1 to 9.5 %; R_{Rec} ranging from 93 to 110 %; LOQ of 0.3 mg/kg and for *egg yolk* of 0.5 mg/kg;
- for *fish flesh*: RSD_r and RSD_R ranging from 4.1 to 9.2 %; R_{Rec} of 95.8% and LOQ of 0.01 mg/kg.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified methods, based on NP-HPLC-VIS, submitted by the Applicant, to determine *Canthaxanthin* in *poultry tissues*, *egg yolk* and the CEN/TS 16233-1:2011 method, based on NP-HPLC-VIS to determine *Canthaxanthin* in *fish flesh*.

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
Canthaxanthin, sensory additives, colourants, chickens for fattening, chickens reared for laying, laying hens, other poultry, salmon, trout, other fish, pets and other non-food producing animals, MRLs.

1. BACKGROUND

Canthaxanthin is already authorised as *feed additive* under the category 'sensory additives' in the functional group 'colourants' [1, 2]. In the current application authorisation is sought under articles 4(1) and 10(2) for *Canthaxanthin* under the category/functional group 2(a) "sensory additives"/"colourants", subgroups

(i) "substances that add or restore colour in *feedingstuffs* (for dogs and cats)",

(ii) "substances which, when fed to animals, add colours to food of animal origin" and

(iii) "substances, which favourably affect the colour of ornamental fish or birds", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [2, 3].

According to the Applicant, the *feed additive* is a deep violet crystals or crystalline powder, sensitive to oxidation, light and heat [4, 5]. Therefore, to ensure stability of the additive *per se* as well in *premixtures* and *feedingstuffs, Canthaxanthin* is placed on the market as a formulated product. The *active substance* in the formulation is the 10 % of the complete preparation (with a level of at least 96 % of colouring matters expressed as *Canthaxanthin* and a maximum of 5 % of carotenoids other than *Canthaxanthin*). The formulation is typically complemented by feed materials, solvents, carriers, formulation aids, antioxidants and preservatives [6].

Specifically, authorisation is sought for the use of the *feed additive* for chickens for fattening, chickens reared for laying, laying hens, other poultry, salmon, trout, other fish, pets and other non-food producing animals. The Applicant proposed specific maximum levels for the *feed additive* in *feedingstuffs* (Table 1) and Maximum Residue Limits (MRLs) in tissues (Table 2) [2, 3]. Furthermore, the Applicant derives the maximum levels in *water* applying a specific formula as proposed in the footnote of the conditions of use [3, 7].

Table 1: Specific maximum levels of *Canthaxanthin* in the *feedingstuffs* [2, 3 and 7].

Species or category of animal	Max content mg/kg
Chickens for fattening	25
Chickens for laying	8
Laying hens	8
Salmon and trout	25
Other poultry	25
Other fish	25
Pets and other non food producing animals	-

Table 2: Maximum Residue Limits (MRLs) as proposed by the Applicant [2, 3].

Species or category of animal	Target tissue(s) or food products	Max content (*)
Poultry	Liver	15
Poultry	Egg yolk	30
Poultry	Skin and fat	2.5
Salmon	Flesh	10
Trout	Flesh	5

(*) expressed as: mg Canthaxanthin * kg-1

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 *Canthaxanthin*, 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, mycotoxins, and dioxins) are available from the respective European Union Reference Laboratories [8].

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

For the determination of *Canthaxanthin* in the *feed additive*, the Applicant submitted a spectrophotometric method [9]. The analytical method was single laboratory validated and further verified by a collaborative study that involved six laboratories analysing seven samples in replicate [10]. This method applies for the analysis of powdery or water dispersible *Canthaxanthin preparations* and it is designed for concentrations of *active substance* ranging from 5 to 25%. The *feed additive* is enzymatically treated in water with

protease in order to release the *active substance* from the additive. Following a dilution with acetone, the mass fraction of *Canthaxanthin* is determined photometrically at the isobestic wavelength of 426 nm. The mass fraction is calculated by an absolute measurement considering the specific absorbance of the *active substance* at this wavelength. The performance characteristics derived from the validation and the collaborative study are presented in Table 3.

Based on the performance characteristics presented, the EURL recommends for official control, the ring trial validated spectrophotometric method, submitted by the Applicant, to determine *Canthaxanthin* in the *feed additive*.

Table 3: Method performance characteristics for the determination of *Canthaxanthin* in the *feed additive* (FA) [10].

FA	validation	intercomparison study
RSD_r (%)	0.3	0.2 - 0.8
RSD_{ip} (%)	0.3	-
RSD_R (%)	-	1.3 - 4.0
R_{rec} (%)	99.3	96.2 - 105

RSD_r, RSD_{ip} - relative standard deviation for *repeatability* and *intermediate precision*, respectively; RSD_R - relative standard deviation for *reproducibility*; R_{rec} - *recovery* rate.

For the determination of *Canthaxanthin* in *premixtures* and *feedingstuffs*, the Applicant submitted a normal phase high performance liquid chromatographic method with visible detection (NP-HPLC-VIS) [9]. The analytical method was single laboratory validated and further verified [11]. The method consists of an enzymatic digestion of the sample in order to release the active substance, followed by extraction with ethanol and dichloromethane. The extraction procedure differs in consideration of the nature of the sample and of the *Canthaxanthin* concentration declared. The extract is purified by open-column chromatography on silica gel. Finally, an aliquot is injected into an isocratic normal-phase HPLC system adjusted at 466 nm. The selected chromatographic conditions allow for a full resolution of the cis/trans isomers of *Canthaxanthin,* carotenes and other xanthophylls present in the feed. The *active substance* is expressed as the sum of the all-trans and cis isomers (total *Canthaxanthin*). The separated trans/cis isomers are invidually quantified against a standard solution prepared with the all-trans *Canthaxanthin*. Furthermore, the quantification of the cis isomers of *Canthaxanthin* includes the use of experimentally determined relative response factors, in order to compensate for the different absorbance coefficients of the cis isomers compared to all-trans *Canthaxanthin* [12]. The correspondent performance characteristics are presented in Table 4.

Table 4: Method performance characteristics for the determination of *Canthaxanthin* in *premixtures* (PM) and *feedingstuffs* (FS) [11].

PM & FS	Premix		Layer Feed		Broiler Feed		Fish Feed	
	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.
Content, *mg/kg*	1017 - 3268		3.8 - 43.3		8.4 - 108.1		30.5 - 45.9	
RSD_r (%)	1.4 - 6.9	2.1 - 2.7	8.5 - 15.0	5.4 - 12.9	2.6 - 9.6	2.6 - 8.3	8.7 - 9.5	6.4 - 9.2
RSD_{ip} (%)	2.1	2.3	14.8	12.6	8.0	8.2	9.1	7.0
R_{rec} (%)	-	99.8 - 105	-	85.5 - 86.5	-	94.9 - 102	-	107
LOD (mg/kg)	-	20	0.03	0.3	0.01	0.3	0.01	0.3
LOQ (mg/kg)	-	60	1	1	1	1	1	1

RSD_r, RSD_{ip} - relative standard deviation for *repeatability* and *intermediate precision*, respectively;
R_{rec} - *recovery* rate; LOD – limit of detection; LOQ – limit of quantification

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified NP-HPLC-VIS method, submitted by the Applicant, to determine *Canthaxanthin* in *premixtures* and *feedingstuffs*.

For the determination of *Canthaxanthin* in *water*, the Applicant submitted the above mentioned NP-HPLC-VIS method [9]. However no experimental data have been provided. Therefore the EURL could not evaluate nor recommend a method for official control to determine *Canthaxanthin* in *water.*

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.

Description of the analytical methods for the determination of the residues of the additive or of its metabolites in food.

For the quantification of *Canthaxanthin* in *poultry tissues (liver, skin, fat)* the Applicant proposed a single laboratory validated and further verified method, based on NP-HPLC coupled to VIS detection measuring at 466 nm [13, 14]. The sample (1 to 2 g of homogenate poultry tissue) and equal amount of magnesium sulphate are placed in a solid phase extraction (SPE) column equipped with a 10 µm frit and extracted four times with acetone. The filtered extract is evaporated at reduced pressure using a rotary evaporator or a flow of nitrogen at 50°C. The residue is dissolved in cyclohexane and analysed by NP-HPLC at 466 nm. The quantification is done against external standard solutions The reported performance characteristics are presented in Table 5.

For the quantification of *Canthaxanthin* in <u>*egg yolk*</u> the Applicant proposed a single laboratory validated and further verified method, based on NP-HPLC coupled to VIS detection measuring at 466 nm [13, 15]. The sample (10 g) is diluted with water. The emulsion is mixed with ethanol and extracted by shaking with n-heptane. An aliquot of the n-heptane phase is injected and analysed by NP-HPLC at 466 nm. The quantification is done against external standard solutions. The reported performance characteristics are presented in Table 5.

Table 5: Method performance characteristics for the determination of the *residues* in *poultry tissues* and *egg yolk*

Tissue	Content (mg/kg)	LOQ (mg/kg)	Validation			Verification		
			RSD_r (%)	RSD_{ip} (%)	R_{rec} (%)	RSD_r (%)	RSD_{ip} (%)	R_{rec} (%)
Liver [15]	0.7 – 91	0.3	1.9 - 5.5	5.5 – 8.7	93 - 99	4.2 - 8.1	8.5	110
Skin [15]	0.9 – 101	0.3	2.6 - 5.9	-	98 - 105	1.8 - 2.7	7.3	100
Fat [15]	1.2 – 114	0.3	5.8	-	104 - 106	1.1 -2.7	9.5	102
Egg yolk [16]	2.5 – 40	0.5	1.7 - 2.7	0.7 - 2.8	98 - 110	2.2 - 2.3	2.9	107

LOQ – Limit of Quantification; RSD_r, RSD_{ip} - relative standard deviation for *repeatability* and *intermediate precision*, respectively; R_{rec} - *recovery* rate

For the quantification of *Canthaxanthin* in <u>*fish flesh*</u> *(salmon* and *trout)* the Applicant proposed the CEN/TS 16233-1:2011 analytical method, based on NP-HPLC coupled to VIS detection measuring at 470 nm [16]. The method can be applied at a range above 0.02 mg/kg. This method was further ring trial validated by six laboratories in a collaborative study organised by the University of Trondheim (HIST) on behalf of the Norwegian Seafood Federation (FHL). It consists in a homogenization of the sample with butylated hydroxytoluene (BHT) and magnesium sulphate diluted with acetone. The procedure of extraction is slightly different in function of the scale of the sample analysed. The mixture is filtered through a frit using vacuum. The filtered extract is evaporated at reduced pressure, using a rotary evaporator, or under flow of nitrogen at 50°C. The residue is dissolved in a mixture of n-heptane and acetone (86:14 V:V) and transferred into a HPLC vial. The vial is centrifuged and an aliquot of the clear supernatant phase is injected and analysed by isocratic NP-HPLC coupled to VIS detection measuring at 470 nm. The quantification is done against external standard solutions. The performance characteristics reported in the collaborative study are [16]:

- a relative standard deviation for *repeatability* (RSD_r) of 4.1%;
- a relative standard deviation for *reproducibility* (RSD_R) of 9.2%; and
- a *recovery* rate (R_{Rec}) of 95.8%.

Furthermore, for *Canthaxanthin* in *fish flesh*, the Applicant reported limit of quantification (LOQ) of 0.01 mg/kg.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on NP-HPLC-VIS, submitted by the Applicant, to determine *Canthaxanthin* in *poultry tissues*, *egg yolk* and the CEN/TS 16233-1:2011 method (and further ring-trail validated by the HIST), based on NP-HPLC-VIS to determine *Canthaxanthin* in *fish flesh*.

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:

- a single laboratory validated and further ring trial verified method based on spectrophotometry to determine *Canthaxanthin* in the *feed additive*;
- a single laboratory validated and further verified method using HPLC with VIS detection to determine *Canthaxanthin* in *premixtures* and *feedingstuffs*;
- a single laboratory validated and further verified method using HPLC with VIS detection to determine *Canthaxanthin* in *poultry tissues (liver, skin, fat) egg yolk;*
- the CEN/TS 16233-1:2011 method using HPLC with VIS detection to determine *Canthaxanthin* in *fish flesh*

Recommended text for the register entry (analytical method)

For the quantification of *Canthaxanthin* in the *feed additive*:

- Spectrophotometry at 426 nm

For the quantification of *Canthaxanthin* in the *premixtures* and *feedingstuffs*:

- Normal Phase High Performance Liquid Chromatography coupled to visible detection (NP-HPLC-VIS, 466 nm)

For the quantification of *Canthaxanthin* in *poultry tissues (liver, skin, fat)* and *egg yolk:*

- Normal Phase High Performance Liquid Chromatography coupled to visible detection (NP-HPLC-VIS, 466 nm)

For the quantification of *Canthaxanthin* in *fish flesh:*

- Normal Phase High Performance Liquid Chromatography coupled to visible detection (NP-HPLC-VIS, 470 nm) - CEN/TS 16233-1:2011

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Canthaxanthin* 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] Council Directive 70/524/EEC concerning additives in feedingstuffs - List of authorised additives in feedingstuffs (2004/C50/01)
[2] *Application, Reference SANCO/D/2 Forw. Appl. 1831/005-2009
[3] *Application, Proposal of Register Entry – Annex A
[4] *Technical dossier, Section II: 2.1.1 Name of the additive
[5] *Technical dossier, Section II: 2.2 Characterisation of the active substance
[6] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[7] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[8] 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
[9] *Technical dossier, Section II: 2.6.1 Method of analysis for the active substance
[10] *Technical dossier, Section II: 2.6.1.c
[11] *Supplementary information: Canthaxanthin Premix Feed Verification.pdf
[12] *Technical dossier, Section II: 2.6.1.d
[13] *Technical dossier, Section II: 2.6.2 Method(s) of analysis for the determination of the residues of the additive or of its metabolites in food
[14] *Supplementary information: 2010-11-26_CARAC_Canthaxanthin_Poultry tissue.pdf
[15] *Supplementary information: 2011-01-05_CARAC_Canthaxanthin_Egg yolk.pdf
[16] *Supplementary information: 2011-06-30_CARAC_Canthaxanthin_Fish tissue.pdf
* Refers to Dossier No. FAD-2008-0048

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

The following National Reference Laboratories contributed to this report:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Kmetijski inštitut Slovenije, Ljubljana (SI)