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JOINT RESEARCH CENTRE
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 Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

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| Dossier related to: | FAD-2010-0036<br>EURL/ 100048 |
| Name of product: | Optimun |
| Active Agent (s): | *Nucleotides (adenine, cytosine, guanine, thymine, uracil and purified RNA)* |
| Rapporteur Laboratory: | European Union Reference Laboratory for Feed Additives (EURL-FA)<br>Geel, Belgium |
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| Report approved by:<br>Date: | Christoph von Holst<br>07/02/2011 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for new product *Optimun* under the category 'zootechnical additives', functional group 4(d) 'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. The product *Optimun* is a brownish powder, consisting of a minimum 15% of total *nucleotides (adenine, cytosine, guanine, thymine, uracil and purified RNA)* and *Saccharomyces cerevisiae* yeast as a carrier. Specifically, the authorisation is sought for the use of *Optimun* for salmon and trout. It is intended to be mixed to complete *feedingstuffs* at a dose of 2000 mg/kg.

For the determination of *nucleotides* in the *feed additive* the Applicant proposed a single laboratory validated and further verified method, based on Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) coupled to UV detector. The Applicant reported the following performance characteristics: - a relative standard deviation of *repeatability* ($RSD_r$) and relative standard deviation of *intermediate precision* ($RSD_{ip}$) ranging from 3.6 to 4.2%; and - a *recovery* rate ($R_{Rec}$) ranging from 83.5 - 89.5%.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) coupled to UV detector, to determine *nucleotides (adenine, cytosine, guanine, thymine, uracil and purified RNA)* in *feed additive.*

The Applicant did not provide any experimental method or data for the determination of *nucleotides* in *feedingstuffs*. Furthermore, the unambiguous determination of the content of *nucleotides* added to *feedingstuffs* via *Optimun* is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *nucleotides* in *feedingstuffs*.

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**

*nucleotides, adenine, cytosine, guanine, thymine, uracil, purified RNA*, zootechnical additives, other zootechnical additives, salmon, trout.

## 1. BACKGROUND

In the current application authorisation is sought under article 4(1) for new product *Optimun* under the category 'zootechnical additives', functional group 4(d) 'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003 [1]. The product *Optimun* is a brownish powder, consisting of a minimum 15% of total *nucleotides (adenine, cytosine, guanine, thymine, uracil and purified RNA)* and 81% of yeast *Saccharomyces cerevisiae*, acting as a carrier [2, 3]. Specifically, the authorisation is sought for the use of *Optimun* for salmon and trout. The product is intended to be mixed to complete *feedingstuffs* at a dose of 2000 mg/kg [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 (EFSA) for each application or group of applications. For this dossier, the methods of analysis submitted in connection with *Optimun*, 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, mercury, lead, dioxins and PCBs) are available at the respective Community Reference Laboratories [4].

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

For the determination of *nucleotides (adenine, cytosine, guanine, thymine, uracil and purified RNA)* in the *feed additive* the Applicant proposed a single laboratory validated

spectrophotometric method, based on measuring absorption at 270 nm [5]. The data supplied by the Applicant does not prove the transferability of the method [6].

However, the Applicant also proposed, for the determination of *nucleotides (adenine, cytosine, guanine, thymine, uracil and purified RNA)* in the *feed additive,* a single laboratory validated Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) method [7], as described by Lassalas et al. [8]. In this method nucleotides are hydrolysed with acid to purine and pyrimidine bases and separated on a reverse phase HPLC system. The method allows the quantification of every single nucleotide in the product and the total nucleotide content. The sample (50 mg) is extracted with 70 % perchloric acid at 95$^{o}$C for 60 minutes. After cooling to room temperature, ammonium dihydrogenphosphate (28.5 mM) is added and the solution is incubated for additional 60 minutes at 95$^{o}$C. The solution is then cooled to room temperature and centrifuged for 15 minutes at 13000 rpm. An aliquot is injected in the HPLC system, with the following mobile phase: potassium acetate buffer (50 mM, pH 6.0) and methanol. As the bases are eluted from the column, they are detected by spectrophotometry at 254 nm and quantified using external calibration curves. Commercially available bases (adenine, cytosine, guanine, thymine and uracil; all with a purity of >99%) are used as calibrants. This single laboratory validated method was further verified by a second independent laboratory [9]. The following method performance characteristics were recalculated by the EURL, based on the experimental data from both laboratories and provided by the Applicant:

- a relative standard deviation of *repeatability* ($RSD_r$) and a relative standard deviation of *intermediate precision* ($RSD_{ip}$) ranging from 3.6 to 4.2%; and

- a *recovery* rate ($R_{Rec}$) ranging from 83.5 - 89.5%.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) coupled to UV detector method, submitted by the Applicant to determine *nucleotides (adenine, cytosine, guanine, thymine, uracil and purified RNA)* in *feed additive.*

The Applicant did not provide any experimental method or data for the determination of *nucleotides* in *feedingstuffs*. Furthermore, the unambiguous determination of the content of *nucleotides* added to *feedingstuffs* is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *nucleotides* in *feedingstuffs*.

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 single laboratory validated and further verified Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) coupled to UV detector method, submitted by the Applicant, for the determination of *nucleotides* in *feed additive*.

The Applicant did not provide any experimental method or data for the determination of *nucleotides* in *feedingstuffs*. Furthermore, the unambiguous determination of the content of *nucleotides* added to *feedingstuffs* is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *nucleotides* in *feedingstuffs*.

***Recommended text for the register entry (analytical method)***

For the determination of *nucleotides* in *feed additive*:

- the single laboratory validated and further verified Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) coupled to UV detector

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Optimun* 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/Ref:SANCO/D/2:Forw.Appl.1831/0027-2010.
[2] *Application, Proposal for Register Entry, Annex A
[3] *Technical dossier, Section II/2.1.3. Qualitative and quantitative composition
[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, Annex_II_18_SOP_006
[6] *Technical dossier, Annex_II_27_Validation_Verification_SOP_006
[7] *Technical dossier, Annex_II_11_SOP_005
[8] *Technical dossier, Annex_II_2_Lassalas_1993
[9] *Technical dossier, Annex_II_26_Validation_Verification_SOP_005
*Refers to Dossier no: FAD-2010-0036

## 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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- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)