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

D08/FSQ/CVH/GS/D(2007)18791

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-2006-0032 EFSA-Q-2007-018
Formulated product:	Carophyll® Stay Pink
Active Substance(s):	Astaxanthin dimethyldisuccinate
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
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EXECUTIVE SUMMARY

Astaxanthin dimethyldisuccinate (AXN-DMDS) is a product for which authorisation is sought as feed additive under the category "sensory additives", functional group "colorants: substances which, when fed to animals, adds colours to food of animal origin", according to the classification system of Annex I of Regulation (EC) No 1831/2003. The active substance is all-trans astaxanthin dimethyldisuccinate which is a specific geometrical isomer of AXN-DMDS. The applicant intends to place the product on the market in the specific beadlets formulation Carophyll® Stay Pink.

The product contains in addition to all-trans AXN-DMDS about 1.5 % of two cis isomers of AXN-DMDS and less than 4 % of carotenoids other than AXN-DMDS. It is intended to add the feed additive to fish feed for salmon and trout at a recommended rate ranging from 55 to 97 mg/kg of the active substance in complete feedingstuff. The recommended maximum concentration of the active substance is 138 mg/kg of complete feedingstuff. The concentration of the active substance in all matrices is measured as the sum of the concentration of all-trans and two cis isomers of AXN-DMDS.

The applicant proposes analytical methods for the determination of the active substance that are specifically designed for this particular product formulation (beadlets). The analytical procedures for the analysis of AXN-DMDS in the formulated product, premixtures and feedingstuffs are similar and are comprised of the following steps: (1) The enzymatic release of AXN-DMDS from the beadlets formulation, (2) the extraction of AXN-DMDS with dichloromethane and ethanol, (3) the purification of the extract with solid phase extraction columns when analysing feedingstuffs and (4) the determination of AXN-DMDS by normal phase high performance liquid chromatography (HPLC) coupled to ultraviolet detection (UV detection) measuring at about 470 nm. The method allows for the simultaneous determination of the all-trans and cis isomers of AXN-DMDS and the other carotenoids present in the matrix, since these substances are well separated in the HPLC chromatogram.

Method performance characteristics were determined on *feedingstuffs* containing AXN-DMDS of about 50 mg/kg, obtaining a percentage recovery rate of 98 % and a relative within laboratory reproducibility standard deviation of about 2%. The limit of quantification was 0.2 mg/kg. Performance characteristics for other carotenoids have not been provided. Based on the results from the validation study the method is considered suitable for official control purposes to determine the active substance in *feedingstuffs* within the frame of this application.

The applicant proposed a Maximum Residue Limit (MRL) for astaxanthin in the target *fish tissue* of 25 mg/kg, for which an analytical method has been proposed. The astaxanthin content is expressed in terms of the concentration of the sum of the measured geometrical isomers of this compound. The method is comprised of two steps, which are the extraction of the sample with acetone and the determination of the target analytes with HPLC coupled to UV detection. The parameters of the HPLC detection of the target analytes in all matrices (i.e fish tissue, formulations, premixtures and feedingstuffs) are identical.

Method performance characteristics were determined on *fish flesh* fortified with astaxanthin. The percentage recovery rate was 98 % obtained on samples containing 10 mg/kg of the target analyte and the relative within laboratory reproducibility standard deviation was about 3 %, obtained on samples containing about 6.5 mg/kg of the target analyte. The limit of quantification was 0.2 mg/kg. Based on the results from the validation study the method is considered suitable for official control purposes.

The applicant proposed limits of various impurities in the feed additive including some heavy metals for which appropriate methods are available.

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

KEYWORDS

Astaxanthin dimethyldisuccinate, sensory additives, feed additive, coulorants.

BACKGROUND

Astaxanthin dimethyldisuccinate (AXN-DMDS) (Carophyll® Stay Pink) is a product for which authorisation is sought as feed additive 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. The active substance is *all-trans* astaxanthin dimethyldisuccinate which is a specific geometrical isomer of AXN-DMDS [1]. The applicant intends to place the product on the market in the specific beadlets formulation Carophyll® Stay Pink [2]. The product contains in addition to all-trans AXN-DMDS about 1.5 % of two cis isomers of AXN-DMDS (9 cis- AXN DMDS , 13 cis- AXN DMDS) and less than 4 % of carotoneids other than AXN-DMDS such as adonirubin methylsuccinate [3].

The intended use of the current application is for incorporation into feed for salmon and trout at a recommended rate ranging from 55 to 97 mg/kg of complete feedingstuff [4]. The recommended maximum concentration of the active substance is 138 mg/kg of complete feedingstuff [4].

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 astaxanthin dimethyldisuccinate (Carophyll® Stay Pink) 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

The applicant proposes an analytical method [5] for the determination of astaxanthin dimethyldisuccinate (AXN-DMDS) that is specifically designed for this particular product formulation (Carophyll® Stay Pink). The analytical procedure utilised for the analysis of AXN-DMDS in the product (beadlets formulation) is comprised of the following steps: (1) The enzymatic release of AXN-DMDS from the beadlets, (2) the extraction of AXN-DMDS with dichloromethane and ethanol and (3) the determination of AXN-DMDS by normal phase high performance liquid chromatography (HPLC) coupled to ultraviolet detection (UV detection) measuring at about 470 nm. 100 mg of the sample are treated with demineralised water and the enzyme at 50^0C for 30 minutes in an ultrasonic water bath. The target analyte and other carotenoids are extracted with ethanol and dichloromethane added to a portion of the sample/water suspension. Finally, an aliquot of the ethanol/dichloromethane solution is subjected to HPLC analysis without further clean-up.

The method allows for the simultaneous determination of the all-trans and the cis isomers of AXN-DMDS and other carotenoids including astaxanthin, adonirubin, astacene, canthaxanthin, lutein and zeaxanthin, since these substances are well separated in the chromatogram. However, method performance characteristics for these substances are not specified in the dossier.

Limits for various impurites in the product, namely lead, mercury, arsenic and triphenylphosphine oxide have been proposed, for which appropriate methods have been submitted.

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

For the determination of AXN-DMDS in *premixtures* [5] the applicant proposed a very similar method as utilised for the analysis of the formulated product, just the ratio of the sample amount to the volume of the extraction solutions was adjusted to account for the lower content of AXN-DMDS in premixtures compared to the content of AXN-DMDS in the formulated product.

The method for the determination of AXN-DMDS in *feedingstuffs* [5] is based on the same principle as the methods for the analysis of the formulated product and premixtures. However, an additional clean-up step has been introduced in the analytical procedure. Pellets need to be ground in frozen state. 10 g of the feed samples are treated with demineralised water and the enzyme at 50^0C for 30 minutes in an ultrasonic water bath. The target analyte and other carotenoids are extracted with ethanol and dichloromethane added to a portion of the sample/water suspension. Prior to analysis with normal-phase HPLC the extract is passed through a silicagel column to remove polar substances.

For the validation studies of the methods for determination of the target analyte in premixtures and in feedingstuffs [5], samples were individually fortified with organic solutions of AX-DMDS and afterwards subjected to analysis.

The target AX-DMDS concentration of the experiments for the study on *premixtures* was 2 g/kg and the following performance characteristics were obtained: the percentage recovery rate was 99 % and the relative within laboratory reproducibility standard deviation was about 2.3 %. The validation study also revealed a limit of detection (LOD) of 2 mg/kg and a limit of quantification (LOQ) of 20 mg/kg. Based on the obtained method performance characteristics the method is considered suitable for the intended purpose.

The target AX-DMDS concentration of the experiments for the study on *feedingstuffs* was 50 mg/kg and the following performance characteristics were obtained: The percentage recovery rate was 98 % and the relative within laboratory reproducibility standard deviation was about 2.0 %. The validation study also revealed a limit of detection (LOD) of 0.02 mg/kg and a limit of quantification (LOQ) of 0.2 mg/kg. Based on the obtained method performance characteristics the method is considered suitable for official control purposes.

The applicant also submitted a *reversed phase* HPLC [6] method which laboratories generally prefer to apply compared to *normal* phase HPLC. However, validation data were not provided and therefore this method could not be evaluated against its suitability for official control.

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)

The method for the determination of astaxanthin [7] as the marker residue [8] in fish flesh foresees that about 100 g of the sample is homogenised and a sub-sample of about 1 g is taken and mixed with the same amount of magnesium sulphate. This mixture is placed in an empty SPE cartridge and AX-DMDS and other carotenoids are extracted from this mixture with acetone. An aliquot of the extract is subjected to HPLC analysis without further clean-up.

Method performance characteristics were determined on *fish flesh* fortified with astaxanthin. The percentage recovery rate was 98 % obtained on samples containing 10 mg/kg of the target analyte and the relative within laboratory reproducibility standard deviation was about 3 %, obtained on samples containing about 6.5 mg/kg of the target analyte. The limit of quantification was 0.2 mg/kg. Based on the results from the validation study the method is considered suitable for official control purposes.

CONCLUSIONS AND RECOMMENDATIONS

The applicant proposed suitable methods for the determination of astaxanthin dimethyldisuccinate in beadlets formulations (Carophyll® Stay Pink), premixtures and feedingstuffs based on HPLC. In addition, the applicant submitted a HPLC based method for the determination of astaxanthin in fish flesh. Based on the reported method performance characteristics all methods are considered suitable for the intended purpose.

Since the application is for astaxanthin dimethyldisuccinate, whereas the analytical methods are designed for the determination of the active substance when present in beadlets formulations (Carophyll® Stay Pink), this restriction should be included in the conditions of use.

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 the formulated product Carophyll® Stay Pink have been sent to the Community Reference Laboratory for Feed Additives. The sample set provided by the applicant is a specific beadlets formulation (CAROPHYLL® Stay-Pink) which contains the feed additive. 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.

[1] Technical dossier, 1_Astaxanthin_DMDS.pdf, chapters 2.2.1 and 2.2.2

[2] Technical dossier, 1_Astaxanthin_DMDS.pdf, chapter 5.1.1

[3] Technical dossier, 1_Astaxanthin_DMDS.pdf, chapter 2.2.3, Tables 2-1 and 2-2

[4] Technical dossier, 1_Astaxanthin_DMDS.pdf, chapter 2.4.4.

[5] Technical Annex, Annex 2.18

[6] Technical Annex, Annex 2.16

[7] Technical Annex, Annex 2.19

[8] Technical dossier, 1_Astaxanthin_DMDS.pdf, chapter 5.6

RAPPORTEUR LABORATORY

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

ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Veterinary Faculty-National Veterinary Institute, Slovenia
- National Veterinary Research Institute, Poland
- VITO, Belgium
- Laboratory Agroalimentari - Department of Agriculture of the Generalitat of Catalonia, Spain
- Central Institute for Supervising and Testing in Agriculture, Czech Republic
- TLL Jena, Germany