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EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for the Authorisation of Feed Additives according to Regulation (EC) No 1831/2003

Dossier related to:	FAD-2010-0144 CRL/100150 FAD-2010-0225 CRL/100112
Feed additive:	L-Carnitine L-Carnitine L-tartrate (LCLT)
Active Substance(s):	L-Carnitine L-Carnitine L-tartrate (LCLT)
Rapporteur Laboratory:	European Reference Laboratory for Feed Additives, IRMM, Geel, Belgium
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Report approved by: Date:	Christoph von Holst 22/11/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) and 10(2) for *L-Carnitine*[1,2] and *L-Carnitine L-Tartrate (LCLT)*[2] under the category/functional group 3(a) "nutritional additives"/"vitamins, pro-vitamins and chemically well defined substances having similar effect", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the two *feed additives* for all animal species and categories. According to the Applicants, *L-Carnitine* and *LCLT* have a minimum purity of 97 %. Both *feed additives* are intended to be used in *premixtures* or added directly into the *feedingstuffs* and *water*. None of the Applicants proposed any minimum or maximum concentration of *L-Carnitine* or *LCLT* in *feedingstuffs* or *water*, as set in the previous regulations.

For the determination of *L-Carnitine* in the *feed additive*, Applicant[1] submitted the US Pharmacopoeia method. The EURL identified instead the European Pharmacopoeia method. Even though no performance characteristics are provided, the EURL recommends for official control the European Pharmacopoeia method (Ph. Eur. 6th Edition, monographs 1339), for the identification and quantification of *L-Carnitine* in the *feed additive*.

For the determination of *L-Carnitine* in *premixtures*, *feedingstuffs* and *water* Applicant[1] submitted an enzymatic method, specific for the *L-Carnitine* isomer. This single-laboratory validated method was further verified by a second independent laboratory. The relative precisions (i.e. relative standard deviations of *repeatability* (RSD_r) and relative standard deviations of *intermediate precision* (RSD_{ip}) ranging from 4.5 to 5.1 % were recalculated by the EURL using the experimental data provided by the Applicant[1]. Furthermore, the Applicant[1] reported a recovery rate (R_{Rec}) ranging from 94 to 102 % and a limit of detection (LOD) of 15 mg *L-Carnitine* /kg *feedingstuffs*, which is well below the usual concentrations of *L-Carnitine* in *feedingstuffs* or *water*.The experiments were carried out at 5 g *L-Carnitine* /kg *premixtures* and 100-165 mg *L-Carnitine* /kg *feedingstuffs* or *water*.

For the determination of *L-Carnitine* in *premixtures*, Applicant[2] proposed an ion chromatography method with electrical conductivity detection (IC-ECD), which does not distinguish between two enantiomers *L-* and *D-Carnitine*. This single-laboratory validated method was further verified by a second independent laboratory. The relative precisions ranging from 4.2 to 4.6 % were recalculated by the EURL using the experimental data provided by the Applicant[2]. Applicant[2] reported R_{Rec} ranging from 92 to 100 %. The experiments were carried out at 8-10 g *L-Carnitine* /kg *premixtures*.

For the determination of *L-Carnitine* in *feedingstuffs*, Applicant[2] proposed a Reversed-Phase High Performance Liquid Chromatography method using a fluorimetric detector (RP-HPLC), which does not distinguish between two enantiomers *L-* and *D-Carnitine*. The relative precisions ranging from 6.9 to 12.4 % and LOD of 6.3 mg *L-Carnitine* /kg *feedingstuffs* were

recalculated by the EURL using the experimental data provided by the Applicant[2]. Applicant[2] reported R_{Rec} ranging from 88 to 115 %. The experiments were carried out at 40-60 mg *L-Carnitine* /kg *feedingstuffs*.

For the determination of *L-Carnitine* in *water*, Applicant[2] proposed a potentiometric titration. RSD_r of 0.1 % was reported by Applicant[2].

Based on the above considerations and the performance characteristics presented, the EURL recommends for official control the following single-laboratory validated and further verified methods based on the spectrophotometric method after enzymatic reaction with carnitine-acetyl-transferase enzymatic reaction for the determination of *L-Carnitine* in *premixtures*, *feedingstuffs* and *water*,
together with

- ion chromatography method with electrical conductivity detection (IC-ECD) for the determination of *L-Carnitine* in *premixtures*;
- Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using a fluorimetric detector for the determination of *L-Carnitine* in *feedingstuffs*;
- potentiometric titration with hydrochloric acid for the determination of *L-Carnitine* in the *water*.

For the determination of *L-Carnitine L-Tartrate (LCLT)* in the *feed additive*, Applicant[2] submitted a potentiometric back-titration. The method was single-laboratory validated and further verified. RSD_r ranging from 0.72 to 1.19 % and RSD_{ip} of 1.19 were recalculated by the EURL using the experimental data provided by the Applicant[2]. R_{Rec} ranging from 99.8 to 101 % were reported by the Applicant[2].

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified potentiometric back-titration method to determine *L-Carnitine L-Tartrate (LCLT)* in the *feed additive*.

L-Carnitine is released from the *LCLT* salt during sample treatment. Therefore, the EURL considers that all the analytical methods recommended for the determination of *L-Carnitine* in the matrices investigated are suitable for official control to determine *LCLT* (expressed as *L-Carnitine*) in *premixtures*, *feedingstuffs* and *water*.

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) is not considered necessary.

KEYWORDS
L-Carnitine, *L-Carnitine L-Tartrate (LCLT)*, nutritional additive, vitamins, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use in water) and 10(2) (re-evalutation of *feed additives* already authorized under provisions of Council Directive 70/524/EEC) for *L-Carnitine*[1,2] and *L-Carnitine L-Tartrate (LCLT)*[2] under the category/functional group 3(a) "nutritional additives"/"vitamins, pro-vitamins and chemically well defined substances having similar effect" [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the two *feed additives* for all animal species and categories [1].

According to the Applicants, *L-Carnitine* and *LCLT* have a minimum purity of 97 % [2,3]. *L-Carnitine* is a synthetic, white to beige powder, soluble in water (0.1 g/mL at 20 °C), while *LCLT* is a white crystalline powder, very soluble in water (1 g/mL at 20 °C) [4,5]. Both *feed additives* are intended to be used in *premixtures* or added directly into the *feedingstuffs* and *water*. These water-soluble *feed additives* can be used in *water* only when the carrier substances are also water-soluble, in order to avoid sedimentation [4,5]. None of the Applicants proposed any minimum or maximum concentration of *L-Carnitine* or *LCLT* in *feedingstuffs* or *water* [2,3], as set in the previous regulations [6]. However, a typical concentration ranging from 20 to 100 mg L-Carnitine /kg *feedingstuffs* and *water* is suggested by the Applicants [4-5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 these particular dossiers, the methods of analysis submitted in connection with *L-Carnitine* and *L-Carnitine L-Tartrate (LCLT)*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

[1] FAD-2010-0225; [2] FAD-2010-0144

3. EVALUATION

Identification /Characterisation 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, mycotoxins, PAHs and dioxins) are available from the respective European Union Reference Laboratories [7].

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

L-Carnitine

For the determination of *L-Carnitine* in the *feed additive*, Applicant[1] submitted the US Pharmacopoeia method [8]. The EURL identified instead the European Pharmacopoeia method [9]. Identification is based on specific optical rotation and infrared absorption, while quantification is based on titration with 0.1 M perchloric acid using crystal violet as indicator. Even though no performance characteristics are provided, the EURL recommends for official control the European Pharmacopoeia method (Ph. Eur. 6th Edition, monographs 1339), for the identification and quantification of *L-Carnitine* in the *feed additive*.

For the determination of *L-Carnitine* in *premixtures*, *feedingstuffs* and *water* Applicant[1] submitted a spectrophotometric method after enzymatic reaction with carnitine-acetyl-transferase, specific for the *L-Carnitine* isomer [10-12]. The determination of *L-Carnitine* is based on a transfer of acetyl from the added acetyl-coenzyme to *L-Carnitine* by the enzyme carnitine-acetyl-transferase. The released Coenzyme A then reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) to produce a yellow coloured complex measured spectrophotometrically at 412 nm [10]. In order to remove potential interferences, *feedingstuffs* samples are treated with sodium hydroxide for 1 hour at 50 ^{o}C, then neutralised by hydrochloric acid and centrifuged before analysis [12].

This single-laboratory validated [10] method was further verified by a second independent laboratory [13,14]. As the enzymatic methods for *premixtures* and *feedingstuffs* are substantially the same, Applicant[1] submitted complete validation and verification studies for *premixtures* only. The few performance characteristics provided for *feedingstuffs* are in good agreement with those obtained for *premixtures* (see Table 1), thus demonstrating the applicability of this method to both matrices. The relative precisions (i.e. relative standard deviations of *repeatability* (RSD_r) and relative standard deviations of *intermediate precision* (RSD_{ip}) were recalculated by the EURL [15] using the experimental data provided by the Applicant[1] [10]. The performance characteristics are presented in Table 1. Furthermore,

Applicant[1] reported a LOD of 15 mg *L-Carnitine* /kg *feedingstuffs* [12], well below the usual concentrations of *L-Carnitine* in *feedingstuffs* or *water*.

For the determination of *L-Carnitine* in *premixtures*, Applicant[2] proposed an ion chromatography method with electrical conductivity detection (IC-ECD) [16], which does not distinguish between two enantiomers *L-* and *D-Carnitine*. The sample is extracted in an aqueous mixture of acetonitrile and perchloric acid in an ultrasonic bath. The supernatant is then filtered through a 0.45 µm membrane, diluted 10 times, and measured by IC-ECD. The sum of *(L+D)-Carnitine* is quantified using an external calibration curve [16]. This method was single-laboratory validated [17] and further verified by an independent laboratory [18]. The reported performance characteristics recalculated by the EURL [15] are presented in Table 1.

For the determination of *L-Carnitine* in *feedingstuffs*, Applicant[2] proposed a Reversed-Phase High Performance Liquid Chromatography method using a fluorimetric detector (RP-HPLC) [19], which does not distinguish between two enantiomers *L-* and *D-Carnitine*. The feed sample (5 g) is extracted with 5 mL of acetonitrile and 25 mL of water. An aliquot of the sample extract is then purified on an ion-exchange column. The fraction containing *Carnitine* is then derivatized with a solution of fluorenyl methyl chloroformate. After separation by HPLC, it is detected by fluorimetry, using an excitation wavelength at 260 nm and an emission at 315 nm. The sum of *(L+D)-Carnitine* is quantified with the standard addition technique, to compensate for the matrix influences on the detection [19]. This method was single-laboratory validated [20] and further verified by an independent laboratory [21]. The performance characteristics recalculated by the EURL [15] are presented in Table 1. Furthermore, a limit of detection (LOD) of 6.3 mg *(L+D)-Carnitine* /kg *feedingstuffs* was recalculated by the EURL [15] using the experimental data provided by the Applicant[2] [21].

For the determination of *L-Carnitine* in *water*, Applicant[2] proposed a titration method derived from the above mentioned European Pharmacopoeia method, based on potentiometric titration using a solution of hydrochloric acid [22]. A relative standard deviation for *repeatability* (RSD_r) of 0.1 % was reported [23], when only *L-Carnitine* was added to water (with no other *feed additive* present).

[1] FAD-2010-0225; [2] FAD-2010-0144

Table 1 Performance characteristics of analytical methods for the determination *L-Carnitine* in *premixtures* and *feedingstuffs*, within concentration ranging from 5 to 10 g/kg *premixtures* and from 40 to 165 mg/kg *feedingstuffs*, as recalculated by the EURL [15]

		Chromatography methods		Enzymatic method	
		Validation	Verification	Validation	Verification
Premixtures	RSD_r (%)	4.6	4.2	4.5	4.9
	RSD_{ip} (%)	4.6	4.2	4.5	5.0
	R_{rec} (%)	98 - 100 [17]	92 [18]	101 [10]	94 [13]
Feedingstuffs	RSD_r (%)	12.4*	6.9	5.1	n.d.
	RSD_{ip} (%)	12.4	7.7	n.d.	n.d.
	R_{rec} (%)	88 -115 [20]	107 [21]	102 [12]	n.d.

RSD_r and RSD_{ip}: relative standard deviation for repeatability and reproducibility, respectively
R_{rec} : recovery rate
n.d. not detected
* the high RSD_r is due to a high value reported on day 1

Based on the above considerations and the performance characteristics presented in Table 1, the EURL recommends for official control the following single-laboratory validated and further verified methods based on:

- Spectrophotometric method after enzymatic reaction with carnitine-acetyl-transferase for the determination of *L-Carnitine* in *premixtures*, *feedingstuffs* and *water*;
  together with
- ion chromatography method with electrical conductivity detection (IC-ECD) for the determination of *L-Carnitine* in *premixtures*;
- Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using a spectrofluorometric detector for the determination of *L-Carnitine* in *feedingstuffs*;
- potentiometric titration with hydrochloric acid for the determination of *L-Carnitine* in the *water*.

L-Carnitine L-Tartrate (LCLT)

For the determination of *L-Carnitine L-Tartrate (LCLT)* in the *feed additive*, Applicant[2] submitted a potentiometric back-titration using 0.1M hydrochloric acid and 0.1M sodium hydroxide [24]. The method was single-laboratory validated [25] and further verified [26]. The relative standard deviation of *repeatability* (RSD_r) ranging from 0.72 to 1.19 % and relative standard deviation of *intermediate precision* (RSD_{ip}) of 1.19 were recalculated by the EURL [15] using the experimental data provided by the Applicant[2] [25,26]. Furthermore, a *recovery* rate (R_{Rec}) ranging from 99.8 to 101 % was reported by the Applicant[2] [25,26].

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified potentiometric back-titration

method to determine *L-Carnitine L-Tartrate (LCLT)* in the *feed additive*, within the concentration range covered by the experimental data.

L-Carnitine is released from the *LCLT* salt during sample treatment. Therefore, the EURL considers that all the analytical methods recommended for the determination of *L-Carnitine* in the matrices investigated are suitable for official control to determine *LCLT* (expressed as *L-Carnitine*) in *premixtures*, *feedingstuffs* and *water*.

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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- the European Pharmacopoeia method (Ph. Eur. 6th Edition, monographs 1339) using titration with perchloric acid for the determination of *L-Carnitine* in the *feed additive*;
- the single-laboratory validated and further verified method using potentiometric back-titration with hydrochloric acid and sodium hydroxide, to determine *L-Carnitine L-Tartrate (LCLT)* in the *feed additive*;
- the single-laboratory validated and further verified method submitted by the Applicant, based on the spectrophotometric method after enzymatic reaction with carnitine-acetyl-transferase for the determination of *L-Carnitine* in *premixtures*, *feedingstuffs* and *water*;
  together with
- the single-laboratory validated and further verified method, based on ion chromatography method with electrical conductivity detection (IC-ECD) for the determination of *L-Carnitine* in *premixtures*;
- the single-laboratory validated and further verified method, based on Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using a fluorimetric detector for the determination of *L-Carnitine* in *feedingstuffs*;
- the single-laboratory validated and further verified method using potentiometric titration with hydrochloric acid for the determination of *L-Carnitine* in *water*;

Recommended text for the register entry (analytical method)

For the determination of *L-Carnitine* in the *feed additive*:

Titration with perchloric acid (Ph. Eur. 6th edition, monograph 1339)

For the determination of *L-Carnitine L-Tartrate (LCLT)* in the *feed additive*:

Potentiometric back-titration

For the determination of L-Carnitine in *premixtures*:

Ion chromatography method with electrical conductivity detection (IC-ECD)
or
Spectrophotometric method after enzymatic reaction with carnitine-acetyl-transferase

For the determination of *L-Carnitine* in *feedingstuffs*:

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) with fluorimetric detector
or
Spectrophotometric method after enzymatic reaction with carnitine-acetyl-transferase

For the determination of *L-Carnitine* in *water*:

Potentiometric titration
or
Spectrophotometric method after enzymatic reaction with carnitine-acetyl-transferase

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-Carnitine* and *L-Carnitine L-Tartrate (LCLT)* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

[1] FAD-2010-0225; [2] FAD-2010-0144

6. REFERENCES

[1] Reference Forw. Appl. SANCO/D/2/MGR/ci/250965

[2] *Application, Proposal for Register Entry – Annex A

[3] +Application, Proposal for Register Entry – Annex A

[4] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis

[5] +Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis

[6] Official Journal of the European Union, C 50 of 25.2.2004, p. 1, *List of the authorised additives in feedingstuffs (1) published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs*

[7] 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

[8] +Technical Dossier, Section II, Annex II-01 USP 32 specification L-Carnitine

[9] Pharmacopoeia 6th Ed. - Monograph 1339, Levocarnitine

[10] +Technical Dossier, Section II, Annex II-26 SOP plus Validation

[11] +Technical Dossier, Section II, Annex II-21 stability test in water

[12] +Technical Dossier, Section II, Annex II-27 extension of scope report

[13] +Technical Dossier, Section II, Annex II-35 Verification study report

[14] +Technical Dossier, Section II, Annex II-36 Verification report

[15] *Supplementary information – Precision data as recalculated by the EURL

[16] *Technical Dossier, Section II, Annex II-21 Method premix

[17] *Technical Dossier, Section II, Annex II-22 Validation method premix

[18] *Supplementary information provided by the Applicant upon request EURL – EURLFA-Verification Premix

[19] *Technical Dossier, Section II, Annex II-24 Method Feed

[20] *Technical Dossier, Section II, Annex II-25 Validation method feed

[21] *Supplementary information provided by the Applicant upon request EURL – EURLFA-Verification Feed

[22] *Technical Dossier, Section II, Annex II-19 SOP

[23] *Technical Dossier, Section II, Annex II-31 CHVI-29873

[24] *Technical Dossier, Section II, Annex II-20 L-Carnitine L-Tartrate (LCLT)

[25] *Technical Dossier, Section II, Annex II-32 Validation L-Carnitine in LCLT

[26] *Supplementary information provided by the Applicant upon request EURL – EURLFA-Verification L-Carnitine

* Refers to Dossier No. FAD-2010-0144
+ Refers to Dossier No. FAD-2010-0225

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Cabrils (ES)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava (SK)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)