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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

Preparation of carvacrol, thymol, D-carvone, methyl salicylate and L-menthol

(FAD-2020-0070; CRL/200050)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2020-0070 - CRL/200050**
Name of Feed Additive:	***Preparation of carvacrol, thymol, D-carvone, methyl salicylate and L-menthol (Biomin® DC-P)***
Active Agent (s):	**carvacrol, thymol, D-carvone, methyl salicylate and L-menthol**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Zigmas Ezerskis** **09/06/2021**
Report approved by: Date:	**Christoph von Holst** **11/06/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for a currently authorised *preparation of carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol (Biomin® DC-P)* under the category/ functional group (4 d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for the use of the *feed additive* for all poultry species. The *feed additive* is an off-white powder composed of a mixture of the following active substances: 12 to 16 % (w/w) of *carvacrol*, 0.1 to 0.3 % (w/w) of *thymol*, 0.3 to 0.6 % (w/w) of *D-carvone*, 1.0 to 3.5 % (w/w) of *methyl salicylate* and 3.0 to 5.5 % (w/w) of *L-menthol*. In addition, *Biomin® DC-P* contains hydrogenated vegetable oil and silica as carriers.

The *feed additive* is intended to be incorporated through *premixtures* or directly into *feedingstuffs* at a recommended *Biomin® DC-P* content ranging from 65 to 105 mg/kg *feedingstuffs.*

For the quantification of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified multi-analyte method based on gas chromatography coupled to flame ionisation detection (GC-FID).

Based on the experimental evidence available the EURL recommends for the official control the GC-FID method for the quantification of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* in the *feed additive*.

Additionally, the Applicant submitted a single-laboratory validated and further verified multi-analyte method based on gas chromatography coupled to mass spectrometry (GC-MS) for the quantification of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* in *premixtures* and *feedingstuffs*. The EURL considers the GC-MS method proposed by the Applicant fit-for-purpose for the quantification of the total content of each active substances, namely *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol,* in *premixtures* and *feedingstuffs* at the content levels investigated in the frame of the validation and verification studies.

However, as the accurate determination of the *Biomin® DC-P* content added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate or recommend any method for official control to determine *Biomin® DC-P* 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

Preparation of carvacrol, thymol, D-carvone, methyl salicylate and *L-menthol (Biomin® DC-P),* zootechnical additives, other zootechnical additives, all poultry species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new use of a *feed additive*) for the *preparation of carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol (Biomin® DC-P)* under the category/ functional group (4 d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for the use of the *feed additive* for all poultry species [1].

Biomin® DC-P is a *feed additive* currently authorised (4d20) for chickens for fattening, chickens reared for laying and minor poultry species reared for laying by Commission Regulation (EC) No 2020/996 [2].

The *feed additive* is an off-white powder composed of a mixture of the following active substances: 12 to 16 % (w/w) of *carvacrol*, 0.1 to 0.3 % (w/w) of *thymol*, 0.3 to 0.6 % (w/w) of *D-carvone*, 1.0 to 3.5 % (w/w) of *methyl salicylate* and 3.0 to 5.5 % (w/w) of *L-menthol*. In addition, *Biomin® DC-P* contains hydrogenated vegetable oil and silica as carriers [3].

The *feed additive* is intended to be incorporated through *premixtures* or directly into *feedingstuffs* at a recommended *Biomin® DC-P* content ranging from 65 to 105 mg/kg *feedingstuffs* [4].

Note: The analytical methods for the determination of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* were already evaluated by the EURL in the frame of previous dossiers [5].

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 the *preparation of carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol (Biomin® DC-P)* 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 quantification of the five active substances (*carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol*) in the *feed additive* the Applicant submitted a single-laboratory validated and further verified multi-analyte method based on gas chromatography coupled to flame ionisation detection (GC-FID) [6].

The *Biomin® DC-P* sample (0.1 g) is mixed with 1.5 ml of ethyl acetate and shaken for 10 min. The extract is centrifuged at 10000 *g* for 10 min and the supernatant is collected and further diluted with ethyl acetate up to a final volume of 5 ml. An aliquot of the resulting solution is analysed by GC-FID. The quantification of the active substances is performed by external matrix-free calibration using standard solutions containing the mixture of all the relevant analytes [6].

Table 1 presents the performance characteristics for the quantification of the five analytes of interest in the *feed additive* based on experimental data obtained by the Applicant in the frame of the validation [7] and verification [8] studies and as recalculated by the EURL [9,10].

Based on the experimental evidence available the EURL recommends for the official control the single-laboratory validated and further verified multi-analyte GC-FID method for the quantification of *carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol* in the *feed additive*.

Table 1 Performance characteristics of the single-laboratory validated and verified multi-analyte GC-FID method for the quantification of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* in the *feed additive (Biomin® DC-P)* as recalculated by the EURL [9,10] from the validation (Val) [7] and verification (Ver) [8] data.

	carvacrol		D-carvone		L-menthol		methyl salicylate		thymol	
	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.
Mass fraction, g/kg	144		5.0		18.9		24.8		2.3	
RSD_r, % (**)	1.8	0.9	1.9	0.8	2.0	0.7	2.2	0.8	1.7	0.8
RSD_{ip}, % (**)	2.6	1.0	4.5	1.0	3.9	1.2	7.2	1.1	4.7	1.1
R_{rec}, %	101(*)	88	97(*)	85	97(*)	82	96(*)	91	97(*)	92

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec} - *recovery* rate;
(*) Recoveries are based on blank feed additive samples spiked with the analytes at low concentration levels (3 to 293 times lower than the minimum content of the analytes in the feed additive);
(**) Recalculated by the EURL based on validation and verification data.

In addition, the Applicant submitted a single-laboratory validated and further verified multi-analyte method based on gas chromatography coupled to mass spectrometry (GC-MS) for the quantification of *carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol* in *premixtures* and *feedingstuffs* [11].

The samples of *premixtures* and *feedingstuffs* are treated with ethyl acetate using an automated extraction device (a pressurised fluid extraction system). The obtained extracts are then centrifuged at 32000 *g* for 5 min and the supernatants are analysed by GC-MS. The quantification of the active substances is performed by external matrix-free calibration using standard solutions containing the mixture of all the relevant analytes [11].

Tables 2 and 3 present the relevant performance characteristics for the quantification of the five analytes of interest in *premixtures* (table 2) and *feedingstuffs* (table 3) based on experimental data obtained by the Applicant in the frame of the validation [13,14] and verification [15,16] studies and as recalculated by the EURL [17,18]. Based on the experimental evidence available EURL considers GC-MS method proposed by the Applicant fit-for-purpose for the quantification of the total content of each active substances, namely *carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol* in *premixtures* and *feedingstuffs* at the content levels investigated in the frame of the validation and verification studies.

However, as the accurate determination of the content of *Biomin® DC-P* added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate or recommend any method for official control to determine the content of *Biomin® DC-P* in *premixtures* and/or *feedingstuffs*.

Table 2 Performance characteristics of the single-laboratory validated and verified multi-analyte GC-MS method for the quantification of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* in *premixtures* as recalculated by the EURL [17] from the validation (Val) [13] and verification (Ver) [15] data.

	carvacrol		D-carvone		L-menthol		methyl salicylate		thymol	
	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.
Mass fraction, mg/kg	633.3	660	18.8		140.6	130	89.9	88.6	10.2	10.3
RSD_r, % (**)	1.4	3.1	1.4	3.2	0.9	3.4	0.1	3.9	2.4	2.71
RSD_{ip}, % (**)	2.8	3.1	12.3	3.2	3.7	3.4	17.4	3.9	14.4	2.7
R_{rec}, %	102*	106	80*	80	94*	87	71*	71	99*	97

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec} - *recovery* rate;
(*) recoveries are based on blank premixture samples spiked with the feed additive at 4.34 g/kg;
(**) Recalculated by the EURL based on validation [13] and verification [15] data.

Table 3 Performance characteristics of the single-laboratory validated and verified multi-analyte GC-MS method for the quantification of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* in *feedingstuffs* as recalculated by the EURL [18] from the validation (Val) [14] and verification (Ver) [16] data.

	carvacrol		D-carvone		L-menthol		methyl salicylate		thymol	
	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.
Mass fraction, mg/kg	10.3	9.4	0.4		2.4	1.9	1.9	1.5	0.2	
RSD_r, % (**)	6.5	14.7	5.2	6.3	7.8	14.7	5.3	12.1	9.8	11.5
RSD_{ip}, % (**)	6.5	16.3	7.1	14.9	15.6	17.1	5.3	13.5	11.5	14.0
R_{rec}, %	89*	90	100*	95	86*	74	100*	72	116*	113

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec} - *recovery* rate;
(*) recoveries are based on blank feed samples spiked with the feed additive at 85 mg/kg;
(**) Recalculated by the EURL based on validation [14] and verification [16] data.

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)

The Applicant submitted a single-laboratory validated and further verified multi-analyte methods based on gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) for the quantification of the content of *carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol* in tissues (chicken muscle, chicken skin and fat) [19-21].

However, as maximum residue limits (MRLs) have not been established and/or proposed by the Applicant in the frame of this dossier, the EURL considers that 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 the single-laboratory validated and further verified multi-analyte method based on gas chromatography coupled to flame ionisation detection (GC-FID) for the quantification of *carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol* in the *feed additive*.

In addition, the EURL considers the GC-MS method proposed by the Applicant fit-for-purpose for the quantification of the total content of each active substances, namely *carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol* in *premixtures* and *feedingstuffs* at the content levels investigated in the frame of the validation and verification studies.

However, as the accurate determination of the *Biomin® DC-P* content added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate or recommend any method for official control to determine *Biomin® DC-P* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol* in the *feed additive*:

– Gas chromatography coupled to flame ionisation detection (GC-FID)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the *preparation of carvacrol*, *thymol*, *D-carvone*, *methyl salicylate* and *L-menthol (Biomin® DC-P)* 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. 1831-0078-2020 & Annex 1, Application form Submission No. 1601456589858-2682

[2] Commission Implementing Regulation (EU) 2020/996 of 9 July 2020 concerning the authorisation of the preparation of carvacrol, thymol, D-carvone, methyl salicylate and L-menthol as a feed additive for chickens for fattening, chickens reared for laying and minor poultry species reared for laying (holder of authorisation Biomin GmbH) O.J. L 221, 10.7.2020

[3] Technical dossier, Section II: 2.1 Identity of the additive

[4] Technical dossier, Section II: 2.5 Conditions of use of the additive

[5] EURL Evaluation Report:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0023-biomin-dc-p.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2017-0026_biomin.pdf

[6] *Technical dossier, Section II – Annexes II_63-64

[7] *Technical dossier, Section II – Annex II_68 Validation Reports-feed additive

[8] *Technical dossier, Section II – Annex II_71-75 Verification Reports-feed additive

[9] Supplementary information- "eurl_anova_val-fa.pdf"

[10] Supplementary information-"eurl_anova_ver-fa.pdf"

[11] *Technical dossier, Section II – Annexes II_65, 66 &67

[12] *Technical dossier, Section II – Annexes II_63-64

[13] *Technical dossier, Section II – Annex II_69 Validation Reports-premixtures

[14] *Technical dossier, Section II – Annex II_70 Validation Reports-feed

[15] *Technical dossier, Section II – Annex II_76-80 Verification Reports- premixtures

[16] *Technical dossier, Section II – Annex II_81-85 Verification Reports- feed

[17] Supplementary information, "eurl_anova_val-ver-pm.pdf"

[18] Supplementary information, "eurl_anova_val-ver-fs.pdf

[19] *Technical dossier, Section II – Annexes II_86-89

[20] *Technical dossier, Section II – Annexes II_92-93

[21] *Technical dossier, Section II – Annexes II_96-105

*Refers to Dossier no: FAD-2020-0070

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)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)
- Wageningen Food Safety Research[1] (WFSR) (NL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)

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