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



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



D08/FSQ/CVH/AMJ (2005) D 24894

**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-2005-007

Name of Additive: VevoVital[®]

Active Substance(s): Benzoic Acid

Rapporteur Laboratory: OMMI Kozponti Laboratorium - Budapest
Hungary

Report prepared by: Jozsef Dömsödi

Report checked by: A. M. Jensen (CRL-FAA)
Date: 03 October 2005

Report approved by: C. von Holst (CRL-FAA)
Date: 07 October 2005

1. EXECUTIVE SUMMARY

VevoVitall[®] is a feed additive belonging to the 'other zootechnical additives' group (4d), according to the classification system of Annex I of Regulation (EC) No 1831/2003. VevoVitall[®] is a high purity benzoic acid product ($\geq 99.9\%$ on anhydrous basis). Benzoic acid is widely used in food. The appearance is white flakes.

In the current application authorisation is sought for use of VevoVitall[®] as a performance enhancer for weaned piglets. The feed additive is intended to be mixed into compound feedingstuffs at a concentration of 5000 mg/kg .

For the determination of the active substance (benzoic acid) in the feed additive a titrimetric method is proposed, which is considered suitable for samples containing more than 90 % (w/w) benzoic acid. This method is considered suitable for routine control for the field of application that is sought.

For the determination of the active substance (benzoic acid) in feedingstuffs a Reversed Phase High Performance Liquid Chromatography (RP HPLC) method with Ultraviolet (UV) detection is submitted. The method's performance characteristics include a recovery rate above 95 %, a relative repeatability standard deviation of 2 % and a relative within-laboratory reproducibility standard deviation 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 routine control for the field of application that is sought. By utilising another type of detector which measures absorbance across a spectrum of wavelengths simultaneously the specificity of the method can be significantly improved. However, since the validation did not include a wide range of different feed matrices and since there are no practical experiences available regarding the use of a more specific detector, the suitability of the proposed method for official control purposes cannot be confirmed.

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.* the Annex of Directive 2001/79/EC) have not been reported and therefore the suitability of the method for official control could not be evaluated.

Control methods are submitted for determination of possible contaminants and impurities (heavy metals, arsenic, sulphate) in the feed additive. These methods are based on well known principles and widely applied techniques and they are considered fit for routine and official control purposes without further validation.

Further testing or validation is not considered necessary.

2. KEYWORDS

VevoVital[®], benzoic acid, zootechnical additives, feed additive, acidity regulator, piglets

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4. BACKGROUND

VevoVital[®] is a feed additive belonging to the 'other zootechnical additives' group (4d). It contains benzoic acid: purity of ≥ 99.9 % (on anhydrous basis) as the active ingredient. Benzoic acid is already provisionally authorized as acidity regulator for urine of fattening pigs cf. Regulation (EC) No 877/2003. The provisional authorisation is granted by the EC until 25 May 2007.

The intended use (*cf.* EFSA-Q-2005-007) of the current application is to enhance the performance of weaned piglets, by mixing the feed additive into compound feedingstuffs in a concentration of 5000 mg/kg.

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 requested 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 and validation studies submitted in connection with VevoVital[®], *cf.* EFSA-Q-2005-007, was evaluated.

6. 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). The method protocols and corresponding validation data – if applicable – are given in Appendix Section II of the dossier [1], whereas the method protocol for the determination of residues in animal tissue is specified in another file of the dossier [2]. For methods regarding impurities see [3] and [4].

Description of the methods used for the determination of the criteria listed (cf. pt. 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. This method is considered suitable for routine and official control.

(*Cf.* the requirements listed in point 2.1.3 of the Annex)

Determination of heavy metals

A colorimetric comparison method from the Food Chemicals Codex is proposed. Its limit of detection of is 10 mg/kg for various heavy metals. The sample is evaporated, dissolved in nitric acid and sodium carbonate. After evaporation of the solution to dryness, the residue is dissolved in acetic acid and water and hydrogen sulphide is added. The resulting colour is compared with colour of a standard lead solution. For the determination of arsenic a Food Chemicals Codex method, based on spectrophotometry, is proposed. It has a limit of detection of 2 mg/kg. The proposed methods are commonly used and are considered suitable for routine and official control, since the referred source (Food Chemicals Codex) is generally accepted and since the limits of detection are considered acceptable.

(*Cf.* the requirements listed in point 2.2.3 of the Annex)

Particle size distribution

Examination is performed by sieve analysis. The method is commonly used and is considered suitable as routine and official method.

(Cf. the requirements listed in point 2.1.4 of the Annex)

Relevant properties (melting point, boiling point etc.)

The proposed methods are commonly used for determination of the relevant properties and are considered suitable as routine and official method.

(Cf. the requirements listed in point 2.2.4 of the Annex)

Stability of the additive

The applied analytical method is commonly used and is considered suitable for this purpose (see below in the chapter “Quantitative analysis of active substance (benzoic acid) in the feed additive”).

(Cf. the requirements listed in point 2.3.1 of the Annex)

Stability during the preparation and storage of the feed

The applied analytical method is identical with the method proposed by the applicant as control method according to pt. 2.5.2 of the Annex and is considered suitable for this purpose.

(Cf. the requirements listed in point 2.3.2 of the Annex)

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

Since VevoVital[®] is a high purity benzoic acid product, points 2.1.3 (Qualitative and quantitative composition) and 2.2.3 (Purity) of the Annex are considered together in the evaluation (see below).

For the determination of benzoic acid in benzoic acid flakes a titrimetric method is applied which is considered suitable for samples containing more than 90 % (w/w) benzoic acid.

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. The sample is dissolved in dimethylformamide and injected on the GC. The target analytes are quantified using an 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 isormers) in benzoic acid are determined by isocratic Reverse Phase (RP) High Performance Liquid Chromatography (HPLC) and Ultraviolet (UV) detection measuring at 254 and 296 nm. In the chromatogram the majority of the peaks of target analytes are well separated from each other. The methods are considered suitable for routine control.

(Cf. the requirements listed in point 2.1.3 and 2.2.3 of the Annex)

Description of the qualitative and quantitative analytical methods for routine control of the active substance in premixtures and feedingstuffs.

In the Summary for Publication submitted by the Applicant in May 2005 it is stated, that VevoVital[®] is placed on the market for use in premixtures and compound feed. However, according to EFSA's Annex III (Proposal of Annex Entry), a letter to the CRL from the Applicant dated 7 July 2005, and the Applicant's Summary for publication dated April 2005, authorisation for use in premixtures is not sought. Methods for determination of the active substance in premixtures and validation hereof have therefore not been considered during the evaluation.

Description of the method to determine benzoic acid in feed

An RP HPLC method based on the standard addition technique (spiking) is proposed for the determination of benzoic acid in pig feed. The principle of a standard addition experiment is that a set of identical samples of the homogenised feed is taken. Part of the sample set is analysed as such, but to the other samples known amounts of the standard of the target analyte are added prior to chemical analysis. By subjecting the analytical results of the sample set to statistical assessment, the amount of benzoic acid in the unknown samples is calculated.

In this particular case, pig feed granulates are ground to obtain a homogenous sample. From the homogenised feed sample 6x1 gram samples are taken. Two of the samples are analysed without addition of benzoic acid whereas to the other four samples 10 mg, 20 mg, 30 mg and

40 mg of benzoic acid are added, respectively. The standard is dissolved in an aqueous 10 mM sodium-hydroxide solution and added to the samples prior to the extraction procedure. To each of these 1 gram samples aqueous 10 mM sodium-hydroxide solution is added to obtain a final volume of the extraction solution of 100 ml. After an extraction time of 1 hour at 100 °C and at pH 11 to 12 the pH of the suspension is adjusted to a pH of 0 to 1 with perchloric acid. The extracts are filtered and an aliquot of the clear solutions is injected on the HPLC. The HPLC analysis is performed on a 250*4mm Nucleosil C18 RP column with an eluent mixture containing aqueous 10mM phosphoric acid solution and acetonitrile, and modifying the composition of the eluent by a gradient. Benzoic acid is measured with an UV detector at 278 nm. The chromatogram of a sample containing 20000 mg/kg benzoic acid shows a symmetric peak of benzoic acid at about 6 minutes without any interferences.

The relative *repeatability* standard deviation of the method is 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 representing the precision when the method is performed on different days, by different technicians and using different instruments is 5 %, as determined by analysing three identical feed sample containing 20000 mg benzoic acid / kg feed. The limit of detection is 500 mg/kg and the limit of quantification is 2000 mg benzoic acid / kg feed. The recovery rate was assessed by analysing 6 samples containing 10000 mg/kg and 20000 mg/kg benzoic acid in feed and was in both cases above 95 %. Considering the complexity of feed matrices and the target concentration levels of the active substance in feed the obtained method performance characteristics are acceptable. The method is considered as fit for routine control, since normal laboratory equipment and instrumentation is required to carry out the analysis.

By utilising a diode array detector which measures absorbance across a spectrum of wavelengths simultaneously the specificity of the method can be significantly improved. However, since there are no practical experiences regarding the use of a more specific detector and since the validation did not include a wide range of different feed matrices, the suitability of the proposed method for official control purposes cannot be confirmed.

(cf. pt. 2.5.2 of the Annex of the Commission Directive 2001/79/EC)

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. 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. The overall design of the analytical methods appears to be scientifically sound. With the exception of the LOQ no other performance characteristics have been provided. Therefore the suitability of the method for official control could not be evaluated.

(cf. pt. 2.5.3 of the Annex of the Commission Directive 2001/79/EC)

CHECKLIST

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned on Premixtures accompanied by information on:			X	
	- Sampling Method used				
	- Percentage Recovery				
	- Specificity				
	- Accuracy				
	- Precision				
	- Limits of detection				
	- Limits of quantification				
	- Validation procedure used				
1.2	Is/Are the method(s) mentioned on Feedingstuffs accompanied by information on:	X			Applied validation guide was not specified
	- Sampling Method used		X		
	- Percentage Recovery	X			
	- Specificity		X		
	- Accuracy	X			
	- Precision	X			
	- Limits of detection	X			
	- Limits of quantification	X			
	- Validation procedure used	X			
2.1	Is/Are the method(s) mentioned on Target tissues accompanied by information on:	X			
	- Sampling Method used		X		
	- Percentage Recovery		X		
	- Specificity		X		
	- Accuracy		X		
	- Precision		X		
	- Limits of detection		X		
	- Limits of quantification	X			
	- Validation procedure used		X		Applied validation guide was not specified
2.2	Is/Are the method(s) mentioned on Animal products accompanied by information on:			X	
	- Sampling Method used				
	- Percentage Recovery				
	- Specificity				
	- Accuracy				
	- Precision				
	- Limits of detection				
	- Limits of quantification				
	- Validation procedure used				
3.	If the method(s) has/have been devised, consideration has been given to the fact that their limits of quantification must be below the MRLs.			X	

7. CONCLUSIONS AND RECOMMENDATIONS

For the determination of the active substance (benzoic acid) in the feed additive a titrimetric method is proposed, which is considered suitable for samples containing more than 90 % (w/w) benzoic acid. This method is considered suitable for routine control for the field of application that is sought.

For the determination of the active substance (benzoic acid) in feedingstuffs a Reversed Phase High Performance Liquid Chromatography (RP HPLC) method with Ultraviolet (UV) detection is submitted. The method's performance characteristics include a recovery rate above 95 %, a relative repeatability standard deviation of 2 % and a relative within-laboratory reproducibility standard deviation 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 routine control for the field of application that is sought. By utilising another type of detector which measures absorbance across a spectrum of wavelengths simultaneously the specificity of the method can be significantly improved. However, since the validation did not include a wide range of different feed matrices and since there are no practical experiences available regarding the use of a more specific detector, the suitability of the proposed method for official control purposes cannot be confirmed.

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 for official control could not be evaluated.

Control methods are submitted for determination of possible contaminants and impurities (heavy metals, arsenic, sulphate) in the feed additive. These methods are based on well known principles and widely applied techniques and they are considered fit for routine and official control purposes without further validation.

Further testing or validation is not considered necessary.

8. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of VevoVital[®] have been sent to the Community Reference Laboratory for feed additives authorisation 17 December 2004.

The dossier has been made available to the CRL by EFSA.

9. 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]: 02 Dossier May 1999 Appendix Section II (Identity, characterisation and conditions of use of the additive; methods of control)
- [2]: 01 Dossier May 1999 + ref list Section II (Identity, characterisation and conditions of use of the additive; methods of control)
- [3]: DSM SPR AM 001 (GC), Determination some impurities in benzoic acid and sodium benzoate by GC method
- [4]: DSM SPR AM 002 (HPLC), Determination other group of impurities in benzoic acid and sodium benzoate by HPLC method

10. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was OMMI Kozponti Laboratorium - Budapest Hungary (National Institute for Agricultural Quality Control /NIAQC/, Central Laboratory, Budapest, Hungary.

11. APPENDIX

Not applicable

**Addendum to CRL report (No. D08/FSQ/CVH/AMJ (2005) D 24894) on the dossier
EFSA-Q-2005-007 (VevoVital[®])**

**Additional conclusion regarding the suitability of the analytical method for official
control purposes:**

The analytical method for the determination of the active substance in feedingstuff is, however, 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 weaned piglets) at the target concentration level of the active substance (5000 mg/kg).