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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 oregano oil, caraway oil, carvacrol, methyl salicylate and L-menthol

(FAD-2017-0026; CRL/170002)

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-2017-0026 - CRL/170002**
Name of Product	**Preparation of oregano oil, caraway oil, carvacrol, methyl salicylate and L-menthol (Biomin® DC-C)**
Active Agent (s):	**oregano oil (marker compound – linalool), caraway oil (marker compound – D-carvone), carvacrol, methyl salicylate and L-menthol**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) JRC Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA) 15/11/2017**
Report approved by: Date:	**Christoph von Holst 16/11/2017**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for a *preparation of oregano oil, caraway oil, carvacrol, methyl salicylate* and *L-menthol (Biomin® DC-C)* 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 weaned piglets.

The *feed additive* is an off-white powder consisting of the following active substances (marker compounds) expressed as percentage mass fractions related to the *feed additive*: 9.5 to 14 % of *carvacrol*; 3 to 5.5 % of *L-menthol*; 1 to 4 % of *methyl salicylate*; 6 to 8 % of *oregano oil* (0.18 to 1.6 % of *linalool*); and 0.5 to 1 % of *caraway oil* (0.25 to 0.65 % of *D-carvone*). 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-C* content ranging from 75 to 125 mg /kg *feedingstuffs*.

For the quantification of *carvacrol, L-menthol, methyl salicylate, linalool* and *D-carvone* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified multi-analyte method based on gas chromatography coupled with 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 0.1 to 1.3 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.5 to 4.1 %; and a *recovery* rate (R_{rec}) ranging from 82 to 101 %.

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, L-menthol, methyl salicylate, linalool* and *D-carvone* in the *feed additive*.

Since the accurate determination of the *Biomin® DC-C* 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-C* 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 oregano oil, caraway oil, carvacrol, methyl salicylate and *L-menthol (Biomin® DC-C), linalool, D-carvone,* zootechnical additives, other zotechnical additives, weaned piglets

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new *feed additive*) for the *preparation of oregano oil, caraway oil, carvacrol, methyl salicylate* and *L-menthol (Biomin® DC-C)* 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 weaned piglets [1].

The *feed additive* is an off-white powder consisting of the following active substances (marker compounds) expressed as percentage mass fractions related to the *feed additive*: 9.5 to 14 % of *carvacrol*; 3 to 5.5 % of *L-menthol*; 1 to 4 % of *methyl salicylate*; 6 to 8 % of *oregano oil* (equivalent to 0.18 to 1.6 % of *linalool*); and 0.5 to 1 % of *caraway oil* (equivalent to 0.25 to 0.65 % of *D-carvone*) [2, 3]. In addition, it 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-C* content ranging from 75 to 125 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 the *preparation of oregano oil, caraway oil, carvacrol, methyl salicylate* and *L-menthol (Biomin® DC-C)* 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

For the identification of the *feed additive* the EURL considers the method provided by the Applicant based on gas chromatography coupled with flame ionisation detection (GC-FID) [4, 5] fit-for-purpose for monitoring the major (e.g. *carvacrol, L-menthol, methyl salicylate, linalool* and *D-carvone*) and minor (e.g. *thymol* and *limonene*) components 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 [6].

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

For the quantification of the three active substances (*carvacrol, L-menthol, methyl salicylate)* and the two marker compounds (*linalool* and *D-carvone*) in the *feed additive* the Applicant submitted a single-laboratory validated and further verified multi-analyte method based on gas chromatography coupled with flame ionisation detection (GC-FID) [4, 5].

The *Biomin® DC-C* 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 collected. The extraction of the same sample is repeated 2 more times, the supernatants combined and adjusted with ethyl acetate up to the final volume of 5 ml [4]. An aliquot of the resulting solution is analysed by GC-FID. The quantification of the active/marker substances is performed by external matrix-free calibration using standard solutions containing the mixture of all the relevant analytes [5, 7].

The applicant presented results from the validation study [7] obtained on three days by confirming that the concentration values of the analytes from the 3rd day were outliers. Therefore only the data from the 1st and 2nd days were used for further statistical treatment.

Furthermore, upon request by the EURL, the Applicant provided a new set of data for the verification study where a proper experimental design was applied [8-12]. The corresponding performance characteristics for the quantification of the five analytes investigated in the *feed additive* are presented in Table 1.

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, L-menthol, methyl salicylate, linalool* and *D-carvone* in the *feed additive*.

Since the accurate determination of the content of *Biomin® DC-C* added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine *Biomin® DC-C* in *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 with mass spectrometry (GC-MS) for the quantification of the content of *carvacrol, L-menthol, methyl salicylate, linalool* and *D-carvone* added to *premixtures* and *feedingstuffs* [13, 14].

While acceptable performance characteristics were reported in the frame of the validation study [15, 16], the verification study [17-26] performed using the same samples (used for the validation study) resulted in much lower recovery values for all five analytes in *premixtures* and *feedingstuffs*. Hence, the EURL does not recommend this multi-analyte GC-MS method for official control for the quantification of the total content 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.

Table 1 The performance characteristics of the single laboratory validated (Val) and verified (Ver) multi-analyte GC-FID method for the quantification of *carvacrol, L-menthol, methyl salicylate, linalool* and *D-carvone* in the *feed additive*

	carvacrol		L-menthol		methyl salicylate		linalool		D-carvone	
	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.
Mass fraction, g/kg	120	107	18.6	15.5	19.8	17.4	5.9	5.7	4.0	3.5
RSD_r, %	0.4 - 0.9	0.4 - 0.6	0.4 - 1.3	0.4 - 0.6	0.1 - 1.0	0.4 - 0.6	0.4 - 1.1	0.4 - 0.5	0.2 - 0.7	0.4 - 0.5
RSD_{ip}, %	1.0	0.6	3.7	0.6	2.7	0.6	4.1	0.5	2.3	0.5
R_{rec}, %	101(*)	88(**)	97(*)	82(**)	96(*)	84(**)	92(*)	93(**)	97(*)	86(**)
Reference	[7]	[8]	[7]	[9]	[7]	[10]	[7]	[11]	[7]	[12]

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec} - a *recovery* rate;
(*) recoveries are based on blank feed additive samples spiked with the analytes at low concentration levels (10 to 300 times lower than the real content of the analytes in the feed additive).
(**) the recoveries are based on the "old" feed additive samples analysed after 16 months of storage

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 with flame ionisation detection (GC-FID) for the quantification of *carvacrol, L-menthol, methyl salicylate, linalool* and *D-carvone* in the *feed additive*

Since the accurate determination of *Biomin® DC-C* 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-C* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- Gas chromatography coupled with 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 oregano oil, caraway oil, carvacrol, methyl salicylate* and *L-menthol (Biomin® DC-C)* 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. 1831-0017-2017
[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 – Annex II_64
[5] *Technical dossier, Section II – Annex II_65
[6] 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
[7] *Technical dossier, Section II – Annex II_68
[8] *Supplementary information, Annex II_71 REP Verification_additive_carvacrol
[9] *Supplementary information, Annex II_72 REP Verification_additive_D_carvone
[10] *Supplementary information, Annex II_73 REP Verification_additive_linalool
[11] *Supplementary information, Annex II_74 REP Verification_additive_L_menthol
[12] *Supplementary information, Annex II_75 REP Verification_additive_methyl salicylate
[13] *Technical dossier, Section II – Annex II_66

[14] *Technical dossier, Section II – Annex II_67
[15] *Technical dossier, Section II – Annex II_69
[16] *Technical dossier, Section II – Annex II_70
[17] *Supplementary information, Annex II_76 REP Verification_premix_carvacrol
[18] *Supplementary information, Annex II_77 REP Verification_premix_D_carvone
[19] *Supplementary information, Annex II_78 REP Verification_premix_linalool
[20] *Supplementary information, Annex II_79 REP Verification_premix_L_menthol
[21] *Supplementary information, Annex II_80 REP Verification_premix_methyl salicylate
[22] *Supplementary information, Annex II_81 REP Verification_feed_carvacrol
[23] *Supplementary information, Annex II_82 REP Verification_feed_D_carvone
[24] *Supplementary information, Annex II_83 REP Verification_feed_linalool
[25] *Supplementary information, Annex II_84 REP Verification_feed_L_menthol
[26] *Supplementary information, Annex II_85 REP Verification_feed_methyl salicylate
*Refers to Dossier no: FAD-2017-0026

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

The following National Reference Laboratories contributed to this report:

- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)