EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

Amendment to CRL report (D08/FSQ/CVH/GS/D(2007) 11114) on the dossier EFSA-Q-2006-173 (Panaferd-AX®)

In its report (D08/FSQ/CVH/GS/D(2007) 11114) the CRL-FA evaluated analytical methods based on normal phase High Performance Liquid Chromatography (HPLC) coupled to UV detection for the determination of astaxanthin in feedingstuffs and in fish tissue. Evaluating these methods against their suitability for the determination of other carotenoids showed that the methods also allow for the simultaneous measurement of canthaxanthin and adonirubin – in addition to astaxanthin. Performance characteristics were estimated at various concentrations of the target analytes in feed [1] and fish tissue [2] and the following results were obtained:

For the method for the determination of canthaxanthin in *feedingstuffs* the limit of detection (LOQ) was 0.25 mg/kg, the percentage of the recovery rate (RR) was at least 87 % or higher and the obtained precision values expressed as relative standard deviation (RSD) were below 3.9 %. For the determination of adonirubin in *feedingstuffs* the RR was at least 87 % and the values for the RSD were below 3.3 %. The validation report did not indicate a LOQ for this compound, but it is most likely below 6.7 mg/kg which is the lowest concentration level at which the experiments for assessing the RSD and RR have been conducted.

For the method for the determination of canthaxanthin in *fish tisse* the limit of detection (LOQ) was 0.053 mg/kg. The RR was about 59 % at a concentration of 0.09 mg/kg and above 75 % at 5 mg/kg and at higher concentrations. The values for the RSD were below 14 %. For the determination of adonirubin in *fish tisse* the RR was at least 87 % and the values for the RSD were below 12.7 %. The validation report did not indicate a LOQ for this compound, but it is most likely below 0.24 mg/kg which is the lowest concentration level at which the experiments for assessing the RSD and RR have been conducted.

[1] Technical dossier (EFSA-Q-2006-173), Section II, Appendix II-Z12

[2] Technical dossier (EFSA-Q-2006-173), Section II, Appendix II-Z11

Geel, 06/07/2007

D08/FSQ/CVH/GS/D(2007) 11114

CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	FAD-2006-0021 EFSA-Q-2006-173
Name of Additive:	Panaferd-AX®
Active Substance(s):	Astaxanthin
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
Report prepared by:	María José González de la Huebra (CRL-FA)
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Report approved by:	Christoph von Holst (CRL-FA) 08/05/2007

EXECUTIVE SUMMARY

Panaferd-AX® is a feed additive for which authorisation is sought under the category "sensory additives", functional group "colorants: 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. Panaferd-AX® contains astaxanthin as active substance.

Panaferd-AX® is composed of sterilised dried cells of astaxanthin-rich *Paracoccus carotinifaciens* containing at least 20 g/kg of astaxanthin and is intended to be added to salmonid fish feed at a rate providing up to 85 mg/kg as astaxanthin in the final feedingstuff. Panaferd-AX® will be added directly to the final feedingstuffs and not prepared in pre-mixtures.

An HPLC (high performance liquid chromatography) method with spectrophotometric detection is proposed for the quantification of the active substance (astaxanthin) in the *feed additive*, *feedingstuffs* and *fish tissues*. The validation of the proposed method has been performed according to the requirements laid down by Commission Directive 2001/79/EC in fish pellet feed and fish tissues. For the determination of astaxanthin in *feedingstuffs* the following performance characteristics were obtained. The percentage of the recovery rate was estimated through blank feed samples fortified with the feed additive at different concentrations and ranged between 80 and 93 %. The obtained precision values, expressed as relative standard deviation were below 3.8 %. The limit of detection (LOD) and limit of quantification (LOQ) were 0.124 and 0.412 mg/kg, respectively. These performance characteristics are considered acceptable and the method is therefore considered suitable for official control purposes in *feedingstuffs*. Performance characteristics have also been provided for the method for the determination of the target analyte in *fish tissue*. However, since there are no Maximum Residue Limits (MRLs) for astaxanthin, the CRL cannot evaluate the suitability of the proposed method for official control of astaxathin in *fish tissue*.

Different control methods are proposed for the identification and quantification of impurities. Most of the proposed methods are classical methods that are often part of the relevant legislation, therefore the proposed methodologies can be considered suitable for the intended purposes.

Further testing or validation by the CRL is not considered necessary.

KEYWORDS

Panaferd-AX®, *Paracoccus carotinifaciens*, astaxanthin, sensory additives, feed additive, coulorants.

BACKGROUND

Panaferd-AX® is a feed additive for which authorisation is sought under the category "sensory additives", functional group "coulorants: 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. Panaferd-AX® contains natural carotenoids pigments such as astaxanthin, canthaxanthin, beta carotene, adonixanthin, adonirubin, and low levels of other identified carotenoids being astaxanthin its active substance.

Panaferd-AX® is composed of sterilised dried cells of astaxanthin-rich *Paracoccus carotinifaciens* containing at least 20 g/kg of astaxanthin. Synthetic astaxanthin and astaxanthin-rich *Phaffia rhodozyma* (ATCC 74519) and the mixture of astaxanthin and canthaxanthin are authorised as feed additives for salmon and trout up to a total concentration of 100 mg/kg in the complete feedingstuff, from the age of six months onwards [1]. Astaxanthin-based carotenoids from *Haematococcus pluvialis* are approved as human food supplement in the EU.

The intended use of the current application is for incorporation into salmonid feeds. Panaferd-AX® will be added directly to the final feedingstuffs and not prepared in pre-mixtures at a rate providing up to 85 mg/kg as astaxanthin in the final feedingstuff [2]

TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis submitted in connection with Panaferd-AX® and their suitability to be used for official controls were evaluated.

EVALUATION

The numbering system under this point refers to that of Section II of the Annex of Commission Directive 2001/79/EC (2.5 Control methods).

Description of some of the methods used for the determination of the criteria listed under item 2.5.1 of Commission Directive 2001/79/EC

Qualitative and quantitative analysis of the astaxanthin content in Panaferd-AX® is performed with an isocratic HPLC (High Performance Liquid Chromatography) method using two silica columns serially connected and a spectrophotometer set at 470 nm. The characterisation of astaxanthin in Panaferd-AX® has been carried out by different techniques such as mass spectrometry, UV/Visible spectroscopy, 13C-NMR and 1H-NMR. A comparison of the retention time obtained with commercial standards of astaxanthin analysed as authentic standard has been used as extra means for identification purposes. The information gathered from these tests is considered enough for a reliable identification of astaxanthin in Panaferd-AX®. The quantification of astaxanthin is performed by single point calibration which, even if this is not the preferred one, could be considered as acceptable for control purposes [3].

Routine analyses of arsenic [4], lead [5], mercury [6], sodium, potassium, calcium, magnesium, zinc, manganese, and lead/cadmium/mercury [7] are carried out by methods based on atomic absorption spectrophotometry. An additional test (total heavy metals test) is suggested by the applicant for the determination of the allowable total limit of heavy metals contained as impurities [8]. Other spectrophotometric methods are proposed for iron and phosphorus determinations respectively [7]. A classical method based on thermogravimetry is used for the water determination. Other classical methods (Kjeldahl, ether extraction and direct burning methods) are also used for the determination of crude protein, crude fat and ash content respectively [9]. Most of the proposed methods are classical methods routinely applied by official control laboratories, therefore the proposed methodologies can be considered suitable for the intended purposes.

Description of the qualitative and quantitative analytical methods for routine control of the active substance in feedingstuffs (2.5.2. of the Guidelines)

Minor modifications are introduced in the sample preparation procedure in order to adapt it to the target concentration range of astaxanthin in feedingstuffs. The proposed method [10] involves a multi-extraction procedure with mixtures of organic solvents. The first step is to wet the sample followed by extracting the sample with a mixture of tetrahydrofuran (THF) and methanol (MeOH) (20:1 v/v). After mixing for 3 to 5 minutes, n-hexane is added and the resulting mixture is further mixed and centrifuged at 1800 g for 10 minutes. The upper solvent

layer containing the target analyte is then transferred to a volumetric flask. The whole procedure is successively repeated other two times and the upper organic layers resulting from each extraction step are combined with the first one. Once the three upper organic layers are combined, the volumetric flask is filled up to the mark with the appropriate volume of a mixture of n-hexane, THF and MeOH (40:20:1 v/v/v). An aliquot of this solution is then injected on the HPLC system. The HPLC analysis is performed on two serially connected 250 x 4.6mm Wakosil-II 5 SIL-100 columns using a mixture of n-hexane, THF and MeOH (40:20:1 v:v:v) as mobile phase, measuring the astaxanthin peak at a wavelength of 470 nm.

The validation performed [11] complies with the requirements stated under Commission Directive 2001/79/EC. The analytical method has been in-house validated over the range from 0.04 % to 6 % of the feed additive concentration in formulated fish pellet diet, which correspond to a concentration of astaxanthin in feedingstuffs ranging from 10 to 1536 mg/kg. The validation procedure addressed the following parameters: linearity, precision, accuracy, non-analyte interference, specificity, limit of detection (LOD) and limit of quantification (LOQ). The linearity has been assessed by analyzing standard solutions (6 concentration levels + blank) of astaxanthin in duplicate. The accuracy and precision have been assessed by analyzing blank pellet, previously grounded in a mill, and fortified with the feed additive as powder or as stock solution depending on the final concentration. Five concentration levels were prepared and analyzed in quintuplicate. The percentage of the recovery rate was estimated through blank feed samples fortified with the feed additive at different concentrations and ranged between 80 and 93 %. The LOD and the LOQ were 0.124 and 0.412 mg/kg, respectively. The precision values, expressed as percentage of the relative standard deviations, were below 3.8 % for all tested concentrations.

These performance characteristics are considered acceptable and the analytical method is therefore considered suitable for official control purposes, in the frame of the sought authorisation i.e. in pelleted feedingstuffs as colourant in salmonid fish at a maximum concentration of astaxanthin (85 mg/kg), and a total concentration of astaxanthin and canthaxanthin up to 100 mg/kg.

Description of the qualitative and quantitative analytical methods for determining the marker residue(s) of the active substance in target tissues and animal products. (2.5.3 of the Guidelines)

An HPLC method based on the one applied for assessing the quantitative composition of the additive [2] is proposed for the determination of astaxanthin in fish tissues. Minor modifications are introduced in the sample preparation procedure in order to adapt it to the concentration range expecting in tissues. The method has been in-house validated for astaxanthin in fish fillets (rainbow trout) [12]. However, since there are not MRLs for

astaxanthin set yet, the CRL cannot evaluate the suitability of the proposed method for official control of astaxathin in *fish tissue*.

CONCLUSIONS AND RECOMMENDATIONS

For the determination of astaxanthin in the feed additive, feedingstuffs and tissues a high performance liquid chromatography (HPLC) method with spectrophotometric detection at 470 nm is proposed.

An in-house validation of the proposed method, according to the requirements stated under Commission Directive 2001/79/EC, have been carried out for astaxanthin in fish pellet feed. The performance characteristics are considered to be acceptable and the method is therefore considered suitable for official control purposes.

Regarding the determination of astaxanthin in tissues the in-house validation of the proposed method, according to the requirements stated under Commission Directive 2001/79/EC, has also been carried out for astaxanthin in fish fillets (rainbow trout). Nevertheless, since there are not MRLs for astaxanthin currently set, the CRL cannot evaluate the suitability of the proposed method for official control of astaxathin in *fish tissue*.

Different control methods are proposed for the identification and quantification of impurities. Most of the proposed methods are classical methods that are often part of the relevant legislation, therefore the proposed methodologies can be considered suitable for the intended purposes.

Further testing or validation by the CRL is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

High performance liquid chromatography (HPLC) with spectrophotometric detection (λ = 470 nm)

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of Panaferd-AX® have been sent to the Community Reference Laboratory for feed additives authorisation on 28 July 2006. The dossier has been made available to the CRL by EFSA.

REFERENCES

The dossier provided by the applicant is divided into various documents structured according to the Annex of Commission Directive 2001/79/EC, containing the following files. On request of the CRL the applicant also provided the method description for the determination of astaxanthin and other carotenoids in animal feed (reference 10), which was not included in the dossier.

[1] Commission Regulation (EC) No 1288/2004 (OJ C 50 25.2.2004)

[2] Annex III Proposal of Register entry

[3] Technical dossier, Section II, Appendix II-Z1

[4] Technical dossier, Section II, Appendix II-Z4

[5] Technical dossier, Section II, Appendix II-Z5

[6] Technical dossier, Section II, Appendix II-Z6

[7] Technical dossier, Section II, Appendix II-Z10

[8] Technical dossier, Section II, Appendix II-Z7

[9] Technical dossier, Section II, Appendix II-Z9

[10] Method description: " Method of Analysis for Carotenoids in Fish Feeds"

[11] Technical dossier, Section II, Appendix II-Z12

[12] Technical dossier, Section II, Appendix II-Z11

RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives, Geel, Belgium.

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