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

Dossier related to:	FAD-2010-0197 CRL/100007
Name of Additive:	Folic Acid
Active Substance(s):	Folic Acid
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
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Report revised by: Date:	Piotr Robouch (EURL-FA) 15/09/2011
Report approved by: Date:	Christoph von Holst 16/09/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Folic acid* under the category/functional group 3(a) 'nutritional additives'/'vitamins, pro-vitamins and chemically well defined substances having similar effect' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all animal species and categories. According to the Applicant, *Folic acid* is a yellowish to orange, crystalline powder with a minimum purity of 95 %. The *feed additive* is intended to be incorporated in *feedingstuffs* or complementary *feedingstuffs* through *premixtures* or directly in *water*. The Applicant did not specify any maximum or minimum concentration of *Folic acid* in *feedingstuffs* or *water.*

For the identification and quantification of *Folic acid* in the *feed additive* the Applicant proposes the internationally recognised European Pharmacopoeia method (Ph.Eur. 6th Edition, monograph 0067), based on Liquid Chromatography coupled with UV detection at 280 nm (LC-UV). Even though no performance characteristics are provided, the EURL considers this method suitable to be used within the frame of official control.

For the quantification of *Folic acid* in *premixtures* the Applicant proposed a single laboratory validated and further verified method, based on Reversed Phase High Performance Liquid Chromatography (RP-HPLC) with UV at 280 nm. The following performance characteristics were reported:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.3 to 2.2%
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 1.8 to 2.5%

Based on these performance characteristics, the EURL recommends for official control the validated and further verified RP-HPLC-UV method to determine *Folic acid* in *premixtures* within a concentration range between 200 mg and 200 g/kg.

For the quantification of total *folates* (including added *Folic acid*) in *feedingstuffs* and *water* the Applicant proposed the ring-trial validated method EN 14131 using a microbiological assay with *Lactobacillus Casei*, subsp, *rhamnosus* (ATCC 7469) to extracted folates. The following performance characteristics were determined for folate concentrations ranging from 0.5 to 13 mg/kg:

- RSD_r ranging from 4.9 to 9.2 %, and
- a relative standard deviation for *reproducibility* (RSD_R) ranging from13.8 to 22.3 %.

Even though the Applicant did not provide any data for the quantification of *Folic acid* in water the concentration range covered by the CEN method includes the recommended minimum concentration suggested by the Applicant for w*ater.*

Based on the performance characteristics presented the EURL recommends for official control the CEN ring-trial validated microbiological method (EN 14131) to determine total *folates* (including added *Folic acid*) in *feedingstuffs* and w*ater.*

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

Folic acid, nutritional additive, vitamins, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use in water) and 10(2) (re-evaluation of the additive already authorised under provisions of Council Directive 70/524/EEC) for *Folic acid* under the category/functional group 3(a) 'nutritional additives'/'vitamins, pro-vitamins and chemically well defined substances having similar effect' according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought for the use of the *feed additive* for all animal species and categories [2].

According to the Applicant, the *Folic acid* is a yellowish to orange, crystalline powder with a minimum purity of 95 % [3]. The *feed additive* is intended to be incorporated in complete or complementary *feedingstuffs* through *premixtures* or directly in *water*. The Applicant did not specify any minimum or maximum concentrations of *Folic acid* in *feedingstuffs* or *water*, however, the following doses were recommended ranging from 0.5 to 65 mg/kg in completed *feedingstuffs;* and from 0.8 to 1.2 mg/L for *water* [3].

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 particular dossier, the methods of analysis submitted in connection with *Folic 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, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [4].

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

For the determination of *Folic acid* in the *feed additive* the Applicant proposes the internationally recognised European Pharmacopoeia method (Ph. Eur. 6th Edition, monograph 0067). The *identification* of Folic acid is based on (i) the specific optical rotation and on (ii) liquid chromatography coupled to UV detection at 280 nm (LC-UV) [5]. The same LC-UV method is used for the *quantification* of *Folic acid*. Even though no performance characteristics are provided, the EURL considers this method suitable to be used within the frame of official control.

For the quantification of *Folic acid* in *premixtures* the Applicant proposed a single laboratory validated and further verified method [6], based on Reversed Phase High Performance Liquid Chromatography (RP-HPLC) with UV detection at 280 nm.

Folic acid is extracted using an of aqueous solution composed of 1 ml of 1 % ascorbic acid, 10 ml of 2 % ammonium hydroxide solution and 50 ml of water. The mixture is treated in a swirling ultrasonic bath at 50^{o}C for 20 minutes. Cooled at room temperature and centrifuged, 20 µL of supernatant is then injected into the HPLC. Calibration is performed using an external standard solution of *Folic acid*. The performance characteristics provided by the Applicant are presented in Table 1.

Based on these performance characteristics, the EURL recommends for official control the validated and further verified RP-HPLC-UV method to determine *Folic acid* in *premixtures.*

Table 1: Performance characteristics for the determination of *Folic acid* in *premixtures* [6]

	RSD_r %	RSD_{ip} %	R_{rec}%	LOQ (mg/kg)	LOD (mg/kg)
Validation	0.33	1.79	-	10	3
Verification	2.15	2.45	99.1	23	7

RSD_r and RSD_{ip}= relative standard deviation for *repeatability* and *intermediate precision*;
R_{rec}. = recovery rate; LOD and LOQ = Limit of detection and quantification
Concentration range investigated: from 100 mg/kg to 200 g/kg *folic acid* in *premixtures*

For the quantification of total *folates* (including added *Folic acid*) in *feedingstuffs* and *water* the Applicant proposed a method [7] based on the ring-trial validated method EN 14131 [8] "*Foodstuffs – Determination of folates by microbiological assay*". The samples are suspended in a phosphate buffer and heated for the extraction of folates. These folates are diluted in a medium containing all required growth nutrients except folate. The growth response determined by measuring the turbidity at 620 nm of *Lactobacillus Casei*, subsp, *rhamnosus* (ATCC 7469) to extracted folates is compared to the growth response of calibrant solutions with known concentrations.

The following performance characteristics were determined for folate concentrations ranging from 0.5 to 13 mg/kg [8]:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 4.9 to 9.2 %, and
- a relative standard deviation for *reproducibility* (RSD_R) ranging from13.8 to 22.3 %.

Furthermore, the suitability of the above mentioned CEN method to *feedingstuffs* is supported by the publication of J.F.Gregory *et al*. [9] on the "adequacy of these [microbiological growth] assays for the determination of folates in foods and other biological materials".

Even though the Applicant did not provide any data for the quantification of *Folic acid* in water, the concentration range covered by the CEN method includes the recommended minimum concentration suggested by the Applicant for w*ater.*

Based on the performance characteristics presented, the EURL recommends for official control the CEN ring-trial validated microbiological method (EN 14131) to determine total folates (including added *Folic acid*) in *feedingstuffs* and w*ater.*

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:

- Liquid Chromatography and UV detection (LC-UV) (Ph. Eur. 6th edition, monograph 0067) for the quantification of *Folic acid* in the *feed additive;*
- Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) for the quantification of total folates (including added *Folic acid*) in *premixtures;*
- Microbiology assay with *Lactobacillus casei* subs. *rhamnosus* (based on EN 14131) for the quantification of total folates (including added *Folic acid*) in *feedingstuffs* and w*ater.*

Recommended text for the register entry (analytical method)

For the quantification of *Folic acid* in *feed additive*:

- Liquid Chromatography and UV detection (LC-UV) (Ph. Eur. 01/2008:0067)

For the quantification of *Folic acid* in *premixtures:*

- Reverse Phase High-Performance Liquid Chromatography coupled to UV detection (RP-HPLC-UV)

For the quantification of total *folates* (incl. added *Folic acid*) in *feedingstuffs* and *water*:

- Microbiological assay – based on CEN ring-trial validated method EN 14131

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Folic acid* have been sent to the European Union Reference Laboratory for Feed Additives. However, the Applicant provided reference samples for *Folic acid.* The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANCO/D/2 Forw. Appl. 1831/00110(10039)-2010

[2] *Application, Proposal for Register Entry – Annex A

[3] *Technical dossier, Section II

[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_2_PhEur_67

[6] *Technical Dossier, Section II, Annex_II_41_MoA_Premix

[7] *Supplementary information, *Protocol for the determination of folic acid in feed and water*– VITAC_Folic_Acid_MoA-Feed-Water-11-05-26

[8] *CEN standard EN 14131 "Foodstuffs – Determination of folate by microbiological assays (cf. Technical Dossier, Section II, Annex_II_40_MoA_CEN)

[9] J.F.Gregory et al., Journal of Food Composition and Analysis 3 (1990) 134-144

* Refers to Dossier No. FAD-2010-0197

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

The following National Reference Laboratories contributed to this report:

- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena, DE
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino, IT
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby, DK
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, CZ
- Państwowy Instytut Weterynaryjny, Puławy, PL
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin, PL
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya,Cabrils, ES
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien, AT