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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

Tocopheryl Phosphate Mixture (TPM)

(FAD-2017-0057; CRL/170046)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2017-0057 - CRL/170046**
Name of Product:	***Tocopheryl Phosphate Mixture (TPM)***
Active Agent (s):	**alpha-tocopheryl di-hydrogen phosphate** **alpha-di-tocopheryl hydrogen phosphate**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis (EURL-FA)** **17/07/2019**
Report approved by: Date:	**Christoph von Holst** **17/07/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Tocopheryl Phosphate Mixture (TPM)*, 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. The *feed additive* is a mixture of two different forms of phosphorylated tocopherol namely *alpha-tocopheryl di-hydrogen phosphate (TP)* at a content ranging from 50-70 % w/w and *alpha-di-tocopheryl hydrogen phosphate (T_2P)* at a content ranging from 20-40 % w/w as well as small amounts of residual alpha-tocopherol; phosphoric acid and water. The *feed additive* is intended to be used in *feedingstuffs* through *premixtures* for all animal species without minimum or maximum limits.

Consequently, for the characterisation of *Tocopheryl Phosphate Mixture (TPM)* the Applicant submitted two single-laboratory validated method and based on high performance liquid chromatography (HPLC) with ultraviolet (UV) detection for the determination of *TP* and *T_2P* in the *feed additive*, and HPLC coupled to fluorescence detection (FD) for their determination in *premixtures* and *feedingstuffs*.

Upon request of the EURL the Applicant provided suitable evidences for the transferability of the proposed methods for the determination of *TP* and *T_2P* in the *feed additive*, *premixtures* and *feedingstuffs*.

Consequently the EURL considers the following methods submitted by the Applicant suitable for official control: a HPLC-UV to quantify *TP* and *T_2P* in the *feed additive* and the HPLC-FD method to quantify them in *premixtures* and *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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Tocopheryl Phosphate Mixture, alpha-tocopheryl di-hydrogen phosphate (TP), alpha-di-tocopheryl hydrogen phosphate (T_2P), nutritional additives, vitamins, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *Tocopheryl Phosphate Mixture (TPM)* 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][2][3]. Authorisation is sought for the use of the *feed additive* for all animal species and categories [2][3].

The *feed additive* is a *Tocopheryl Phosphate Mixture (TPM)* of two different forms of phosphorylated tocopherol namely *alpha-tocopheryl di-hydrogen phosphate (TP,)* at a content ranging from 50-70 % w/w, and *alpha-di-tocopheryl hydrogen phosphate (T_2P),* at a content ranging from 20-40 % w/w, as well as small amounts of residual *alpha*-tocopherol; phosphoric acid and water. *TPM* is synthetised from synthetic dl-alpha-tocopherol, also known as all-rac-alpha-tocopherol, in a two-step process, namely phosphorylation and hydrolysis [4].

The *feed additive* is intended to be used in *feedingstuffs* through *premixtures* for all animal species without minimum or maximum restrictions [4].

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 *Tocopheryl Phosphate Mixture (TPM)* 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)

The EURL has previously evaluated and recommended European Pharmacopoiea methods based on gas chromatography (for the *feed additive*) and the Union method using liquid chromatography (for *premixtures* and *feedingstuffs*) in the frame of a different *feed additive* for Vitamin E. However, the methods recommended by the EURL for the official control of

the currently authorised *feed additives* for Vitamin E are not suitable for the control of the *TPM*, because the alkaline ethanolic saponification step required by those methods will lead to a highly water soluble compound in the case of the *TMP*, which is thus not possible to be quantitatively extracted into petroleum ether [5].

Consequently, for the characterisation of *Tocopheryl Phosphate Mixture (TPM)* the Applicant submitted a different single-laboratory validated method based on high performance liquid chromatography (HPLC) with ultraviolet (UV) detection for the determination of *TP* and T_2P in the *feed additive*, and on HPLC coupled to fluorescence detection (FD) for their determination in *premixtures* and *feedingstuffs* [5].

The *feed additive* is mixed with a mixture of acetic acid and *iso*-propanol until completely dissolved. Then, the obtained solution is appropriately diluted with *iso*-propanol, mixed, and injected into the HPLC system. The HPLC analysis is performed using a gradient elution on a C8 column with a mixture of water, phosphoric acid and *iso*-propanol as mobile phase and the signal is measured spectrophotometrically at a wavelength of 286 nm [6]. The target analytes in the *feed additive* are quantified against *TP* and T_2P standards.

Furthermore for the *premixtures* and *feedingstuffs* the sample is extracted with acidified *iso*-propanol and the *tocopherol phosphates (TP and* T_2P*)* are analysed and quantified by HPLC using fluorescence detection (FD) against the correspondent external standards [7].

The method proposed for *premixtures* and *feedingstuffs* [7] involves an extraction procedure with acidified *iso*-propanol. The first step is wetting the sample with a mixture of acetic acid and *iso*-propanol followed by an extraction of the sample in an ultrasonic bath at 40 ^{o}C for 30 min, a mechanical shaking for another 30 min and a 6 min centrifugation. Then the obtained extract is appropriately diluted with alcoholic phosphoric acid, mixed, and filtered through a 0.45 μm syringe filter prior injection into the HPLC system. The HPLC analysis is performed on a C18 column applying a gradient elution with a mixture of acetonitrile, water, phosphoric acid and *iso*-propanol as mobile phase and the signal is measured at an excitation and emission wavelengths of 297 nm and 319 nm, respectively. The analytes are quantified in *premixture* and *feedingstuffs* samples against *TP* and T_2P standards available from the Applicant upon request. Following the EURL request the Applicant also provided the analytical methods for assessing the purity of these in-house standards [8].

The HPLC-UV and HPLC-FD methods proposed by the Applicant were single-laboratory validated for the *feed additive* [9], *premixtures* and *feedingstuffs* [10] according to some international recognised guidelines [11-13].

Table 1. The performance characteristics of the HPLC-UV and HPLC-FD methods for the determination of *TP* and *T_2P* in the *feed additive* (FA); *premixtures* (PM) and *feedingstuffs* (FS) reported in the frame of the validation studies

		Content (units)	RSD_r, %	RSD_{ip}, %	R_{ec} (%)	LOQ (µg/ml)
FA [14]	T_2P	25.48 (% w/w)	1.16*	1.43*	109	134
	TP	58.78 (% w/w)	1.19*	1.66*	98	268
PM [15]	T_2P	7.4 (g/kg)	1.78	-	93	0.5
	TP	15.7 (g/kg)	1.62	-	94	1.0
FS [16]	T_2P	0.13 (g/kg)	4.33	-	79	0.5
	TP	0.29 (g/kg)	7.26	-	87	1.0

RSD_r & RSD_{ip}: relative standard deviation for *repeatability* & *intermediate precision* respectively; R_{Rec}: *recovery* rate (%); (*) recalculated by the EURL

Upon request of the EURL, the Applicant provided the EURL verification forms for the target active substances to deliver relevant information from the validation studies. Furthermore, the Applicant performed some additional experiments and stated that the proposed methods have been transferred and used in different laboratory settings where a formal technical transfer validation was always conducted. The Applicant provided several documents demonstrating this statement [17]. The EURL considers these documents as evidence of the methods' transferability. Furthermore, this approach is a suitable alternative to the verification design proposed by the EURL.

Table 1 depicts the main performance characteristic as provided by the Applicant in the frame of the respective validation studies [14-16].

Additionally, the EURL re-calculated some key performance characteristics based on experimental data obtained by the Applicant in the frame of the homogeneity [18] and stability [19] studies as well as from the additional information provided from the Applicant [20] upon EURL request.

Table 2. Precision values of the HPLC-UV and HPLC-FD methods for the determination of *TP* and *T_2P* in the *feed additive* (FA) *premixtures* (PM) and *feedingstuffs* (FS) reported in the frame of homogeneity [18], stability [19] and transferability [20] studies

		Lab 1		Lab 2	
		RSD_r, %	RSD_{ip}, %	RSD_r, %	RSD_{ip}, %
FA [21]	T_2P	1.22	1.44	0.28	0.28
	TP	1.18	1.65	0.26	0.27
PM [22]	T_2P	2.56	2.56	1.94	2.93
	TP	2.40	6.35	2.48	6.00
FS [23]	T_2P	4.77	4.93	4.40	6.40
	TP	3.55	4.01	4.88	9.12

RSD_r & RSD_{ip}: relative standard deviation for *repeatability* & *intermediate precision* respectively

Table 2 presents the performance characteristics for *TP* and *T_2P* in the *feed additive*, *premixtures* and *feedingstuffs* as provided and/or re-calculated by the EURL [20-23] based on the data provided by the Applicant.

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.

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.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not considered necessary by the EURL.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the high-performance liquid chromatography coupled to UV detection (HPLC-UV), to quantify *alpha-tocopheryl di-hydrogen phosphate (TP)* and *alpha-di-tocopheryl hydrogen phosphate (T_2P)* in the *Tocopheryl Phosphate Mixture* (TPM) and the high performance liquid chromatography coupled to fluorescence detection (HPLC-FD), to quantify *TP* and *T_2P* in *premixtures* and *feedingstuffs.*

Recommended text for the register entry (analytical method)

For the quantification of *alpha-tocopheryl di-hydrogen phosphate (TP)* and *alpha-di-tocopheryl hydrogen phosphate (T_2P)* in *feed additive*:

- high performance liquid chromatography coupled to ultraviolet detection (HPLC-UV)

For the quantification of *alpha-tocopheryl di-hydrogen phosphate (TP)* and *alpha-di-tocopheryl hydrogen phosphate (T_2P)* in *premixtures* and *feedingstuffs*:

- high performance liquid chromatography coupled to fluorescence detection (HPLC-FD)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Tocopheryl Phosphate Mixture (TPM)* 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, Reference SANTE_E5_FWD. APPL. 1831-0048-2017

[2] *Application Form - Annex 1 – Submission No. 1508849684802-2150

[3] *Application, Proposal for Registry Entry – Annex A

[4] *Technical dossier, Section II, 2.1. Identity of the additive & 2.5 Conditions of use

[5] *Technical dossier, Section II, 2.6. Methods of analysis and reference samples

[6] *Technical dossier, Section II, Annexes II_28

[7] *Technical dossier, Section II, Annexes II-32

[8] ICH Guideline Q2 (R1) on Validation of Analytical Procedures

[9] *Technical dossier, Section II, Annexes II_29

[10] *Technical dossier, Section II, Annexes II_33

[11] Supplementary information, POH TM-0024 & POH TM-0025

[12] VICH Guideline GL 1 on Validation of Analytical Procedures: Definition and Terminology

[13] APVMA Guideline on Validation of Analytical Methods for active constituents and agricultural products

[14] Supplementary information, Annexes 3 & 5

[15] Supplementary information, Annexes 7 & 9

[16] Supplementary information, Annexes 11 & 13

[17] *Technical dossier, Section II, Annexes II_30; II-31 & II-35

[18] *Technical dossier, Section II, Annexes II_25a; II-25b

[19] *Technical dossier, Section II, Annexes II_22; II-24a

[20] Supplementary information, Annex D

[21] Supplementary information_EURL_ANOVA-FA.pdf

[22] Supplementary information_EURL_ANOVA-PM.pdf

[23] Supplementary information_EURL_ANOVA-FS.pdf

*Refers to Dossier no: FAD-2017-0057

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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- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Wageningen Food Safety Research[1] (WFSR) (NL)
- Avdelningen för kemi, miljö och fodersäkerhet, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)
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- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)

Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761:

[1] RIKILT Wageningen UR, Wageningen.