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**European Union Reference Laboratory for Feed Additives**

JRC F.5/CvH/MGH/AS/Ares

# 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-2018-0023; CRL/180012)***

European Union Reference Laboratory
Feed Additives

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| Dossier related to: | **FAD-2018-0023 - CRL/180012** |
| Name of Product: | ***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 I** |
| Rapporteur Laboratory: | **European Union Reference Laboratory for Feed Additives (EURL-FA)**<br>**JRC Geel, Belgium** |
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| Report checked by:<br>Date: | **Zigmas Ezerskis**<br>**01/10/2018** |
| Report approved by:<br>Date: | **Christoph von Holst**<br>**01/10/2018** |

## EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) 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, authorisation is sought for the use of the *feed additive* for chickens for fattening and reared for laying and minor avian species other than laying species.

The *feed additive* is an off-white powder consisting of the following active substances (expressed as percentage mass fractions related to the *feed additive*): 10 to 16 % of *carvacrol,* 0.1 to 0.3 % *thymol,* 0.3 to 0.6 % *D-carvone,* 1 to 4.5 % *methyl salicylate* and 3 to 5.5 % *L-menthol*. In addition, it contains hydrogenated vegetable oil and silica as carriers.

The *feed additive* is intended to be incorporated into *feedingstuffs* through *premixtures* with a proposed *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). The following performance characteristics were reported for the five analytes mentioned above: a relative standard deviation for *repeatability* ($RSD_r$) ranging from 0.7 to 1,6 %; a relative standard deviation for *intermediate precision* ($RSD_{ip}$) ranging from 0.9 to 6.5 %; and a *recovery* rate ($R_{rec}$) ranging from 82 to 92 %.

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*.

Since the accurate determination of the *Biomin® DC-P* content added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine *Biomin® DC-P* 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, 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 zotechnical additives, chickens for fattening and reared for laying, minor avian species other than laying species

## 1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new *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, authorisation is sought for the use of the *feed additive* for chickens for fattening and reared for laying, and minor avian species other than laying species [1].

The *feed additive* is an off-white powder composed of a mixture of the following natural or synthetic chemically defined flavourings as active substances (expressed as percentage mass fractions related to the *feed additive*): 10 to 16 % of *carvacrol,* 0.1 to 0.3 % of *thymol,* 0.3 to 0.6 % of *D-carvone,* 1 to 4.5 % of *methyl salicylate* and 3 to 5.5 % of *L-menthol* [2, 3]. In addition, *Biomin® DC-P* contains hydrogenated vegetable oil and silica as carriers [3].

The *feed additive* is intended to be incorporated into *feedingstuffs* through *premixtures* with a proposed *Biomin® DC-P* content ranging from 65 to 105 mg /kg *feedingstuffs* [2,3].

## 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 *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) [4-6].

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 the 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 [4].

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 [5] and verification [6-10] studies and as recalculated by the EURL [11,12].

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** The 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 [11-12] from the validation (Val) and verification (Ver) data.

| | carvacrol | | D-carvone | | L-menthol | | methyl salicylate | | thymol | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Val.** | **Ver.** | **Val.** | **Ver.** | **Val.** | **Ver.** | **Val.** | **Ver.** | **Val.** | **Ver.** |
| **Mass fraction, g/kg** | 144 | 144 | 5.0 | 5.0 | 18.9 | 18.9 | 24.8 | 24.8 | 2.3 | 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 |
| **Reference** | [5] | [6] | [5] | [7]] | [5] | [8] | [5] | [9] | [5] | [10] |

$RSD_r$ 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 244 times lower than the minimum content of the analytes in the feed additive).
(**) Recalculated by the EURL based on validation and verification data

Since the accurate determination of the content of *Biomin® DC-P* added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine *Biomin® DC-P* in *premixtures* and/or *feedingstuffs*.

Furthermore, the accurate quantification of the content of the individual substances of interest added to *premixtures* and *feedingstuffs* is not possible in the frame of official control, due to the potential natural occurrence of these analytes in the mentioned matrices.

Nevertheless, 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 the content of *carvacrol, thymol, D-carvone, methyl salicylate* and *L-menthol* added to *premixtures* and *feedingstuffs* [13].

As the validation [14,15] and verification studies [16-25] in *premixtures* and *feedingstuffs* led to low recovery values (ranging from 50 % to 80 %) for all five analytes, the EURL does not recommend this multi-analyte GC-MS method for the quantification of the analytes of interest 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.

***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)***

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)***

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

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.*

Since the accurate determination of *Biomin® DC-P* content added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor 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 *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. 1831-0031-2018 & Annex 1, Application form Subm. No. 1524642952890-2212
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the feed additive; methods of analysis
[4] *Technical dossier, Section II – Annexes II_68 & 69
[5] *Technical dossier, Section II – Annex II_72 Validation Report_product_DC-P
[6] *Technical dossier, Section II – Annex II_75 Verification Additive Carvacrol
[7] *Technical dossier, Section II – Annex II_76 Verification Additive D-carvone
[8] *Technical dossier, Section II – Annex II_77 Verification Additive L-menthol
[9] *Technical dossier, Section II – Annex II_78 Verification Additive Methyl salicylate
[10] *Technical dossier, Section II – Annex II_79 Verification Additive Thymol
[11] Supplementary information- ANOVa validation
[12] Supplementary information- ANOVa verification
[13] *Technical dossier, Section II – Annexes II_70-71
[14] *Technical dossier, Section II – Annex II_73 Validation Report_premix_DC-P
[15] *Technical dossier, Section II – Annex II_74 Validation Report_feed_DC-P
[16] *Technical dossier, Section II – Annex II_80 Verification Premix Carvacrol
[17] *Technical dossier, Section II – Annex II_81 Verification Premix D-carvone
[18] *Technical dossier, Section II – Annex II_82 Verification Premix L-menthol
[19] *Technical dossier, Section II – Annex II_83 Verification Premix Methylsalicylate
[20] *Technical dossier, Section II – Annex II_84 Verification Premix Thymol
[21] *Technical dossier, Section II – Annex II_85 Verification Feed Carvacrol
[22] *Technical dossier, Section II – Annex II_86 Verification Feed D-carvone
[23] *Technical dossier, Section II – Annex II_87 Verification Feed L-menthol
[24] *Technical dossier, Section II – Annex II_88 Verification Feed Methylsalicylate
[25] *Technical dossier, Section II – Annex II_89 Verification Feed Thymol

*Refers to Dossier no: FAD-2018-0023

## 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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- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)