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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-2010-0224** **CRL/100293**
Feed Additive Name:	***Ethyl ester of beta-apo-8'-carotenoic acid (E 160 f)***
Active Substance(s):	***Ethyl ester of beta-apo-8'-carotenoic acid (E 160 f)***
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 *ethyl ester of beta-apo-8'-carotenoic acid* under the category/functional group 2(a) "sensory additives"/"colourants", subgroup (ii) "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. According to the Applicant, the active substance of the *feed additive* is *ethyl ester of beta-apo-8'-carotenoic acid (apoester)* with a minimum purity of 90 % (expressed as total carotenoids). Specifically, authorisation is sought for the use of the *feed additive* for poultry for fattening and laying (table eggs and liquid eggs for processed food). The Applicant proposed the following maximum levels of the *feed additive* in the *feedingstuffs*: - 8 mg/kg for poultry for laying for table eggs; - 15 mg/kg for poultry for fattening and - 80 mg/kg for poultry for laying for liquid eggs (for processed food). Furthermore, the Applicant proposed the following Maximum Residue Limits (MRL): - 2.5 mg/kg for fat and skin; - 6 mg/kg for liver; - 25 mg/kg for egg yolk in table eggs and - 250 mg/kg for egg yolk in eggs for industrial processing. The limits proposed for *feedingstuffs* and *target tissue* and *egg yolk* refer to the sum of the all-trans and cis isomers of the active substance, expressed as total carotenoids.

For the identification and quantification of *apoester* in the *feed additive,* the Applicant proposed the internationally recognised FAO JECFA monograph for *Beta-apo-8'-carotenoic acid ethyl ester* in food additives, based on spectrophotometry at 449 nm. Even though no performance characteristics are provided, the EURL recommends for official control the JECFA monograph based on spectrophotometry for the quantification of the *apoester* in the *feed additive*.

For the quantification of *apoester* in *premixtures*, *feedingstuffs* and *water*, the Applicant submitted a single laboratory validated and further verified method, based on High Performance Liquid Chromatography (HPLC) with VIS detection at 446 nm. The following performance characteristics were reported:

- for *premixtures:* - a *precision* (*repeatability* and *intermediate precision*) ranging from 0.9 to 3.4 %; and - a *recovery* rate (R_{Rec}) ranging from 100 to 106 %;
- for *feedingstuffs:* - a *precision* ranging from 2.7 to 10.2 %; - R_{Rec} ranging from 77 to 107 %; and a limit of quantification (LOQ) of 0.5 mg/kg *feedingstuffs*; and
- for *water*: LOQ of 0.1 mg/L *water*.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on High Performance Liquid Chromatography (HPLC) with VIS detection, submitted by the Applicant, to determine *apoester* in *premixtures*, *feedingstuffs* and *water*.

For the quantification of *apoester* in *poultry tissues (liver, skin, fat)* the Applicant proposed a single laboratory validated and further verified method, based on High Performance Liquid Chromatography (HPLC) coupled to Diode Array Detector (HPLC-DAD) measuring at 446 nm. For the quantification of *apoester* in *egg yolk* the Applicant proposed single laboratory validated and further verified method, based on HPLC with VIS detection at 446 nm. The methods were validated and verified at the content of *apoester* ranging from 0.5 to 250 mg/kg *tissues* and 0.3 to 250 mg/L *egg yolk*.

The following performance characteristics were reported:

- for *liver*: - a *precision* ranging from 1.9 to 8.9 %; and - R_{Rec} ranging from 88 to 108 %;
- for *skin*: - a *precision* ranging from 2.2 to 4.4 %; and - R_{Rec} ranging from 91 to 101 %;
- for *fat*: - a *precision* ranging from 1 to 5.1 %; and - R_{Rec} ranging from 95 to 104 %; and
- for *egg yolk*: - a *precision* ranging from 1.8 to 7.8 %; and - R_{Rec} ranging from 95 to 109 %; and
- LOQ ranging from 0.1 to 0.3 mg/kg *poultry tissues* and *egg yolk*.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on HPLC-DAD and HPLC-VIS, submitted by the Applicant, to determine *apoester* in *poultry tissues* and *egg yolk*.

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
ethyl ester of beta-apo-8'-carotenoic acid, E 160f, sensory additives, colourants, poultry for fattening and laying, MRL.

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use in water) and 10(2) (re-evaluation of additives already authorised under under provisions of Council Directive 70/524/EEC) for *ethyl ester of beta-apo-8'-carotenoic acid* under the category/functional group 2(a) "sensory additives"/"colourants", subgroup (ii) "substances which, when fed to animals, add colours to food of animal origin" [1, 2], according to the classification system of Annex I of Regulation (EC) No 1831/2003. According to the Applicant, the active substance of the *feed additive* is *ethyl ester of beta-apo-8'-carotenoic acid (apoester)* with a minimum purity of 90 % (expressed as total carotenoids) [2, 3]. The *feed additive* is a red to violet-red crystalline powder, sensitive to light and oxygen. Therefore, the additive is marketed in different formulations (containing carriers, formulation aids, antioxidants and preservatives), to ensure stability of the additive *per se* as well in *premixtures* and *feedingstuffs* [3]. A typical formulation contains approximately 10 % of active substance [3]. Specifically, authorisation is sought for the use of the *feed additive* for poultry for fattening and laying (table eggs and liquid eggs for processed food) [1, 2]. The Applicant proposed the following maximum levels of the *feed additive* in the *feedingstuffs* [2]: - 8 mg/kg for poultry for laying for table eggs; - 15 mg/kg for poultry for fattening; and - 80 mg/kg for poultry for laying for liquid eggs (for processed food). The limits proposed for *feedingstuffs* and *target tissue* and *egg yolk* refer to the sum of the all-trans and cis isomers of the active substance, expressed as total carotenoids. The Applicant also suggested for each species half of the above mentioned levels as maximum in drinking water [3]. Furthermore, the Applicant proposed following Maximum Residue Limits (MRL): - 2.5 mg/kg for fat and skin, - 6 mg/kg for liver, - 25 mg/kg for egg yolk in table eggs and - 250 mg/kg for egg yolk in eggs for industrial processing [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 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 *ethyl ester of beta-apo-8'-carotenoic acid*, 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 [4].

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

For the identification and quantification of *ethyl ester of beta-apo-8'-carotenoic acid (apoester)* in the *feed additive,* the Applicant proposed the internationally recognised FAO JECFA monograph for *Beta-apo-8'-carotenoic acid ethyl ester* in food additives [5], based on spectrophotometry at 449 nm. Even though no performance characteristics are provided, the EURL recommends for official control the JECFA monograph based on spectrophotometry for the quantification of the *apoester* in the *feed additive*.

For the quantification of *apoester* in *formulated products*, the Applicant proposed a spectrophotometric method [6]. This method applies for the analysis of powdery product forms containing chemically synthesized *apoester* containing 5 to 25% *active substance*. The sample is enzymatically treated in water with protease to release the *active substance*. Following a dilution with acetone, the mass fraction of *apoester* is determined spectrophotometrically at 446 nm. The analytical method was validated and further verified by a collaborative study [6] that involved six laboratories analysing six samples in duplicates. The samples contained the target analyte at a concentration ranging from 50 – 250 g/kg. The Applicant reported the following performance characteristics:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.4 to 1 %; and
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 0.7 to 3.4 %.

For the quantification of *apoester* in *premixtures*, *feedingstuffs* and *water*, the Applicant submitted a single laboratory validated and further verified method, based on High Performance Liquid Chromatography (HPLC) with VIS detection [7]. The method consists of an enzymatic digestion of the sample to release the active substance, followed by extraction with ethanol and dichlorometane. [For health and environmental protection, ethylacetate should be used instead of dichlorometane]. The extraction procedure differs depending on the nature of the sample and of the *apoester* concentration declared. The extract is purified by adsorption of polar components on silica gel. Carotenoids are then eluted with n-

hexane/diethyl ether without using vacuum. The eluate is mixed and the solvent is evaporated in a SpeedVac at approx. 40 °C under reduced pressure for 45 minutes. Afterwards 1 mL of n-heptane/acetone is added and the solution is shaken to dissolve the residue. Finally, the aliquots of solution are injected into an isocratic normal-phase HPLC system adjusted at 446 nm, able to resolve cis/trans isomers of *apoester.* The *active substance* is expressed as the sum of the all-trans and cis isomers (total *apoester*). The separated trans/cis isomers are individually quantified against a standard solution prepared with the all-trans *apoester*. Furthermore, the quantification of the cis isomers of *apoester* 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 *apoester*. The reported performance characteristics are presented in Table 1. The Applicant also reported limits of detection and quantification (LOD, LOQ) of 0.2 and 0.5 mg/kg *feedingstuffs,* respectively.

For the determination of *apoester* in *water*, the Applicant proposed to apply the method developed for premixture and feed samples. No performance characteristics were reported for aqueous samples, except an LOQ of 0.1 mg/L [7]. However, the EURL considers the method suitable for official control to determine *apoester* in *water*.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on High Performance Liquid Chromatography (HPLC) with VIS detection (HPLC-VIS), submitted by the Applicant, to determine *apoester* in *premixtures*, *feedingstuffs* and *water*.

Table 1: Method performance characteristics for the determination of *ethyl ester of beta-apo-8'-carotenoic acid* in *premixtures* and *feedingstuffs* [7]

	Premix		Layer Feed		Broiler Feed	
	Validation	Verification	Validation	Verification	Validation	Verification
Content [mg/kg]	982 - 3207		3.5 - 41.6		6.8 - 106.5	
RSD_r (%)	2.9 - 3.0	0.9 - 3.4	7.7 - 9.2	3.9 - 10.2	0.1 - 5.4	2.7 - 8.0
RSD_{ip} (%)	3.1	2.6	8.2	8.9	5.4	6.3
R_{rec} (%)	-	100 – 106	-	77 - 107	-	88 – 90
LOD (mg/kg)	10	10	0.02	0.2	0.02	0.2
LOQ (mg/kg)	500	30	1	0.5	1	0.5

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

For the quantification of *apoester* in *poultry tissues (liver, skin, fat)* the Applicant proposed a single laboratory validated and further verified method, based on HPLC coupled to Diode Array Detector (HPLC-DAD) measuring at 446 nm [8]. The sample (1 to 2 g of homogenous poultry tissue) and equal amount of magnesium sulphate are placed in the plastic SPE column and extracted four times with acetone. The extracts are filtered and evaporated using a rotary evaporator or a flow of nitrogen at 50 °C. The residue is dissolved in cyclohexane and analysed by normal-phase HPLC at 446 nm. The quantification is done against external standard solutions. The reported performance characteristics, were recalculated by the EURL [9], and are presented in Table 2. Furthermore, the Applicant reported LOQ ranging from 0.1 to 0.3 mg/kg *poultry tissues*.

For the quantification of *apoester* in *egg yolk* the Applicant proposed a single laboratory validated and further verified method, based on HPLC with VIS detection [10, 11]. The sample (5 to 10 g) is diluted with water. The emulsion is mixed with ethanol and extracted by shaking with n-heptane. The aliquots of the n-heptane phase are injected and analysed by normal phase HPLC at 446 nm. The quantification is done against external standard solutions. The reported performance characteristics were recalculated by the EURL [9], and are presented in Table 2. Furthermore, the Applicant reported LOQ of 0.1 mg/kg *egg yolk*, respectively.

Table 2: Method performance characteristics for the determination of the *residues* in *tissues* and *egg yolk*

		Validation			Verification		
Tissue	Content [mg/kg]	RSD_r (%)	RSD_{ip} (%)	R_{rec} (%)	RSD_r (%)	RSD_{ip} (%)	R_{rec} (%)
Liver	0.5 – 250	1.9 – 8 [8]	3.4 – 8.9 [8]	88 - 102 [8]	3.0 [9]	3.6 [9]	108 [8]
Skin	0.5 – 250	2.2 – 4.4 [8]	-	91- 99 [8]	3.5 [9]	3.5 [9]	101 [8]
Fat	0.5 – 250	1 – 5.1 [8]	-	95 – 104 [8]	1.5 [9]	1.5 [9]	103 [8]
Egg yolk	0.3 – 250 mg/L	-	1.8 – 7.8 [11]	95 – 101 [11]	3.7 [9]	4.4 [9]	109 [11]

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

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on HPLC-DAD and HPLC-VIS, submitted by the Applicant, to determine *apoester* in *poultry tissues* and *egg yolk*.

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:

- the identification and quantification assay described in the JECFA monograph *Beta-apo-8'-carotenoic acid ethyl ester* in the *feed additive*;
- the single laboratory validated and further verified method using High Performance Liquid Chromatography (HPLC) with VIS detection to determine *apoester* in *premixtures, feedingstuffs* and *water*;
- the single laboratory validated and further verified method using HPLC with diode array detector to determine *apoester* in *poultry tissues (liver, skin, fat);*
- the single laboratory validated and further verified method using HPLC with VIS detection to determine *apoester* in *egg yolk*

Recommended text for the register entry (analytical method)

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

- spectrophotometry at 449 nm (JECFA monograph *Beta-apo-8'-carotenoic acid ethyl ester)*

For the quantification of *apoester* in the *premixtures, feedingstuffs* and *water*:

- High Performance Liquid Chromatography with VIS detection at 446 nm

For the quantification of *apoester* in *poultry tissues (liver, skin, fat):*

- High Performance Liquid Chromatography with VIS detection at 446 nm

For the quantification of *apoester* in *egg yolk:*

- High Performance Liquid Chromatography with VIS detection at 446 nm

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *ethyl ester of beta-apo-8'-carotenoic acid* 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 SANCO/D/2 Forw. Appl. 1831/00132/(10191)/2010
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the 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_26_FAO
[6] *Technical dossier, Section II, Annex_II_27_Fefana_Spectro
[7] *Technical dossier, Section II, Annex_II_28_FEFANA_2010
[8] *Technical dossier, Section II, Annex_II_32_FEFANA_2010
[9] *Supplementary information, Precision data EURL
[10] *Technical dossier, Section II, Annex_II_30_FEFANA_2011
[11] *Supplementary information, Schierle_Frey_2010
* Refers to Dossier No. FAD-2010-0224

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

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- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)

- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)