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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**

**Lemon extract (*Citrus limon* Burm)**  
*(FAD-2021-0070; CRL/210018)*





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in connection with the Application for Authorisation of a  
Feed Additive according to Regulation (EC) No 1831/2003**

Dossier related to: **FAD-2021-0070 - CRL/210018**

Name of Product: ***Lemon extract (Citrus limon Burm)***

Active Agent (s): ***total polyphenols, eriocitrin***

Rapporteur Laboratory: **European Union Reference Laboratory for  
Feed Additives (EURL-FA)  
JRC Geel, Belgium**

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Date: **20/05/2022**

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Date: **20/05/2022**

## EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *lemon extract (Citrus limon Burm)* 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 *feed additive* to be used for weaned piglets and all growing poultry species.

The Applicant proposed as phytochemical markers for the characterisation of the *feed additive* (i) *total polyphenols* and (ii) *eriocitrin*. The product contains respectively a minimum content of 1 % of *total polyphenols* and 4000 mg/kg product of *eriocitrin*. The *feed additive* (Citrozest<sup>®</sup>) is a liquid extract from the peel and the seeds of *Citrus limon (L.) Burm.* fruit. The product is to be used through *premixtures* or directly into *feedingstuffs* at proposed inclusion levels ranging from a minimum of 120 to a maximum 1000 mg/kg *feedingstuffs*.

For the determination of the *total polyphenols* in the *feed additive* the Applicant proposed a colourimetric method described in the European Pharmacopoeia monograph 2.8.14. Upon request of the EURL, in the frame of the similar "FAD-2010-0130 – Lemon extract" dossier, the Applicant provided experimental data for the analysis of the *total polyphenols* content in two different batches of *lemon extract*. Based on the acceptable performance characteristics presented the EURL recommends for official control the colourimetric method described in the European Pharmacopoeia 2.8.14 monograph for the determination of the *total polyphenols* content in the *feed additive*.

For the determination of the *eriocitrin* content in the *feed additive* the Applicant presented an extension of scope of the ring-trial validated method from the "International Fruit and Vegetable Association" (IFU 580) dedicated to the determination of hesperidin and naringin in juices. In the frame of the similar "FAD-2010-0130 – Lemon extract" dossier, the Applicant provided experimental data for the analysis of the *eriocitrin* content in two different batches of *lemon extract*. According to the results provided, the EURL calculated a relative standard deviation for repeatability (RSD<sub>r</sub>) of 0.1 % and 1.8 %, and a relative standard deviation for intermediate precision (RSD<sub>ip</sub>) of 8.1 % and 8.5 %. Based on the experimental evidences provided, the EURL considers the above mentioned HPLC-UV method fit-for-purpose for the determination of the *eriocitrin* content in the *feed additive*.

For the determination of the *eriocitrin* content in *premixtures* the Applicant submitted a single-laboratory validated and further verified method based on HPLC-UV. For the determination of the *eriocitrin* content in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on ultra-high performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS).

Based on the experimental evidences provided, the EURL considers the proposed HPLC-UV and UPLC-ESI-MS/MS methods fit-for-purpose for the potential official control of *eriocitrin* content respectively in *premixtures* and *feedingstuffs*.

Furthermore, the Applicant did not provide experimental data or analytical methods for the determination of *lemon extract* in *premixtures* and *feedingstuffs*, as the unambiguous determination of the *feed additive* added to the matrices is not achievable experimentally. Therefore, the EURL cannot evaluate nor recommend any method for official control for the determination of *lemon extract* 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

*Lemon extract (Citrus limon Burm)*, Citrozest<sup>®</sup>, total polyphenols, eriocitrin, zootechnical additives, weaned piglets, all growing poultry species

## 1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *lemon extract (Citrus limon Burm)* 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 [1,2]. Specifically, the authorisation is sought for the *feed additive* to be used for weaned piglets and all growing poultry species [2].

The *feed additive*, marketed under the registered name Citrozest<sup>®</sup>, is a natural dark orange to yellow liquid extract containing only water as excipient and propionic acid as preservative (1 %) [3-5]. The product is extracted from the peel and the seeds of *Citrus limon (L.) Burm.* fruit [6].

The Applicant proposed as typical phytochemical markers of the *feed additive* (i) *total polyphenols* content and among them (ii) *eriocitrin* [5]. Citrozest<sup>®</sup> contains a minimum 1 % of *total polyphenols* and a minimum content of *eriocitrin* of 4000 mg/kg product [5].

*Lemon extract* is to be used through *premixtures* or directly into *feedingstuffs* at inclusion levels ranging from a minimum of 120 to a maximum 1000 mg/kg *feedingstuffs* content [7].

Note: The EURL has previously evaluated the analytical methods for the determination of *lemon extract* in the frame of a similar dossier for a product intended as sensory additive [8].

## 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 *lemon extract (Citrus limon Burm)* 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 the *total polyphenols* in the *feed additive* the Applicant proposed a colourimetric method described in the European Pharmacopoeia monograph 2.8.14 [9,10].

The polyphenols are extracted with water and mixed with a mixture of phosphomolybdate and phosphotungstate solutions (Folin–Ciocalteu reagent (FCR)). The coloured complex formed with the polyphenols is then measured at 760 nm and the total polyphenols content is expressed as pyrogallol equivalent [10].

In the frame of the “FAD-2010-0130 – Lemon extract” dossier, upon request of the EURL, the same Applicant provided experimental data for the analysis of total polyphenols content in two different batches of *lemon extract* [8]. According to the results provided by the Applicant in the frame of the analysis, the EURL calculated relative standard deviations for repeatability ( $RSD_r$ ) and for intermediate precision ( $RSD_{ip}$ ) ranging from 2.1 to 5.2 % and from 3.3 to 6.5 %, respectively [8]. Furthermore, the Applicant applied this method to five different batches of the *feed additive* leading to *total polyphenols content* ranging from 1.1 to 1.2 % [11-16].

Based on the experimental evidences provided, the EURL recommends for official control the colourimetric method described in the European Pharmacopoeia for the determination of *total polyphenols* (phytochemical marker) in the *feed additive*.

For the determination of the *eriocitrin* content in the *feed additive* the Applicant presented an extension of scope of the ring-trial validated method from the “International Fruit and Vegetable Association” (IFU 580) dedicated to the determination of hesperidin and naringin in juices [9,17]. This method is based on high performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV) [17].

10 ml of sample is dissolved in ammonium oxalate solution with addition of dimethylformamide (DMF). The solution is heated at 90 °C for 10 min in a water bath. After cooling, a filtered aliquot of the sample is injected in HPLC and *eriocitrin* is detected by UV at 280 nm. The determination is performed using the external standard method by integration of the peak areas or by measuring the peak heights [17].

In the frame of the “FAD-2010-0130 – Lemon extract” dossier, the Applicant provided experimental data for the analysis of the *eriocitrin* content in two different batches of *lemon extract* [8]. According to the results provided, the EURL calculated a  $RSD_r$  of 0.1 % and 1.8 %, and a  $RSD_{ip}$  of 8.1 % and 8.5 % [8].

Based on the experimental evidences provided, the EURL considers the proposed HPLC-UV method fit-for-purpose for the determination of the *eriocitrin* content in the *feed additive*.

For the determination of the *eriocitrin* content in *premixtures* the Applicant submitted a single-laboratory validated and further verified method based on HPLC-UV [9,18-20].

The sample is extracted with a solution methanol/water 50/50 (v/v) in an ultrasonic bath. After centrifugation an aliquot of the supernatant is injected in HPLC and detected by UV at 283 nm. The determination is performed using the external standard method by integration of the peak areas [18]. The performance characteristics reported for the determination of *eriocitrin* content in *premixtures* are listed in Table 1.

Based on the experimental evidences provided, the EURL considers the proposed HPLC-UV method fit-for-purpose for the potential official control of *eriocitrin* content in *premixtures*.

For the determination of the *eriocitrin* content in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on ultra high performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) [9,21-23]. The method has been validated for a content of the *feed additive* corresponding to 150 mg/kg *feedingstuffs* [21].

*Feedingstuffs* sample are grinded and spiked with camelliaside A. The sample is then extracted with methanol in ultrasonic bath. The extract is filtrated and evaporated into dryness. The extract is solved in methanol and centrifuged. After centrifugation, aliquots of supernatant are spiked with known amount of camelliaside A and *eriocitrin*. Aliquots are filtered and injected in UPLC-ESI-MS/MS. The areas corresponding to camelliaside A and *eriocitrin*, determined in single reaction monitoring, are used to determine the content of camelliaside A in feed by the standard addition method. Camelliaside A allows to obtain the recovery of the analysis and it is used in the calculation for the determination of *eriocitrin* [21]. The performance characteristics reported for the determination of *eriocitrin* content in *feedingstuffs* are listed in Table 1.

**Table 1:** Method performance characteristics obtained in the frame of validation and verification studies for the determination of *eriocitrin* in *premixtures* and *feedingstuffs*.

	<i>Premixtures</i> <i>HPLC-UV</i>		<i>Feedingstuffs</i> <i>UPLC-ESI-MS/MS</i>	
	Validation	Verification	Validation	Verification
<b>RSD<sub>r</sub> %</b>	3.8	1.1	8.7	10.8
<b>RSD<sub>ip</sub> %</b>	4.9	1.9	8.7	11.9
<b>R<sub>rec</sub> %</b>	92.7	92.8	97	98.2
<b>Reference</b>	[19]	[20]	[22]	[23]

RSD<sub>r</sub> and RSD<sub>ip</sub>: relative standard deviations for *repeatability and intermediate precision*, respectively; R<sub>rec</sub>: a *recovery rate*.

Based on the experimental evidences provided, the EURL considers the proposed UPLC-ESI-MS/MS method fit-for-purpose for the potential official control of *eriocitrin* content in *feedingstuffs*.

Furthermore, the Applicant did not provide experimental data or an analytical method for the determination of *lemon extract* (Citrozest<sup>®</sup>) in *premixtures* and *feedingstuffs*, as also the unambiguous determination of the *feed additive* added to these matrices is not achievable experimentally. Therefore, the EURL cannot evaluate or recommend any method for official control for the determination of *lemon extract* in *premixtures* and *feedingstuffs*.

***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 colourimetric method described in the European Pharmacopoeia monograph 2.8.14 for the determination of the *total polyphenols* (phytochemical marker) in the *feed additive* and (ii) the HPLC-UV method based on a ring-trial validated method from the "International Fruit and Vegetable Association" (IFU 580) dedicated to the determination of hesperidin and naringin in juices and further verified for the determination of *eriocitrin* (phytochemical marker) in the *feed additive*.



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***Recommended text for the register entry (analytical method)***

For the determination of the *total polyphenols* in the *feed additive*:

- spectrophotometry at 760 nm expressing the total polyphenols content as pyrogallol equivalent (European Pharmacopoeia monograph 2.8.14)

For the determination of the *eriocitrin* in the *feed additive*:

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

## **5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL**

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *lemon extract (Citrus limon Burm)* 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: FORW. APPL. 1831-0066-2021
- [2] \*Application, Annex I - Submission number 1616662049962-2968
- [3] \*Technical dossier, Section II: 2.1.5. Physical state of each form of the product
- [4] \*Technical dossier, Section II: 2.3. Manufacturing process, including any specific processing procedures
- [5] \*Technical dossier, Section II: 2.2 Characterisation of the active substance(s)
- [6] \*Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition (active substance/agent, other components, impurities, batch to batch variation)
- [7] \*Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
- [8] FAD-2010-0130, Lemon extract Ref. Ares(2020)7664520 – 16/12/2020
- [9] \*Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance
- [10] European Pharmacopoeia monograph 2.8.14 Tannins in herbal drugs 01/2008:20814
- [11] \*Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition (active substance/agent, other components, impurities, batch to batch variation)
- [12] \*Technical dossier, Section II – Annex\_II.27
- [13] \*Technical dossier, Section II – Annex\_II.28
- [14] \*Technical dossier, Section II – Annex\_II.29
- [15] \*Technical dossier, Section II – Annex\_II.30
- [16] \*Technical dossier, Section II – Annex\_II.31
- [17] \*Technical dossier, Section II – Annex\_II\_75\_SOP Eriocitrin IFU
- [18] \*Technical dossier, Section II – Annex\_II\_61\_Quantification of eriocitrin in premixture\_Conf

- [19] \*Technical dossier, Section II – Annex\_II\_76\_Validation report eriocitrin in premixture\_Conf
- [20] \*Technical dossier, Section II – Annex\_II\_77\_EURL verification report premixture\_Conf
- [21] \*Technical dossier, Section II – Annex\_II\_67\_Quantification of eriocitrin in complete feed\_Conf
- [22] \*Technical dossier, Section II – Annex\_II\_78\_Validation report eriocitrin in complete feed\_Conf
- [23] \*Technical dossier, Section II – Annex\_II\_79\_EURL verification report feed\_Conf
- \*Refers to Dossier no: FAD-2021-0070

## **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**

The following National Reference Laboratories contributed to this report:

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

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