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

Vitamin K_1 (phytomenadione)

(FAD-2020-0006; CRL/190066)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2020-0006 - CRL/190066**
Name of Product:	***Vitamin K_1 (phytomenadione)***
Active Agent:	**Phytomenadione**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis** **07/01/2021**
Report approved by: Date:	**Christoph von Holst** **07/01/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *vitamin K_1 (phytomenadione)* as *feed additive* under the category/functional group 3(a) "nutritional additive"/"vitamins, provitamins and chemically well-defined substances having a similar effect" according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for horses.

The product presented by the Applicant contains as *active substance* a mixture of *trans-phytomenadione* (not less than 75 %, w/w), *cis-phytomenadione* (approx. 20 %, w/w) and *trans-epoxy-phytomenadione* isomers (not more than 4 %, w/w). The sum of the three components is not less than 97 % (w/w). The product is placed on the market as preparation with a typical content of 42 g *vitamin K_1*/kg of the preparation. It is intended to be used in complementary feed with a minimum content of 140 mg *vitamin K_1* /kg feed.

For the determination of *vitamin K_1 (trans-* and *cis-isomers of phytomenadione*, and *trans-epoxy-phytomenadione)* in the *feed additive* the Applicant proposed the method presented within the *phytomenadione* monograph of the European Pharmacopoeia, where quantification is based on the generic high performance liquid chromatography (HPLC) method as described in the test for related substances. The EURL recommends for official control the European Pharmacopoeia method described in the *phytomenadione* monograph to determine *vitamin K_1 (trans-* and *cis-isomers of phytomenadione*, and *trans-epoxy-phytomenadione)* in the *feed additive*.

For the determination of *vitamin K_1* in the additive preparation and in complementary feed the Applicant presented a procedure based on the ring-trial validated CEN method (EN 14148) intended for foodstuffs. The analytical method is based on HPLC coupled with fluorescence detection (FLD). The Applicant's method is a slightly modified EN 14148 method in order to reach the same concentration range as specified in the CEN method and is routinely used by the Applicant.

In the frame of the ring-trial validation studies supporting the CEN method the following performance characteristics were reported for the quantification of *vitamin K_1* in milk and infant formula samples with a content ranging from 0.05 to 1.2 mg/kg: a relative standard deviation for *repeatability* (RSD_r) from 2.6 to 9.0 % and a relative standard deviation for reproducibility (RSD_R) from 4.3 to 10.9 %.

Based on the findings above, the EURL recommends for official control the Applicant's method based on the HPLC-FLD ring-trial validated method EN 14148 to determine *vitamin K_1* in the additive preparation and in complementary feed.

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, as last amended by Regulation (EU) 2015/1761), is not considered necessary.

KEYWORDS

Vitamin K_1 (phytomenadione), nutritional additives, vitamins, pro-vitamins and chemical well-defined substances having a similar effect, horses.

1. BACKGROUND

In the current application an authorisation is sought under Article 4 (authorisation of a new *feed additi*ve) for *vitamin K_1 (phytomenadione)* as *feed additive* under the category/functional group 3(a) "nutritional additive"/"vitamins, provitamins and chemically well-defined substances having a similar effect" according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for horses [1-3].

The product presented by the Applicant is a clear, yellow-to-amber, viscous and odourless liquid produced by chemical synthesis. According to the Applicant, the *active substance* is a mixture of *trans-phytomenadione* isomers, *cis-phytomenadione* isomers and *trans-epoxy-phytomenadione* isomers. It contains not less than 75 % (w/w) of *trans-phytomenadione* isomers and not more than 4 % (w/w) of *trans-epoxyphytomenadione* isomers. In addition, *cis-phytomenadione* isomers are present at approx. 20 % (w/w). The sum of the three components is not less than 97 % (w/w). The product is placed on the market in the form of solid preparation (Quinaquanone®) with a typical content of 42 g *vitamin K_1* /kg of the preparation [4]. The product is intended to be used in complementary feed containing not less than 140 mg *vitamin K_1*/kg feed (i.e. Bonekare® - complementary feed used by the Applicant for the studies supporting the present dossier) [3].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *vitamin K_1 (phytomenadione)* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the determination of *vitamin K_1 (trans-* and *cis-isomers of phytomenadione; trans-epoxy-phytomenadione)* in the *feed additive* the Applicant proposed the method presented within the *phytomenadione* monograph of the European Pharmacopoeia where quantification is based on the generic high performance liquid chromatography (HPLC) method as described in the test for related substances [5,6,7]. Furthermore, specific modifications are listed in the *phytomenadione* monograph [6]. The procedure has been successfully applied in the frame of the supporting studies presented within the current dossier (i.e. batch-to-batch variation and stability studies) [4,8,9]. The Applicant's product has been analysed by HPLC coupled with a Diode Array Detector (DAD) (detection 249 nm, reference 360 nm) [10].

The EURL recommends for official control the European Pharmacopoeia method described in the *phytomenadione* monograph to determine *vitamin K_1 (trans- and cis-isomers of phytomenadione; trans-epoxy-phytomenadione)* in the *feed additive.*

For the determination of *vitamin K_1* in the additive preparation and in complementary feed the Applicant presented a single-laboratory validated method based on HPLC-DAD [5,11,12].

Alternatively, on request of the EURL, the Applicant submitted the ring-trial validated CEN method intended for foodstuffs (EN 14148) based on HPLC coupled with fluorescence detection (FLD) [13]. The CEN method has been validated for milk and infant formula and it is mentioned in the scope of the standard itself that "*laboratory experiences exist which show that the method is also applicable to other type of foodstuffs*" [13-15]. According to the Applicant, the ring-trial tested "dry blended infant formula" is a matrix similar in composition to the typical *vitamin K_1* preparation and therefore additional experiments for extending the scope of the standard are not considered necessary [14]. The method of the Applicant is slightly modified from EN 14148 in order to reach the same concentration range as specified in the CEN method and is routinely used by the Applicant for the analysis of *vitamin K_1* in the additive preparation with a typical content of 42 g *vitamin K_1*/kg (w/w) (i.e. Quinaquanone®) and in complementary feed containing not less than 140 mg/kg (i.e. Bonekare®) [16].

The homogenised sample (0.5 to 1 g) is accurately weighed into a 100 ml sealable amber test tube. 15 ml of water are added and the tube content is vortexed for 20 min. 5 ml of phosphate buffer and 1 g of lipase are added to the sample solution and mixed. The sample is enzymatically digested during incubation at 37 °C for 2 h under vigorous shaking. Once the sample has been cooled down at room temperature, 10 ml of reagent alcohol solution and 1 g

of potassium carbonate are added and the tube content is mixed. 30 ml of hexane are added and the sample tube is vigorously shaken. The vessel is left in the dark for 10 min allowing the separation of the phases. 1 ml of the hexane phase is pipetted into a test tube, hexane is evaporated under nitrogen and the precipitate is dissolved in 10 ml of methanol. The Applicant's protocol modifies the original EN protocol and foresees that the solution is diluted in methanol at the ratio 1:100 (v/v) for the analysis of complementary feed samples. This dilution should be further diluted with methanol at the ratio 1:200 (v/v) for the analysis of the additive preparation. 20 µl of the diluted solution are injected into the HPLC and *vitamin K_1* is determined by FLD (excitation: 243 nm, emission: 430 nm) using *vitamin K_1 (Phytomenadione)* as an external standard for calibration. *Vitamin K_1* isomers are quantified as a single unresolved peak [16].

In the frame of the ring-trial validation studies supporting the CEN method (where different dilutions were applied compared to the modified method), the following performance characteristics were reported for the quantification of *vitamin K_1* in milk and infant formula samples with a content ranging from 0.05 to 1.2 mg/kg: a relative standard deviation for *repeatability* (RSD_r) ranging from 2.6 to 9.0 % and a relative standard deviation for reproducibility (RSD_R) ranging from 4.3 to 10.9 % [13].

Based on these findings, the EURL recommends for official control the Applicant's method based on the HPLC-FLD ring-trial validated method EN 14148 to determine *vitamin K_1* in the additive preparation and in complementary feed.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control: (i) the High Performance Liquid Chromatographic method presented within the phytomenadione monograph of the European Pharmacopoeia (8.0, 01/2014:1036) to determine *vitamin K_1* in the *feed additive*; and (ii) the Applicant's method based on High Performance Liquid Chromatography coupled with fluorescence detection (HPLC-FLD) based on the ring-trial validated method EN 14148 to determine *vitamin K_1* in the additive preparation and in complementary feed.

Recommended text for the register entry (analytical method)

For the determination of *vitamin K_1* in the *feed additive*:

- High Performance Liquid Chromatography - European Pharmacopoeia (8.0, 01/2014:1036)

For the determination of *vitamin K_1* in the additive preparation and in complementary feed:

- High Performance Liquid Chromatography with Fluorescence detection (HPLC-FLD)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *vitamin K_1 (phytomenadione)* 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, Annex I – submission number 1579863293020-2518

[2] *Technical dossier, Section II: 2.1.2 Proposal for classification

[3] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[4] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition (active substance/agent, other components, impurities, batch-to-batch variation)

[5] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance

[6] *European Pharmacopoeia monograph - Ph. Eur. 8.0, 01/2014:1036

[7] *European Pharmacopoeia monograph - Ph. Eur. 8.0, 01/2014:20229

[8] *Technical dossier, Section II: Annex_II_1_CoAs.pdf

[9] *Technical dossier, Section II: Annex_II_16_Stability_additive.pdf

[10] *Technical dossier, Section II: Annex_II_2_Chromatograms.pdf

[11] *Technical dossier, Section II: Annex_II_23

[12] *Technical dossier, Section II: Annex_II_24

[13] EN 14148:2003 – Foodstuffs: Determination of vitamin K_1 by HPLC

[14] *Supplementary information: 2020-09-10-SIn letter May 11-draft reply EURL.pdf

[15] Woolard, D.C., Indyk H.E., Bertram, Y.F and Cook, K.K.: Determination of Vitamin K_1 Isomers in Food by liquid Chromatography with C 30 Bonded-Phase Column, J. AOAC intern. 85, 2002, 682-691

[16] *Supplementary information: SIn_EURL_Sept_2020_Annex_I_Analytical_Method.pdf

*Refers to Dossier no: FAD-2020-0006

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- Wageningen Food Safety Research (WFSR), Wageningen (NL)[1]
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)

[1] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen (NL).