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Feed Additives

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

Botanically defined flavourings group BDG 16 - Rosales

(FAD-2010-0325; CRL/100218)

European Union Reference Laboratory
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Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2010-0325 - CRL/100218**
Name of Product:	***Hawthorne tincture, dropwort tincture, hop tincture, tormentill tincture, rose tincture, black berry tincture, common nettle extract (sb), common nettle extract (wb) and dwarf nettle tincture from botanically defined flavourings Group (BDG 16) - Rosales***
Phytochemical marker(s):	**Hyperoside, isoquercitrin, ethyl salicylate, rutin, xanthohumol, tannins, ellagic tannins, ellagic acid, quercetin, silica and punicalagin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Zigmas Ezerskis** **09/07/2021**
Report approved by: Date:	**Christoph von Holst** **09/07/2021**

EXECUTIVE SUMMARY

In the current grouped application an authorisation is sought under Articles 4 and 10 for *hawthorne tincture, dropwort tincture, hop tincture, tormentill tincture, rose tincture, black berry tincture, common nettle extract (sb), common nettle extract (wb) and dwarf nettle tincture from botanically defined flavourings Group (BDG 16) - Rosales*[1], under the category/functional group 2(b) 'sensory additives'/flavouring compounds', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for all animal species. For each preparation the Applicant indicated the corresponding phytochemical marker(s) and the range of content. The *feed additives* are intended to be incorporated into *feedingstuffs* or drinking *water* directly or through flavouring *premixtures* with no proposed minimum or maximum levels. However, the Applicant suggested the typical maximum inclusion level of the *feed additives* of 25 mg/kg *feedingstuffs*.

For the quantification of the phytochemical markers *hyperoside* and *isoquercitrin* in *hawthorne tincture*, *rutin* and *xanthohumol* in *hop tincture*, *ellagic acid* and *quercetin* in *rose tincture* and the *ellagic acid* in *black berry tincture* the Applicant proposed a single-laboratory validated and further verified method based on high performance liquid chromatography coupled with diode array detection (HPLC-DAD). The Applicant provided validation and verification studies demonstrating satisfactory performance characteristics (i.e. precision expressed as relative standard deviation for *repeatability* (RSD_r) and *reproducibility* (RSD_R) ranging from 0.1 to 5.0 %). Based on the experimental evidences provided, the EURL recommends for official control the single-laboratory validated and further verified method based on HPLC-DAD for the quantification of the phytochemical markers *hyperoside* and *isoquercitrin* in *hawthorne tincture*, *rutin* and *xanthohumol* in *hop tincture*, *ellagic acid* and *quercetin* in *rose tincture* and the *ellagic acid* in *black berry tincture*.

For the identification of the phytochemical marker *ethyl salicylate* in *dropwort tincture*, the Applicant submitted a method based on gas chromatography coupled to mass spectrometry (GC-MS) using a retention time locking (RTL) methodology. The EURL recommends for official control the method based on GC-MS using RTL methodology for the identification of the phytochemical marker *ethyl salicylate* in *dropwort tincture.*

For the determination of the phytochemical markers *tannins* and *ellagic tannins* in *tormentill tincture* the Applicant submitted a thin-layer chromatographic method (TLC) described in the European Pharmacopeia monograph for *tormentill tincture*. However, the method is not suitable for the quantification of the indicated markers. Nevertheless, the EURL identified in

the same monograph a specific spectrophotometric method (absorbance at 760 nm) for the determination of *tannins* in herbal drugs. The EURL recommends for official control the spectrophotometric method specified in the European Pharmacopeia monograph for the quantification of the phytochemical marker *tannins* in *tormentill tincture*. Furthermore, the different methods described within the European Pharmacopeia monograph for *tormentill tincture* are considered by the EURL suitable for the identification of the *feed additive* preparation.

For the determination of the phytochemical marker *silica* in *common nettle extract (sb)* and *common nettle extract (wb)* the Applicant submitted a single-laboratory validated and further verified method based on inductively coupled plasma-optical emission spectrometry (ICP-OES). Precision values from 1.9 to 4.1 % were derived for the analysis of samples of *nettle* leaves containing 1.5 % of silica. Based on the experimental evidences available the EURL recommends for official control the single-laboratory validated and further verified method based on ICP-OES for the quantification of the phytochemical marker *silica* in *common nettle extract (sb)* and *common nettle extract (wb).*

For the determination of the phytochemical marker *punicalagin* in *dwarf nettle tincture* the Applicant submitted a TLC method described in the French Pharmacopoeia monograph for "Urtica urens pour préparations homéopathiques". However, the TLC method described in the monograph is not suitable for the quantification of the phytochemical marker *punicalagin* in *dwarf nettle tincture*. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify *punicalagin* in *dwarf nettle tincture*. Nevertheless, the different methods described within the French Pharmacopoeia monograph for "Urtica urens pour préparations homéopathiques" are considered by the EURL suitable for the identification of the *feed additive* preparation *dwarf nettle tincture.*

Due to the intrinsic nature of the BDG 16, the accurate quantification of the *feed additives* in *premixtures* and *feedingstuffs* is not achievable experimentally. Furthermore, the Applicant did not provide any experimental data to determine the *feed additives* in *water*. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify the *feed additives* 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

[1] The application for "Apple concentrate" as feed additive was included in the original application BDG 16 and lately withdrawn (SANTE/E5/AR/bvp/ddg2.E.5(2019) - Ref. Ares(2019)1299322 - 26/02/2019).

KEYWORDS

Hawthorne tincture, dropwort tincture, hop tincture, tormentill tincture, rose tincture, black berry tincture, common nettle extract (sb), common nettle extract (wb) and dwarf nettle tincture from botanically defined flavourings group 16 (BDG 16), rosales, sensory additives, flavouring compounds, all animal species.

1. BACKGROUND

In the current grouped application an authorisation is sought under Articles 4(1) (new use in water) and 10(2) (re-evaluation of additives already authorised under the provisions of the Council Directive 70/524/EEC) for *hawthorne tincture*, *dropwort tincture*, *hop tincture, tormentill tincture, rose tincture*, *black berry tincture*, *common nettle extract (sb), common nettle extract (wb) and dwarf nettle tincture* from *botanically defined flavourings group 16 (BDG 16) - Rosales*, under the category/functional group 2(b) 'sensory additives'/flavouring compounds', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for all animal species [1,2].

Table 1. Specifications for additives belonging to the botanically defined group BDG 16 [5]

Additive	Plant species	Description	Phytochemical marker	Mass fraction [%](*)
Hawthorne tincture	Crataegus oxyacantha L.p.p. et auct.	colourless/pale yellow-brown liquid	hyperoside	0.001 - 0.01
			isoquercitrin	0.0005 - 0.005
Dropwort tincture	Filipendula ulmaria (L.) Maxim.	Brown liquid	ethyl salicylate	-
Hop tincture	Humulus lupulus L.	pale yellow-brown to reddish liquid	rutin	0.01 - 0.1
			xanthohumol	0.0003 - 0.003
Tormentill tincture	Potentilla erecta L.	brown liquid	tannins	15 - 20
			ellagic tannins	10 - 15
Rose tincture	Rosa canina L.	colourless/pale yellow-brown liquid	ellagic acid	0.001 - 0.002
			quercetin	0.0001 - 0.0003
Black berry tincture	Rubus spp., e.g. Rubus fructicosus L.)	colourless/pale yellow-brown liquid	ellagic acid	0.05 - 0.12
Common nettle extract (sb)	Urtica dioica L.	brown powder	silica	-
Common nettle extract (wb)	Urtica dioica L.	green-brown powder	silica	1 - 5
Dwarf nettle tincture	Urtica urens L.	brown orange liquid	punicalagin	0.05 - 0.15

(*) The content of phytochemical markers expressed in terms of i.e. mass fraction.

The flavouring preparations described in this dossier have a natural origin (botanically defined) and are derived from plant species belonging to the botanical order "*Rosales"* [3]. The application of *apple concentrate* preparation as *feed additive*, included in the original dossier, has been withdrawn [4]. For each preparation the Applicant indicated the corresponding phytochemical marker(s) and the correspondent amount [5]. Nevertheless, the Applicant did not indicate the corresponding range for the markers in *dropwort tincture* and *common nettle extract (sb)*. The proposed specifications of the additives are presented in Table 1.

The *feed additives* are intended to be incorporated into *feedingstuffs* or drinking *water* directly or through flavouring *premixtures* with no proposed minimum or maximum levels [6]. However, the Applicant suggested the typical maximum inclusion level of the *feed additives* of 25 mg/kg *feedingstuffs* [6].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *botanically defined flavourings group BDG 16 - Rosales* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of the phytochemical markers *hyperoside* and *isoquercitrin* in ***hawthorne tincture***, *rutin* and *xanthohumol* in ***hop tincture***, *ellagic acid* and *quercetin* in ***rose tincture*** and the *ellagic acid* in ***black berry tincture*** the Applicant proposed a single-laboratory validated and further verified method based on high performance liquid chromatography coupled with diode array detection (HPLC-DAD) [7,8].

According to the procedure, the tinctures diluted in methanol are injected into the HPLC system. The individual compounds are separated by gradient elution and detected by spectrophotometric detection at 350 nm (*ellagic acid* is detected at 370 nm).

Table 2. The performance characteristics of the HPLC-DAD method obtained in the frame of validation and verification studies for the quantification of the corresponding phytochemical markers in *hawthorne tincture*, *hop tincture*, *rose tincture* and *black berry tincture* [11].

Phytochemical markers	RSD_r %	RSD_R %
hyperoside	0.7 - 1.4	3.0 - 4.0
isoquercitrin	0.4 - 1.4	2.9 - 3.3
rutin	1.1 - 2.2	1.9 - 4.2
xanthohumol	0.1 - 3.3	4.3 - 4.7
ellagic acid	1.2 - 5.0	4.4 - 4.5
quercetin	0.5 - 2.3	1.3 - 2.6

RSD_r, RSD_R - relative standard deviation for *repeatability* and *reproducibility,* respectively.

The amount of the phytochemical markers in the tinctures are calculated on the basis of the calibration curves obtained from the HPLC analysis of the corresponding standard substances [8]. The Applicant provided satisfactory performance characteristics from the validation and verification studies for the quantification of the different phytochemical markers in the *feed additives*, which are presented in Table 2 [9-11].

Based on the experimental evidences provided, the EURL recommends for official control the single-laboratory validated and further verified method based on HPLC-DAD for the quantification of the phytochemical markers *hyperoside* and *isoquercitrin* in *hawthorne tincture*, *rutin* and *xanthohumol* in *hop tincture*, *ellagic acid* and *quercetin* in *rose tincture* and the *ellagic acid* in *black berry tincture*.

For the identification of the phytochemical marker *ethyl salicylate* in ***dropwort tincture***, the Applicant submitted a method based on gas chromatography coupled to mass spectrometry (GC-MS) using a retention time locking (RTL) methodology [7,12].

Before the analysis, the inlet pressure of the GC-MS system is adjusted in a way to match a retention time of 6.7 or 8.3 min for polar and non-polar columns, respectively, by using the reference substance for RTL – limonene. By making the proper adjustment to the inlet pressure, the retention times can closely match those of the corresponding reference databases. Then, the sample (with or without preparation) is used for chromatographic analysis. The phytochemical marker is identified by using the reference databases/libraries of retention times and of mass spectra of flavourings, available from the Applicant on request (i.e. *ethyl salicylate* retention time of 23.28 or 15.19 min for polar and non-polar columns, respectively) [12]. The method submitted is compliant with the general method for the determination of volatile flavouring substances as adopted by JECFA and the Food Chemicals

Codex and is the same that was already positively evaluated by the EURL for chemically defined flavourings [7,13].

The EURL recommends for official control the method based on GC-MS using RTL methodology for the identification of the phytochemical marker *ethyl salicylate* in *dropwort tincture.*

For the determination of the phytochemical markers *tannins* and *ellagic tannins* in ***tormentill tincture*** the Applicant submitted a thin-layer chromatographic method (TLC) described in the European Pharmacopeia monograph for *tormentill tincture* [7,14].

The method proposed is suitable for the identification of the *feed additive* and not for the quantification of the specific phytochemical markers. Nevertheless, for the determination of *tannins* in *tormentill tincture* a spectrophotometric method at 760 nm for the determination of tannins in herbal drugs is specified in the mentioned monograph [15].

The EURL recommends for official control the spectrophotometric method specified in the European Pharmacopeia monograph for the quantification of the phytochemical marker *tannins* in *tormentill tincture.* The Applicant did not provide any analytical method or experimental data to determine the *ellagic tannins* in *tormentill tincture*. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify the *ellagic tannins in tormentill tincture*.

However, the different methods described within the European Pharmacopeia monograph for *tormentill tincture* are considered by the EURL suitable for the identification of the *feed additive* preparation of *tormentill tincture.*

The EURL recommends for official control the methods described in European Pharmacopeia monograph for *tormentill tincture* for the identification of *tormentill tincture.*

For the determination of the phytochemical marker *silica* in ***common nettle extract (sb)*** and ***common nettle extract (wb)*** the Applicant submitted a single-laboratory validated and further verified method based on inductively coupled plasma-optical emission spectrometry (ICP-OES) [7,16].

According to the method, 500 mg of sample is transferred into a microwave vessel where 2 ml of nitric acid, 8 ml of hydrofluoric acid and 10 ml of 4 % aqueous boric acid solution dedicated for inorganic trace analysis are added for mineralisation. The mixture is mineralised in a microwave oven at 170 ºC for 15 min and held for 10 min. The clear cooled liquid is transferred into a 100 ml volumetric flask and filled to the mark with water for inorganic trace analysis. In parallel, solutions for establishing a calibration curve are prepared with a silicon (Si) standard. The digested sample is analysed via ICP-OES, where 251.611 nm is the

wavelength chosen for the analysis. The content of Si in a sample is directly estimated via a calibration curve.

The Applicant validated and further verified the above mentioned method for the analysis of the phytochemical marker following the "CRL–FA verification form" [17]. Relative precision values from 1.9 to 4.1 % were derived for the analysis of samples of *nettle* leaves containing 1.5 % of silica [16].

Based on the experimental evidences available the EURL recommends for official control the single-laboratory validated and further verified method based on ICP-OES for the quantification of the phytochemical marker *silica* in *common nettle extract (sb)* and *common nettle extract (wb).*

For the determination of the phytochemical marker *punicalagin* in ***dwarf nettle tincture*** the Applicant submitted a TLC method described in the French Pharmacopoeia monograph for "Urtica urens pour préparations homéopathiques" [7,18].

However, the TLC method described in the monograph is not suitable for the quantification of the phytochemical marker *punicalagin* in *dwarf nettle tincture*. Furthermore, the Applicant did not provide any additional analytical method or experimental data to determine the phytochemical marker in *dwarf nettle tincture*. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify *punicalagin* in *dwarf nettle tincture*. However, the different methods described within the French Pharmacopoeia monograph for "Urtica urens pour préparations homéopathiques" are considered by the EURL suitable for the identification of the *feed additive* preparation of *dwarf nettle tincture.*

The EURL recommends for official control the methods described in the French Pharmacopoeia monograph for "Urtica urens pour préparations homéopathiques" for the identification of *dwarf nettle tincture.*

Due to the intrinsic nature of the BDG 16, the accurate quantification of the *feed additives* in *premixtures* and *feedingstuffs* is not achievable experimentally. Furthermore, the Applicant did not provide any experimental data to determine the *feed additives* in *water* [7]. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify the *feed additives* in *premixtures*, *feedingstuffs* and *water*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not relevant for the present application.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control: (i) the single-laboratory validated and further verified method based on HPLC-DAD for the quantification of *hyperoside* and *isoquercitrin* in *hawthorne tincture*, *rutin* and *xanthohumol* in *hop tincture*, *ellagic acid* and *quercetin* in *rose tincture* and the *ellagic acid* in *black berry tincture*; (ii) the GC-MS method using RTL for the identification of the phytochemical marker ethyl *salicylate* in *dropwort tincture*; (iii) the spectrophotometric method specified in the European Pharmacopeia monograph for *tormentill tincture* for the quantification of the phytochemical marker *tannins* in *tormentill tincture*; (iv) the European Pharmacopeia monograph for *tormentill tincture* for the identification of *tormentill tincture*; (v) the single-laboratory validated and further verified method based on ICP-OES for the quantification of the phytochemical marker *silica* in *common nettle extract (sb)* and *common nettle extract (wb)*; and (vi) the French Pharmacopoeia monograph for "Urtica urens pour préparations homéopathiques" for the identification of *dwarf nettle tincture*.

Recommended text for the register entry (analytical method)

For the quantification of *hyperoside* and *isoquercitrin* (phytochemical markers) in the *feed additive* (*hawthorne tincture*), *rutin* and *xanthohumol* (phytochemical markers) in the *feed additive* (*hop tincture*), *ellagic acid* and *quercetin* (phytochemical markers) in the *feed additive* (*rose tincture*) and *ellagic acid* (phytochemical marker) in the *feed additive* (*black berry tincture):*

- high performance liquid chromatography coupled with diode array detection (HPLC-DAD)

For the identification of *ethyl salicylate* (phytochemical marker) in the *feed additive* (*dropwort tincture*):

- gas chromatography coupled to mass spectrometry (GC-MS) using retention time locking (RTL)

For the quantification of *tannins* (phytochemical marker) in the *feed additive* (*tormentill tincture*):

- spectrophotometry at 760 nm – European Pharmacopoeia monograph 1895

For the identification of *tormentill tincture*:

- European Pharmacopoeia Monograph for *tormentill tincture* – European Pharmacopoeia monograph 1895

For the quantification of *silica* (phytochemical marker) in the *feed additives* (*common nettle extract (sb)* and *common nettle extract (wb)*):

- inductively coupled plasma-optical emission spectrometry (ICP-OES)

For the identification of *dwarf nettle tincture*:

- Pharmacopée française monographie "Urtica urens pour préparations homéopathiques"

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Botanically defined flavourings group BDG 16 - Rosales* 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/D2/MGR/ng/(2010)913322
[2] *Annex I – submission number 1288339849229-1274
[3] *Technical dossier, Sect_II_Identity.pdf : introduction
[4] *Supplementary information: Withdrawal I.pdf "Partial withdrawal of applications for various Botanically Defined Groups"; Ref. Ares(2019)1299322 - 26/02/2019
[5] *Technical dossier, Section II: 2.1.4.2. Additives whose authorisation is not linked to a holder of autorisation – Table II.3
[6] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition
[7] *Technical dossier, Section II: 2.6.1.2. Analysis of the botanically defined flavourings from BDG16
[8] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_I_IAB Method_Description_Matrix tinctures
[9] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_III_IAB Method_Validation Report.pdf
[10] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_IVa_IAB Method_Verification Study Reports.pdf
[11] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_IVb_IAB Method_Verification Comparison Reports.pdf
[12] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_I_GCMS_Introduction_Matrices BDG16.pdf
[13] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_II_Methods assay.pdf
[14] European Pharmacopoeia monograph Tormentil tincture, 01/2008:1895
[15] European Pharmacopoeia monograph Tannins in herbal drugs, 01/2008:20814

[16] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_I_ICP-OES_B16-3_Protocols_Analytical results.pdf
[17] https://ec.europa.eu/jrc/en/eurl/feed-additives/guidance-for-applicants
[18] *Supplementary information BDG16_MISSING PARTS_Jan13: Annex_I_TLC_B16-4_Description.pdf
*Refers to Dossier no: FAD-2010-0325

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Wageningen Food Safety Research (WFSR), Wageningen (NL)[2]
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Państwowy Instytut Weterynaryjny, Puławy (PL)

[2] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen (NL)