EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

EURL
European Union Reference Laboratory
Feed Additives

JRC.F.5/UV/SB/AS/Ares

Subject: Amendment to EURL evaluation reports

References:

FAD-2010-0029 Benzoic acid - JRC.DG.D.6/CvH/GB/mds/Ares(2011)721019
FAD-2006-0012 Benzoic acid - D08/FSQ/(2006) D/29039 - 23/11/2006
FAD-2004-0006 Benzoic acid – D08/FSQ/CVH/AMJ (2005) D24894 - 07/10/2005

In the frame of the above-mentioned *feed additive* dossier, upon publication of the ring-trial validated method EN 17298 for the analysis of *benzoic acid* in feed additives, premixtures, feed materials, compound feed and water, the EURL, under the frame of article 5 of Regulation (EC) No 378/2005, considered appropriate to perform a new evaluation of the methods of analysis for official control [1,2].

For the determination of *benzoic acid* in the *feed additive*, *premixtures* and *compound feed,* the EURL evaluated the ring-trial validated EN 17298 method based on high performance liquid chromatography (HPLC) coupled to spectrophotometric (UV) detection at 230 nm [1]. This method is designed for the determination of *benzoic* and sorbic acid and their salts (as total individual acids) in *feed additives*, *premixtures*, feed materials, *compound feed* and water (for *benzoic acid* only).

5 g of the sample is mixed with 100 ml of the extraction solution containing acetate buffer (pH 4.6) and methanol (60:40, v/v) and treated in ultrasonic bath for 30 min. The resulting extract is filtered using an ash free paper filter or centrifuged at 5000 *g* for 3 min. The filtrate or the supernatant after dilution with the extraction solution, is passed through a membrane filter (0.45 µm) before the chromatographic analysis. The individual analytes are detected by spectrophotometry (UV) at 230 nm and the quantification is performed using an external standard calibration curve prepared from the standard solutions of the above-mentioned acid [1].

Table 1. The performance characteristics obtained in the frame of the ring-trial validation studies of the EN 17298 method for the quantification of total *benzoic acid* in *premixtures*, and *feedingstuffs* (feed material, complimentary feed and compound feed) [1].

	Premixtures	Feedingstuffs
Mass fraction, mg/kg	60121 - 90577	870 - 12668
RSD_r, %	1.8	1.0 – 2.5
RSD_R, %	3.0 – 3.2	1.6 – 5.5
Reference	[1]	

RSD_r and RSD_R: relative standard deviations for *repeatability and reproducibility, respectively*.

The performance characteristics obtained in the frame of the ring-trial validation studies of the EN 17298 method for the quantification of total *benzoic acid* in *premixtures* and *feedingstuffs* (feed material, complimentary feed and compound feed) are presented in Table 1. In addition, a limit of quantification (LOQ) of 200 mg *benzoic* acid /kg *feedingstuffs* is reported [1].

Based on the performance characteristics available and the scope of the method in terms of matrices, the EURL recommends for official control the ring-trial validated EN 17298 method based on high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection for the determination of *benzoic acid* (as total *benzoic acid*) in the *feed additives*, *premixtures* and *compound feed*.

Recommended text for the registry entry (analytical method) (replacing the previous recommendations)

For the determination of *benzoic acid* in the *feed additive*, *premixtures* and *compound feed*:

- High performance liquid chromatography with ultraviolet detection (HPLC-UV) – EN 17298

References

[1] EN 17298 Animal feeding stuffs: Methods of sampling and analysis – Determination of benzoic and sorbic acid by High Performance Liquid Chromatography (HPLC)

[2] Commission Regulation (EC) No 378/2005 of 4 March 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, OJ L 059 5.3.2005, p. 8

Amendment
- Prepared by Stefano Bellorini
- Reviewed by María José González de la Huebra and approved by Ursula Vincent (EURL-FA), respectively, Geel, 26/02/2024

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

D08/FSQ/(2006) D/29039

CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2006-056
Name of Additive:	VevoVitall®
Active Substance(s):	Benzoic Acid
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives Authorisation, IRMM, Geel, Belgium
Report prepared by:	Giuseppe Simone (CRL-FAA)
Report checked by:	Federica Serano (CRL-FAA)
Report approved by:	Christoph von Holst (CRL-FAA)
Date:	23/11/06

EXECUTIVE SUMMARY

VevoVitall® is a feed additive for which authorisation is sought under the category "zootechnical additives", functional groups "other zootechnical additives" and "substances which favourably affect the environment", according to the classification system of Annex I of Regulation (EC) No 1831/2003. VevoVitall® contains high purity benzoic acid (≥ 99.9 % on anhydrous basis) as active substance.

In the current application authorisation is sought for use of VevoVitall® for pigs for fattening. The feed additive is intended to be mixed into compound feedingstuffs at a concentration of 5000 mg to 10000 mg/kg feedingstuffs.

For the determination of the benzoic acid in the *feed additive* the CRL recommends a titrimetric assay as specified by the corresponding monograph of the European Pharmacopoeia.

For the determination of the active substance in *feedingstuffs* a Reversed Phase High Performance Liquid Chromatography (RP HPLC) method with diode array detection (DAD) is submitted. The method's performance characteristics include a recovery rate between 94 % and 113 %, a relative repeatability standard deviation (RSD_r) of 2 % and a relative within-laboratory reproducibility standard deviation (RSD_R) of 5 %. The limit of detection of the method is 500 mg/kg and the limit of quantification is 2000 mg/kg. These performance characteristics are considered acceptable and the method is therefore considered suitable for official control purposes, if the analysis aims at the quantification of the target analyte in feedingstuffs samples in the frame of the sought authorisation, i.e. in target feed samples (feedingstuffs for pigs for fattening) at the target concentration range of benzoic acid (5000 to 10000 mg/kg).

Control methods are submitted for determination of possible contaminants and impurities (heavy metals, arsenic, organic impurities) in the feed additive which are considered suitable for the intended purposes. For official controls of heavy metals various standard methods, based on the same analytical technique and routinely applied by official control authorities are available and recommended by the CRL.

Further testing or validation by the CRL is not considered necessary.

KEYWORDS
VevoVitall®, benzoic acid, zootechnical additives, feed additive, acidity regulator, pigs

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BACKGROUND

VevoVitall® is a feed additive for which authorisation is sought under the category "other zootechnical additives", functional groups "other zootechnical additives" and "substances which favourably affect the environment" according to Annex I of Regulation (EC) No 1831/2003 [1]. VevoVitall® contains benzoic acid with a purity of ≥ 99.9 % (on anhydrous basis) as the active substance [1]. Benzoic acid is already provisionally authorised as acidity regulator for urine of fattening pigs [2] and the authorisation is granted by the EC until 25 May 2007.

The intended use (*cf*. EFSA-Q-2006-056) of the current application is for pigs for fattening by incorporating the feed additive directly into compound feeds at a concentration of minimum 5000 to maximum 10000 mg/kg complete feedingstuff [1].

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 requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis submitted in connection with VevoVitall® dossier (EFSA-Q-2006-056) and their suitability to be used for official controls were evaluated.

EVALUATION

The numbering system under this point refers to that of Section II of the Annex of Commission Directive 2001/79/EC (2.5 Control methods).

Description of some of the methods used for the determination of the criteria listed under item 2.5.1 of Commission Directive 2001/79/EC

Determination of water content

A method based on Karl Fischer titration is proposed for determination of water [3]. This method is considered suitable for official controls.

Determination of heavy metals

For the analysis of heavy metals, including arsenic (As), the applicant proposed an inductively coupled plasma mass spectrometry (ICP-MS) method after microwave digestion [3]. The method is considered suitable for the intended purpose. For official controls various standard methods, based on the same analytical technique and routinely applied by official control authorities are available and recommended by the CRL.

Determination of organic impurities

For the determination of organic impurities (benzyl benzoate, diphenyl and three methyldiphenyl isomers) in benzoic acid a Gas Chromatography (GC) method is proposed which utilised a capillary column with chemically bonded free fatty acid phase and a flame ionisation detector (FID) [5]. The sample is dissolved in dimethylformamide and injected on the GC. The target analytes are quantified using n-propyl benzoate as internal standard and the chromatogram shows well separated peaks of the compounds. Traces of other impurities (phtalic acid, three hydroxybenzoic acid isomers and three methylbenzoic acid isomers) in benzoic acid are determined by isocratic Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and Ultraviolet (UV) detection measuring at 254 and 296 nm [6]. In the chromatogram the majority of the peaks of target analytes are well separated from each other. These methods are considered suitable for the intended purposes.

Quantitative analysis of active substance (benzoic acid) in the feed additive

The applicant proposed to calculate the purity of the dry benzoic acid by the equation [7]:

(100% - % total organic impurities)

Since the specifications [8] of VevoVitall® are in compliance with the requirements of the European Pharmacopoeia (Ph. Eur.) for benzoic acid, the CRL recommends determining the purity of the product by the titrimetric assay using sodium hydroxide prescribed by the corresponding monograph of the European Pharmacopoeia [9].

Description of the qualitative and quantitative analytical methods for routine control of the active substance in feedingstuffs (2.5.2. of the Guidelines)

An RP-HPLC method based on the standard addition technique (spiking) is proposed for the determination of benzoic acid in pig feed [10]. According to the method protocol pig feed granulates are ground to obtain a homogenous sample. From the homogenised feed sample 6 x 1 gram samples are taken. To five of these samples a known amount of benzoic acid is added, usually 2 mg, 10 mg, 20 mg, 30 mg and 40 mg, whereas one sample is analysed as such. To all samples 50 mM sodium hydroxide solution is added to obtain a final volume of the extraction solution of 40 ml. After an extraction time of 1 hour at 100 ^{0}C and at pH > 10, the volume is adjusted to 100 ml with an aqueous perchloric acid solution. An aliquot of the top layer clear solution is filtered and injected on the HPLC. The HPLC analysis is performed on a 250*4mm Nucleosil C18 column with an eluent mixture containing an aqueous 10mM phosphoric acid/acetonitrile solution [90+10(v+v)], and modifying the composition of the eluent by a gradient, obtaining a final aqueous 10mM phosphoric acid/acetonitrile solution [25+75(v+v)]. Benzoic acid is quantified at a wavelength of 278 nm and the purity of the peak is determined with a diode array detector (DAD) measuring from 200 to 300 nm. The chromatogram of a sample containing 10000 mg/kg benzoic acid shows a symmetric peak of this analyte at about 5.5 minutes without any interference.

The relative *repeatability* standard deviation (RSD_r) of the method was 2 %, as calculated from the analysis of 6 feed samples that contained 9000 mg benzoic acid / kg feed. The relative within-laboratory *reproducibility* standard deviation (RSD_R) representing the precision when the method was performed on different days, by different technicians and

using different instruments was 5 %, as determined by analysing three identical feed sample containing 20000 mg benzoic acid / kg feed [11]. The limit of detection is 500 mg/kg and the limit of quantification is 2000 mg benzoic acid / kg feed [11]. The recovery was assessed *via* stability tests, in which samples containing 2500, 5000 and 10000 mg sodium benzoate/kg (corresponding to 2119, 4237 and 8474 mg benzoic acid/kg, respectively), were analysed 6 times, respectively. The recovery rate was between 94% and 113% [12]. Considering the complexity of feed matrices and the target concentration levels of the active substance in feed the obtained method performance characteristics are considered acceptable.

The analytical method is considered suitable for official control purposes, if the analysis aims at the quantification of the target analyte in feedingstuffs samples in the frame of the sought authorisation, i.e. in target feed samples (feedingstuffs for pigs for fattening) at the target concentration range of benzoic acid (5000 to 10000 mg/kg).

Description of the qualitative and quantitative analytical methods for determining the marker residue(s) of the active substance in target tissues and animal products.

An RP HPLC method was proposed in order to determine benzoic acid and hippuric acid in the following types of tissue: liver, kidney, fat, loin muscle and leg muscle [13]. The samples are extracted with acetonitrile containing 0.1 M formic acid. Without further sample preparation an aliquot of the filtered and centrifuged extract was injected on the HPLC and measured with a UV detector at 228 nm. The limit of quantification (LOQ) for benzoic acid is 5 mg/kg tissue and for hippuric acid 10 mg/kg tissue. With the exception of the LOQ no other performance characteristics have been provided. Therefore the suitability of the method could not be evaluated.

CONCLUSIONS AND RECOMMENDATIONS

For the determination of benzoic acid in the feed additive the CRL recommends the assay prescribed by the corresponding Ph. Eur. Monograph. This is possible, since the specifications of VevoVitall® are in compliance with the requirements of the European Pharmacopoeia (Ph. Eur.) for benzoic acid.

For the determination of benzoic acid in feedingstuffs a Reversed Phase High Performance Liquid Chromatography (RP HPLC) method with diode array detector (DAD) is submitted.

The performance characteristics are considered acceptable and the method is therefore considered suitable for official control purposes, if the analysis aims at the quantification of the target analyte in feedingstuffs samples in the frame of the sought authorisation, i.e. in target feed samples (feedingstuffs for pigs for fattening) at the target concentration range of the active substance (5000 to 10000 mg/kg).

For determination of benzoic acid and its metabolites in animal tissues obtained from pigs fed feedingstuff containing benzoic acid, an HPLC method is submitted. The reported limits of quantification are 5 mg/kg for benzoic acid and 10 mg/kg for hippuric acid, both of which are considered acceptable. However, other important performance characteristics, as required in the Guidelines (*cf*. Directive 2001/79/EC) have not been reported and therefore the suitability of the method could not be evaluated.

Control methods are submitted for determination of possible contaminants and impurities (heavy metals, arsenic, organic impurities) in the feed additive. The methods are considered suitable for the intended purpose. For official controls of heavy metals various standard methods, based on the same analytical technique and routinely applied by official control authorities are available and recommended by the CRL.

Further testing or validation by the CRL is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Reversed Phase HPLC with Diode Array Detection (DAD)

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of VevoVitall® have been sent to the Community Reference Laboratory for feed additives authorisation on 17 December 2004. The dossier has been made available to the CRL by EFSA.

REFERENCES

The dossier provided by the applicant is divided into various documents structured according to the Annex of Commission Directive 2001/79/EC, containing the following files:

[1] Annex III – Proposal of Register entry

[2] Regulation (EC) No 877/2003

[3] Technical dossier, Section II, Annex 7.2.27e

[4] Technical dossier, Section II, Annex 7.2.27d

[5] Technical dossier, Section II, Annex 7.2.27b

[6] Technical dossier, Section II, Annex 7.2.27a

[7] Technical dossier, Section II, 2.5.1

[8] Technical dossier, Section II, 2.1.2

[9] European Pharmacopoeia 5th Edition

[10] Technical dossier, Section II, Annex 7.2.28

[11] Technical dossier, Section II, Annex 7.2.13

[12] Additional information, Report 2002-2084NC

[13] Technical dossier, Section II, Annex 7.2.31

RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives Authorisation, Geel, Belgium