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Institute for Reference Materials and Measurements
**European Union Reference Laboratory for Feed Additives**

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

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| **Dossier related to:** | FAD-2010-0158<br>CRL/100128 |
| **Product Name:** | *Neohesperidine dihydrochalcone* |
| **Active Substance(s):** | *Neohesperidine dihydrochalcone* |
| **Rapporteur Laboratory:** | European Union Reference Laboratory for Feed Additives (EURL -FA) |
| **Report prepared by:** | Carlos Magno Pinto (EURL -FA) |
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| **Date:** | 14/04/2011 |
| **Report approved by:** | Christoph von Holst |
| **Date:** | 14/04//2011 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Neohesperidine dihydrochalcone* under the category 'sensory additives', functional group 2(b) 'flavouring compounds', according to the Annex I of Commission Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for piglets, pigs, dogs, calves, ovines and fish.

*Neohesperidine dihydrochalcone* is a glycosidic flavonoid, odourless and almost white crystalline powder with a minimum purity of 96% on dried basis. It is intended to be incorporated into *premixtures*, *feedingstuffs* and drinking *water*. The Applicant suggested the following maximum levels in *feedingstuffs* for *Neohesperidin dihydrochalcone*: 35 mg/kg for dogs and piglets; 30 mg/kg for calves, fattening pigs, ovines and fish*;* and 5 mg/kg in *water*.

For the *identification* of *Neohesperidine dihydrochalcone* in the *feed additive,* the Applicant proposed the Commission Directive 2008/60/EC method. For the *determination* of *Neohesperidine dihydrochalcone* in *feed additive,* the Applicant proposed the European Pharmacopoeia method – Ph.Eur. 6.0, method 01/2008:1547, based on Thin Layer Chromatography (TLC). No performance characteristics of these methods are provided. However, the EURL considers these methods suitable to be used within the frame of official control.

For the determination of *Neohesperidine dihydrochalcone* in *premixtures* and *feedingstuffs* the Applicant proposed a single laboratory validated and further verified High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) method. The following performance characteristics were reported for *premixtures* and *feedingstuffs*:

- a relative standard deviation of *repeatability* ($RSD_r$) ranging from 2.6 to 5.1 %;
- a relative standard deviation for *intermediate precision* ($RSD_{ip}$) ranging from 2.6 to 6.2 %
- a *recovery* rate ($R_{Rec}$) ranging from 75 to 106%; and
- a limit of detection (LOD) and quantification (LOQ) of 0.1 and 0.8 mg/kg *feedingstuffs,* respectively.

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

For the determination of *Neohesperidine dihydrochalcone* in *water* the Applicant did not submit any experimental data nor analytical method, therefore the EURL cannot evaluate nor recommend any method for the determination of the active substance in this matrix.

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**

*Neohesperidine dihydrochalcone*, technological additives, sensory additives, piglets, pigs, dogs, calves, ovines, fish.

## 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 Directive 70/524/EC) for *Neohesperidine dihydrochalcone* under the category 'sensory additives', functional group 2(b) 'flavouring compounds', according to the Annex I of Commission Regulation (EC) No 1831/2003 [1]. Authorisation is sought for the use of *Neohesperidine dihydrochalcone* for piglets, pigs, dogs, calves, ovines and fish [1,2].

*Neohesperidine dihydrochalcone* is a glycosidic flavonoid, odourless and almost white crystalline powder with a minimum purity of 96% on dried basis. *Neohesperidine dihydrochalcone* is extracted from the immature fruit of *Citrus aurantium* (bitter orange), or obtained from naringin (the main flavonoid in *Citrus paradisii* (grapefruit) [3].

*Neohesperidine dihydrochalcone* is intended to be incorporated into *premixtures*, *feedingstuffs* and drinking *water*. The Applicant suggested the following maximum levels in *feedingstuffs* for *Neohesperidine dihydrochalcone*: 35 mg/kg for dogs and piglets; 30 mg/kg for calves, fattening pigs, ovines and fish*;* and 5 mg/kg in *water* [4].

## 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 European Union Reference Laboratory concerning applications for authorizations of *feed additives*, as last amended by Regulation (EC) No 885/2009, the EURL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application, or for each group of applications. For this particular dossier, the methods of analysis submitted in connection with the *Neohesperidine dihydrochalcone* 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 any impurities in the additive*

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective Community Reference Laboratories [5].

### *Description of the analytical methods for the determination of active agent(s) in feed additive, premixtures and feedingstuffs*

For the identification of *Neohesperidine dihydrochalcone* in the *feed additive,* the Applicant proposed the Commission Directive 2008/60/EC method [6], base on Neu's test.

For the determination of *Neohesperidine dihydrochalcone* in *feed additive,* the Applicant proposed the method from the European Pharmacopoeia method – Ph.Eur. 6.0, method 01/2008:1547 [7], based on Thin Layer Chromatography (TLC). No performance characteristics of these methods are provided. However, the EURL considers these methods suitable to be used within the frame of official control.

For the determination of *Neohesperidine dihydrochalcone* in *premixtures* and *feedingstuffs* the Applicant proposed a single laboratory validated and further verified High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) method [8], using calibration with external standard solution of *Neohesperidine dihydrochalcone* with a minimum purity of 99.5 %.

For *premixtures* a homogenised 10 g sample is placed into a 100 ml volumetric flask, 70 ml of the extract solution is added and mixed thoroughly and sonicated for 10 minutes. The sample is stirred for 20 minutes at room temperature. A 5 ml aliquot is taken and placed into a 250 ml volumetric flask, filled with the extract solution. The supernatant is filtered through a 0.45µm filter and the content transferred into a suitable HPLC vial and analysed by HPLC.

For *feedingstuffs*, a homogenised 10 g sample is placed into a 50 ml volumetric flask, the 50 ml of the extract solution is added and mixed thoroughly and sonicated for 10 minutes.

The sample is stored for 20 minutes at room temperature. The supernatant is filtered through a 0.45µm filter and the content transferred into a suitable HPLC vial and analysed by HPLC.

**Table 1:** Method performance characteristics for the determination of *Neohesperidine dihydrochalcone* in *premixtures* and *feedingstuffs* for piglets and calves.

| Matrix | $RSD_r$, % [10] | | $RSD_{ip}$, %[10] | | Recovery rate % | |
|---|---|---|---|---|---|---|
| | Validation | Verification | Validation | Verification | Validation[8] | Verification[9] |
| *Premixture* | 2.9 | 2.6 | 4.2 | 3.8 | 99-101 | 103 |
| *Feedingstuffs* | 2.6 | 5.1 | 2.6 | 6.2 | 97-106 | 75-100 |

$RSD_r$,$RSD_{ip}$ - relative standard deviation for *repeatability* and *intermediate precision*

The method performance characteristics derived from the validation and verification studies [8-9], were recalculated by the EURL [10] and are presented in Table 1. Furthermore, a limit of detection (LOD) and a limit of quantification (LOQ) of 0.1 and 0.8 mg/kg in *feedingstuffs* were determined by the Applicant [8].

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

For the determination of *Neohesperidine dihydrochalcone* in *water* the Applicant did not submit any experimental data nor analytical method, therefore the EURL cannot evaluate nor recommend any method for the determination of the active substance in this matrix.

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 European Pharmacopoeia method – Ph.Eur. 6.0, method 01/2008:1547, based on Thin Layer Chromatography (TLC) for the determination of *Neohesperidine dihydrochalcone* in the *feed additive*, and

- the single laboratory validated and further verified method using High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) to determine *Neohesperidine dihydrochalcone* in *premixture* and *feedingstuffs*.

For the determination of *Neohesperidine dihydrochalcone* in *water* the Applicant did not submit any experimental data nor analytical method, therefore the EURL cannot evaluate nor recommend any method for the determination of the active substance in this matrix.

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

For the determination of the *Neohesperidine dihydrochalcone* in the *feed additive*:

- Thin Layer Chromatography (TLC), European Pharmacopoeia 6.0, method 01/2008:1547

For the determination of *Neohesperidine dihydrochalcone* in *premixtures* and *feedingstuffs*:

- High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD).

## 5.DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive *Neohesperidine dihydrochalcone*, have been sent to the European Union Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the EURL by EFSA.

## 6.REFERENCES

[1] *Application/Ref: SANCO/D/2: Forw.Appl.1831/00106 (10019) -2010

[2] *Application, Annex A, Proposal for register entry

[3] *Technical Dossier, Section II.2. Characterisation of the active Substance(s)/ agents(s)

[4] *Technical Dossier, Section II.5.1. Conditions of use

[5] 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

[6] *Technical Dossier, Section II.2.6.1 Methods of analysis for the active substance

[7] *Technical Dossier, Section II. Annex II_11_ PhEur_2008

[8] *Technical Dossier, Section II. Annex II_21_Validation_NHCD

[9] *Technical Dossier, Section II. Annex II_22_Verification_NHCD

[10] *Supplementary information

*Refers to Dossier no: FAD-2010-0158

## 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.

## 8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- 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)
- Kmetijski inštitut Slovenije, Ljubljana (SLO)