EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

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, 29/01/2024

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
European Union Reference Laboratory for Feed Additives

JRC.DG.D.6/CvH/GB/mds/ARES(2011)721019

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-2010-0029 CRL/100039
Feed additive:	Vevo Vitall
Active Substance(s):	Benzoic acid
Rapporteur Laboratory:	European Reference Laboratory for Feed Additives, IRMM, Geel, Belgium
Report prepared by:	Gerhard Buttinger
Report revised by: Date:	Piotr Robouch (EURL-FA) 29/06/2011
Report approved by: Date:	Christoph von Holst 01/07/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 13(3) for *benzoic acid* under the category of "zootechnical additives" functional group 4d (other zootechnical additives), according to the classification system of Annex I of Regulation (EC) No 1831/2003. The *feed additive Vevo Vitall* is currently authorised under Regulation (EC) No 1730/2006.

According to the Applicant, the *feed additive* is a white powder of flaky consistency. The *feed additive* consists of *benzoic acid*, with minimum purity of 99.9 %. Specifically, authorisation is sought for the use of the *feed additive* for piglets (weaned).

In contrast to the current authorisation where the *feed additive* has to be incorporated into the *complete feeding stuff* via a *complementary feed*, the *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs*. The Applicant suggested a minimum and maximum level of 5 g/kg feedingstuff, as set in the previous regulations.

For the quantification of *benzoic acid* in *feed additive* the Applicant proposed the European Pharmacopoeia Monograph method based on acid/base titration with 0.1 M sodium hydroxide and phenol red as indicator. The EURL recommends for official control the internationally recognised European Pharmacopoeia method (Monograph 0066) to quantify *benzoic acid* in the *feed additive*.

For the quantification of *benzoic acid* in *premixtures* and *feedingstuff* the Applicant proposed a method based on high performance liquid chromatography with UV detection (HPLC-UV) derived from ISO 9231:2008 (benzoic acid in milk and milk products). The Applicant provided validation and verification data demonstrating the applicability of the method and therefore extending the scope of the method to *premixtures* and *feedingstuffs*.

Based on these acceptable method performance characteristics the EURL recommends for official control the Applicant's protocol based on ISO 9231:2008 to quantify *benzoic acid* content using HPLC-UV in the *premixtures* and *feedingstuffs,* within the concentration range covered by the experimental data.

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
benzoic acid, piglets (weaned), zootechnical additive, other zootechnical additives

1. BACKGROUND

In the current application authorisation is sought under 13(3) for *benzoic acid* under the category of "zootechnical additives" functional group 4d (other zootechnical additives) [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003. The feed additive *Vevo Vitall* is currently authorised under Regulation (EC) No 1730/2006 [5].

According to the Applicant, the *feed additive* is a white powder of flaky consistency. The *feed additive* consists of *benzoic acid*, with minimum purity of 99.9 % [2]. A maximum impurity content of phtalic acid and of the sum of bipenhyl and related compounds is specified with 100 mg/kg each.

Specifically, authorisation is sought for the use of the *feed additive* for piglets (weaned) [1].

In contrast to the current authorisation where the *feed additive* has to be incorporated into the *complete feeding stuff* via a *complementary feed*, the *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs* [3]. The Applicant suggested a minimum and maximum level of 5 g/kg feedingstuff [2], as set in the previous regulations [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 this particular dossier, the methods of analysis submitted in connection with *benzoic acid*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

For the identification of *benzoic acid* in *feed additives* the EURL recommends, as suggested by the Applicant, the methods described in the European Pharmacopoeia monograph 0066 [6]. The identification is based on the melting point and selective precipitation reactions of benzoate.

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, PAHs and dioxins) are available from the respective European Union Reference Laboratories [4].

For the quantification of *phthalic acid* impurities in *feed additive* the Applicant proposed a single laboratory validated and further verified method, based on high performance liquid chromatography with UV detection (HPLC-UV).

The method is able to determine phthalic acid, 2-hydroxybenzoic acid, 2-methyl benzoic acid, 3-hydroxybenzoic acid, 3-methyl benzoic acid, 4-hydroxybenzoic acid and 4-methyl benzoic acid. The sample is dissolved in a mixture of tetrahydrofuran, formic acid and water. The solution is directly used for the determination via high performance liquid chromatography with UV detection (HPLC-UV) at 254 nm and 296 nm, applying the standard addition technique [7]. The performance characteristics are summarized in Table 1.

Table 1 Performance characteristics for phthalic acids and analogues [8, 9]

	LOQ [mg/kg]		RSD_r [%]		RSD_{ip} [%]	
	validation	verification	validation	verification	validation	verification
phthalic acid	12	8.1	6.0	5.7	6.1	5.8
2-hydroxybenzoic acid	2	2.1				
2-methylbenzoic acid	12	12	13	7.8	13	8.0
3-hydroxybenzoic acid	5	7.8				
3-methylbenzoic acid	11	17.5	5.1	9.4	6.1	11
4-hydroxybenzoic acid	0.3	0.54				
4-methylbenzoic acid	2	4.4	1.8	1.8	1.8	2.0

For the quantification of *biphenyl and related compounds impurities* in *feed additive* the Applicant proposed a single laboratory validated and further verified method, based on gas chromatography with flame ionisation detection (GC-FID).

The method is able to determine 2-methylbiphenyl, biphenyl, 3-methylbiphenyl, 4-methylbiphenyl, benzylbenzoate and isomers of dimethylbiphenyl. The sample is dissolved in N, N-dimethylformamide and n-propyl benzoate is added as internal standard. The solution is directly used for the determination via gas chromatography with flame ionisation detection (GC-FID) [10]. The performance characteristics are summarized in Table 2.

Table 2 Performance characteristics for biphenyl and analogues [11, 12]

	LOQ [mg/kg]		RSD_r [%]		RSD_{ip} [%]	
	validation	verification	validation	verification	validation	verification
biphenyl	2	3.6				
2-methylbiphenyl	2	3.4				
3-methylbiphenyl	3	3.4	1.6	4.0	1.9	5.0
4-methylbiphenyl	3	4.0	1.7	3.6	4.1	3.6
benzylbenzoate	6	6.2	3.5	4.6	4.6	4.5
isomers of dimethylbiphenyl	3	3.4	4.8	5.6	7.3	6.0

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

For the quantification of *benzoic acid* in *feed additive* the Applicant proposed additionally to the European Pharmacopoeia Monograph [6] to calculate the purity of *benzoic acid* as 100 % minus the sum of impurities found with the above mentioned GC and HPLC methods.

Therefore, the EURL recommends for official control the internationally recognised European Pharmacopoeia method (Monograph 0066) [6], based on acid/base titration with 0.1 M sodium hydroxide and phenol red as indicator, to quantify *benzoic acid* in the *feed additive*.

For the quantification of *benzoic acid* in *premixtures* and *feedingstuffs* the Applicant proposed an adapted version of an internationally standardised method for benzoic acid in milk and milk products (ISO 9231:2008) [13], based on high performance liquid chromatography with UV detection (HPLC-UV).

The sample is extracted with a 0.1 M sodium hydroxide solution and cleared from lipids and proteins by precipitation with Carrez solution. The solution is filtered and directly used for the determination via high performance liquid chromatography with UV detection (HPLC-UV) at 227 nm, using external calibration [14]. The performance characteristics are summarized in Table 3. The provided validation data demonstrate the applicability of the method and therefore extend the scope of the method to *premixtures* and *feedingstuffs*.

Table 3 Performance characteristics for *benzoic acid* in *premixtures* and *feedingstuffs* [15]

benzoic acid	LOQ [g/kg]	RSD_r [%]	RSD_{ip} [%]	Level [g/kg]
premixture	1.4	0.5-1.4	0.5-1.9	50 - 100
feedingstuff	0.48	0.4-0.9	0.4-1.7	3 - 5

Based on the method performance characteristics presented the EURL recommends for official control the Applicant's protocol based on ISO 9231:2008 to quantify *benzoic acid* content by HPLC-UV in the *premixtures* and *feedstuffs,* within the concentration range covered by the experimental data.

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 Monograph method based on acid/base titration to quantify *benzoic acid* in *feed additive,*
- the Applicant's method based on ISO 9231:2008, using method high performance liquid chromatography with UV detection (HPLC-UV) to quantify *benzoic acid* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *benzoic acid* in the *feed additive*:

- titration with sodium hydroxide (European Pharmacopoeia monograph 0066)

For the quantification of the *benzoic acid* in the *premixtures* and *feedingstuffs*:

- reversed phase liquid chromatography with UV detection (RP-HPLC/UV) – method based on ISO9231:2008

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Vevo Vitall* 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 SANCO/D/2 Forw. Appl. 1831/0019-2010

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

[3] *Technical dossier, Section II

[4] 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

[5] Commission Regulation (EC) No 1730/2006 concerning the authorisation of benzoic acid (VevoVitall) as feed additive

[6] *Technical dossier, Section II – Annex-2-23

[7] *Technical dossier, Section II – Annex-2-15

[8] *Technical dossier, Section II – Annex-2-17

[9] *Technical dossier, Section II – Annex-2-19

[10] *Technical dossier, Section II – Annex-2-16

[11] *Technical dossier, Section II – Annex-2-18

[12] *Technical dossier, Section II – Annex-2-20

[13] *Technical dossier, Section II – Annex-2-26

[14] *Technical dossier, Section II – Annex-2-24

[15] *Technical dossier, Section II – Annex-2-25

* Refers to Dossier No. FAD-2010-0029

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

The following National Reference Laboratories contributed to this report:

- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer, DE
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby, DK
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, CZ
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino, IT
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana, SI
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien