EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory - Feed Additives Authorisation

Evaluation Report of the Community Reference Laboratory Feed Additives Authorisation on the Method(s) of Analysis for

Rosemary extract liquid

(Dossier No. FAD-2004-0003)

1. EXECUTIVE SUMMARY

The objective of this report is to evaluate the analytical methods related to the dossier "FAD 2004-0003", which is liquid Rosemary extract. The applicant proposes classification of this feed additive in the category "Technological additives" and in the functional group "Antioxidants".

Liquid rosemary extract is produced by extracting ground rosemary leaves (*Rosmarinus officinalis L.*) with a mixture of acetone, methanol, and tetrafluoroethane. After the extraction the solvent is evaporated and a vegetable oil is added to obtain the final product. Liquid rosemary extract contains mainly carnosic acid (around 10 %) as active substance, sunflower oil with a concentration ranging from 82% to 90 %, as its carrier and a number of other natural compounds that are co-extracted during the processing of rosemeary leaves.

Rosemary extracts have demonstrated to possess a strong antioxidant activity, predominantly due to carnosic acid, which protects fat in the feedingstuff from deterioration by oxidation. The proposed concentration of carnosic acid in feedingstuff ranges from 5 to 50 mg/kg. The target animals are dogs and cats.

For the determination of the target analyte the applicant proposed two methods. One method is suitable for the detection of carnosic acid in rosemary extract and is based on high performance liquid chromatography (HPLC) coupled to a diode array detector (DAD). The limit of detection for this method was about 320 mg/kg which is far below the content of carnosic acid in the extract (around 10 %). The second method has been developed and validated for the determination of carnosic acid in *feedingstuff* and is comprised of two steps which is the derivatisation of carnosic acid by silylation and measuring the derivative by gas chromatography coupled to a Flame Ionisation Detector (FID). The validation study showed

that the limit of detection of carnosic acid in feedingstuff was 1.8 mg/kg and the limit of quantification was 5.4 mg/kg. Though the dossier lacks a full validation of an analytical method suitable for the analysis of carnosic acid in *fat* which is the premixture, in the opinion of the CRL the method validated for the detection of carnosic acid in *feed* also applies to the analysis of *premixtures*. On request of the CRL the applicant delivered additional results indicating that the GC/FID method applied to fat gains acceptable values for the recovery rate and the standard deviation of the method.

In summary, the CRL is of the opinion that both proposed methods fulfil the requirements to determine the concentration of carnosic acid in rosemary extract and in feedingstuff. Taking into account the results from the validation study and given the fact that both instruments (HPLC and GC) are standard techniques in control laboratories, the CRL concludes that both methods are suitable for routine analysis. Therefore, no supplementary experimental work (testing or method validation) is needed.

Date: 24 June 2005

2. TABLE OF CONTENTS

3. KEYWORDS

Rosemary extract, carnosic acid, antioxidants, HPLC, GC

4. BACKGROUND

The feed additive related to dossier FAD-2004-0003 is a rosemary extract of natural origin (*Rosmarinus officinalis L.*) containing between 9 and 10 % of carnosic acid and smaller amounts of other natural compounds such as phenolic diterpenes [1]. These substances are present in sunflower oil which makes up 87 % of the final product and which is added after the extraction of the rosemary plant. Due to the strong antioxidant characteristics of carnosic acid the applicant proposes classifying rosemary extract into the category "Technological Additives" and the functional group "Antioxidants". The target animals are dogs and cats. The target level of addition of rosemary extract ranges from 50 to 500 mg/kg of rosemary extract in feedingstuff corresponding to about 5 to 50 mg/kg of carnosic acid in feedingstuff.

5. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is required to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the suitability of the control methods submitted in connection with FAD – 04 – 003 were evaluated.

6. EVALUATION

The numbering system under this point refers to Section 2.5 (Control Methods) of the Annex of Commission Directive 2001/79/EC. All references to "Annex" refer to this Annex unless otherwise stated.

Description of the methods used for the determination of the criteria as listed under items 2.1.3, 2.1.4, 2.2.3, 2.2.4, 2.3.1, 2.3.2, 2.3.3 and 2.3.4. of the Annex

Qualitative and quantitative composition (active substance, other components, impurities, batch-to-batch variation) according to 2.1.3 of the Annex

For the determination of carnosic acid in the rosemary extract the applicant proposes a method based on HPLC coupled to a DAD detector measuring at 280 nm [2]. The limit of detection and the limit of quantification for the analysis of carnosic acid in rosemary extract are 105 mg/l and 318 mg/l, respectively [5]. According to the protocol the extract is analysed by HPLC after removal of the extraction solvent but without further clean-up procedures. Method performance characteristics have been established on a method protocol that utilises a mixture of tetrafluoroethane and acetone for extracting the target analyte from rosemary leaves, which however deviates from the solvent mixture used in the manufacturing process of the rosemary extract [1]. In the latter process the extraction solvent also contains in addition methanol. Thus, a slightly different composition of the extracts obtained when applying the analytical method and the manufacturing process in terms of the presence of interfering substance and the concentration of carnosic acid cannot be excluded. The overall design of the method proposed by the applicant is straightforward and the HPLC analysis of the rosemary extract shows the main peak corresponding to carnosic acid that is well separated from any interfering substances. It can therefore be concluded that the proposed method is fit for the intended purpose. In addition the applicant conducted extensive studies to establish the present of other naturally occurring compounds such as rosemarinic acid, tocopherols, carotenoids, and other organic acids in the rosemary extract applying HPLC with DAD and mass spectrometry (MS). The concentrations of some trace elements (calcium, cupper, iron, magnesium, phosphorus) are given without specifying the analytical method applied.

The determination of the physical properties of the additive according to 2.1.4 of the Annex

Information is given regarding the physical state and the viscosity but without specifying the analytical method of the latter parameter.

Identification and quantification of occurring chemical impurities and toxic substances according to 2.2.3 of the Annex

The dossier contains information on residues of the extraction solvent from the manufacturing process in the Rosemary extract and the concentration of the following heavy metals that were cadmium, mercury, arsenic and lead and that were all below the detection limit of the method applied (1 mg/kg for cadmium, mercury, arsenic and 5 mg/kg for lead). However, no information on the corresponding method protocol is given in the dossier. In addition no information and therefore no methods are mentioned regarding the presence of persistent organic pollutants such as chloro-organic insecticides in the additive.

Physical properties according to 2.2.4 of the Annex

The dossier contains a number of physical parameters and identification characteristics for ultraviolet (UV) spectroscopy, MS and nuclear magnetic resonance spectroscopy of carnosic acid referring to scientific literature. Though the analytical methods are not explicitly specified it is likely that this information can be found in the scientific papers cited in the dossier.

Stability of each formulation according to 2.3.1 of the Annex

The applicant conducted a study regarding the stability of the active substance in rosemary extract by measuring the concentration of carnosic acid by applying the HPLC method proposed in the dossier. The study revealed a slight decrease of the concentration of the target analyte concluding that the estimated shelf life does not exceed 6 months.

Stability of each formulation according to 2.3.2 of the Annex

The stability of each formulation when added to premixtures and feedingstuff was measured by determining the amount of peroxides in the samples applying a commercially available test kit. The amount of peroxides are expressed in terms of “milli-equivalents” of peroxides without indicating whether this parameter is defined by the method itself and how this response corresponds to a similar response which would be obtained when applying an official method of the American Oil Chemists’ Society (AOCS, Official Method Cd 8b-90) that also measures a peroxide value of samples.

Other information regarding the properties according to 2.3.3 and 2.3.4 of the Annex

The applicant explained in the dossier why no measurements were conducted for the parameters in these chapters. Therefore no analytical methods were described.

Description of the qualitative and quantitative analytical methods for routine control of the active substance in premixtures and feedingstuff, according to 2.5.2 of the Annex.

For the determination of carnosic acid in feedingstuff the applicant proposes a method based on GC coupled to a FID using a non-polar capillary GC column (GC/FID method) [3] . After extraction of the samples with acetonitrile carnosic acid is derivatised with a silylation agent prior to GC analysis owing to its high polarity. The derivative of carnosic acid is less polar and shows a sharp peak in the GC-chromatogram without interferences by other substances. As an optional feature the applicant proposes using a surrogate standard (biphenyl) to control the whole analytical procedure including the derivatisation. The selected method is a classical approach for the measurement of organic acids in complex matrices such as compound feed, especially when the chromatographic system needs to show a high resolution power. The validation study showed that the limit of detection of carnosic acid in

feedingstuff was 1.8 mg/kg and the limit of quantification was 5.4 mg/kg. The recovery rate and relative standard deviation (RSD) of the method were 105 % and 2.4 %, respectively. Taking into account (1) the target level of application which ranges between 50 to 500 mg/kg of rosemary extract in feedingstuff corresponding to 5 to 50 mg/kg of carnosic acid in feedingstuff, (2) the recovery rate and (3) the RSD, it can be concluded that this method is fit for the purpose and suitable for control analysis.

The validation study of this method [4] was carried out on feed matrix samples in which the rosemary extract was introduced in the matrix via fat which is the premixture. However, the study did not explicitly establish whether the method would also be applicable to the determination of carnosic acid in fat. Upon request the applicant provided additional results from various analyses confirming that high recovery rates ranging from 86 % to 101 % of carnosic acid were obtained when applying the above mentioned GC/FID method to fat and oil matrices [6,7]. Therefore no full validation data of the GC/FID method related to the matrix “fat” are available. However, given the general scope of the GC/FID method selected by the applicant and the additional information provided by the applicant it can be assumed that the GC/FID method is also suitable for the determination of carnosic acid in premixtures.

CRL Feed Additives Authorisation

CHECK LIST – PART I

			Y	N	N/A	Comments
1.	A.	Description of the Qualitative and Quantitative analytical method/s for routine control of the active substance in				
		- Premixtures		X		
		- Feedingstuff	X			
	B.	The method has been validated:				
		- In a ring test involving at least four laboratories		X		
		- In-house following harmonised guidelines	X			Another validation guide (see below)
	C.	The validation study contains the following parameters[1]:				
		- Applicability	X			
		- Selectivity	X			
		- Calibration	X			
		- Accuracy	X			
		- Precision	X			
		- Range	X			
		- Limit of detection	X			
		- Limit of quantification	X			

[1] Definition of parameters is given in Annex IV of this document

			Y	N	N/A	Comments
		- Sensitivity	X			
		- Robustness	Y X			
		- Practicability	X			
	D.	Is there evidence available that the characteristics listed above have been assessed?	X			
2.	Description of the Qualitative and Quantitative analytical method/s to determine the marker residue(s) of the active substance:					
	- In target tissue/s				X	
	- In animal products				X	

N/A: Not applicable

CHECK LIST – PART II

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned in Part I (1.- A. Premixtures) accompanied by information on:				
	- Sampling Method used		X		
	- Percentage Recovery	X			
	- Specificity		X		
	- Accuracy		X		
	- Precision		X		
	- Limit of detection		X		
	- Limit of quantification		X		
	- Validation procedure used		X		
1.2	Is/Are the method(s) mentioned in Part I (1.- A. Feedingstuff) accompanied by information on:				
	- Sampling Method used		X		
	- Percentage Recovery	X			
	- Specificity	X			
	- Accuracy	X			
	- Precision	X			
	- Limit of detection	X			
	- Limit of quantification	X			
	- Validation procedure used: Validation of Analytical Procedures: Methodology. in International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. 1996: ICH Steering Committee.				

8. CONCLUSIONS

In this report the analytical methods related to the dossier “FAD 2004-003” have been evaluated, focusing on the determination of carnosic acid which is the active substance. For the determination of carnosic acid the applicant proposes two different methods which are a HPLC based method suitable for the analysis of the active substance in rosemary extract and a GC/FID method for the analysis of the active substance in premixture and in feedingstuff. The limit of detection and the limit of quantification for the analysis of carnosic acid in rosemary extract are 105 mg/l and 318 mg/l, respectively. For the analysis of carnosic acid in feedingstuff the proposed method has a limit of detection of 1.8 mg/kg and a limit of quantification of 5.4 mg/kg.

Both methods require standard laboratory instrumentation and represent classical approaches for the respective analytical tasks. By taking into account the results from the validation study the CRL concludes that the proposed methods are suitable for routine control and that no supplementary experimental work (testing or method validation) is needed.

9. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

The applicant provided the CRL-FAA with the required reference samples.

The dossier provided by the applicant is divided into various documents structured according to the annex of Commission Directive 2001/79/EC. Relevant information regarding Section II (Identity, characterisation and conditions of use of the additive; methods of control) are given in two volumes of the dossier which have the following filenames:

[1] 2[1] Section II-a.pdf

[2] 3[1] Section II-b.pdf

Upon request of EFSA the applicant submitted two additional documents describing the analytical method for the determination of carnosic acid in feedingstuff that have the following filenames:

[3] NCLS_A-28_report.pdf

[4] Gamble_GC_for_CA_white_paper.pdf

Though not explicitly required by the annex of Commission Directive 2001/79/EC additional validation data of the method for the detection of the active substance (carnosic acid) in the feed additive (rosemary extract) are given in this document:

[5] Gamble_RAPID_DETERMINATION_OF_CARNOSIC_ACID.pdf

Upon request of the CRL-FAA the applicant submitted the following documents giving more information regarding the determination of carnosic acid in premixtures:

[6] European Commission REL 27-05-05.pdf

[7] Carnosic Acid Recovery GC II.pdf

10. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the CRL-FAA, IRMM, Geel, Belgium. Responsible person for the evaluation is Christoph von Holst.