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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

Nilablend^{TM} 200G
(FAD-2019-0056; CRL/190036)

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-2019-0056 - CRL/190036**
Name of Product:	***Nilablend^{TM} 200G***
Active Agent (s):	**Lasalocid A sodium and Nicarbazin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **16/01/2020**
Report approved by: Date:	**Christoph von Holst** **16/01/2020**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *NILANBLEND™ 200G*, under Article 4(1) for the category "coccidiostats and histomonostats", according to the classification system of Article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for chickens for fattening.

NILANBLEND™ 200G is a preparation containing as active substances 100 g/kg of *lasalocid A sodium* and 100 g/kg of *nicarbazin. NILANBLEND™* 200G is intended to be incorporated in *feedingstuffs* through *premixtures* for chickens for fattening at levels of *lasalocid A sodium:nicarbazin* from 40:40 to 50:50 mg/kg *feedingstuffs*. The Applicant proposed maximum residue limits (MRLs) in chicken *tissues* ranging from 60 to 300 µg/kg of fresh *tissue* for *lasalocid* and ranging from 4000 to 15000 µg/kg of fresh *tissue* for *4,4-dinitrocarbanilide (DNC)*, which is one of the components of *nicarbazin*.

The proposed MRLs for *nicarbazin* (as *DNC*) are not covered by Commission Regulation (EC) No 37/2010, therefore the corresponding methods of analysis are evaluated by the EURL.

The Applicant proposed two single-laboratory validated and further verified methods based on High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-DAD) for the quantification of *lasalocid A sodium* and *nicarbazin* in the *feed additive*. For the quantification of the active substances in *premixtures* and *feedingstuffs* the Applicant applied the ring-trial validated method AOAC 2008.01 (equivalent to the European Union method described in Regulation (EC) No 152/2009) for *lasalocid A sodium* and a single-laboratory validated and further verified method based on the ring-trial validated method EN 15782 for *nicarbazin*.

Furthermore, the EURL is aware of another ring-trial validated LC/MS-MS method for the determination of various coccidiostats including *lasalocid A* and *nicarbazin* in *feedingstuffs* that has been recently published as CEN standard (EN 17299).

For the quantification of *DNC residues* in chicken *tissues* the Applicant submitted a single-laboratory validated method based on Liquid Chromatography coupled to a triple quadrupole mass spectrometer (LC-MS/MS) that does not fully comply with the confirmatory requirements set by Commission Decision 2002/657/EC. However, in the frame of a previous *nicarbazin* dossier the EURL already evaluated and recommended a similar method (AOAC 2013.07) validated for muscle, kidney, skin/fat and liver that complies with the criteria of Commission Decision 2002/657/EC.

Based on the acceptable method performance characteristics available, the EURL recommends for official control i) the single-laboratory validated and further verified methods based on HPLC-DAD for the quantification of *lasalocid A sodium* and *nicarbazin* in the *feed additive*; ii) the European Union method described in Regulation (EC) No 152/2009 for the quantification of *lasalocid A sodium* in *premixtures* and *feedingstuffs*; iii) the ring-trial validated method EN 15782 for the quantification of *nicarbazin* in *premixtures* and *feedingstuffs*; iv) the ring-trial validated method EN 17299 for the quantification of *nicarbazin* in *feedingstuffs* and v) the AOAC 2013.07 method or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs for *nicarbazin* (as *DNC*) in the target *tissues.*

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

Lasalocid A sodium and *nicarbazin*, *Nilablend^{TM} 200G*, coccidiostats and histomonostats, chickens for fattening

1. BACKGROUND

In the current application authorisation is sought for *NILANBLEND^{TM} 200G*, under Article 4(1) for the category "coccidiostats and histomonostats", according to the classification system of Article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for chickens for fattening [1][2].

NILANBLEND^{TM} 200G is a yellow to brownish-yellow free flowing granular preparation containing 100 g/kg (10 %) of *lasalocid A sodium* and 100 g/kg (10 %) of *nicarbazin* (active substances) [2][3]. *Nicarbazin* is an equimolar complex chemically synthesised from *4,4-dinitrocarbanilide (DNC)* and *2-hydroxy-4,6-dimethyl-pyrimidine (HDP)* [3].

NILANBLEND^{TM} 200G is intended to be incorporated in *feedingstuffs* through *premixtures* for chickens for fattening at levels of *lasalocid A sodium:nicarbazin* ranging from 40:40 to 50:50 mg/kg *feedingstuffs* [2][4].

The Applicant proposed maximum residue limits (MRLs) in chicken for fattening *tissues* (i.e. muscle, kidney, skin/fat and liver) ranging from 60 to 300 μg/kg (60 μg/kg for muscle; 150 μg/kg for kidney and 300 μg/kg for liver and skin/fat) for *lasalocid* and from 4000 to 15000 μg/kg (4000 μg/kg for muscle and skin/fat; 6000 μg/kg for kidney and 15000 μg/kg for liver) for *4,4-dinitrocarbanilide (DNC)* - marker residue for *nicarbazin* [3].

The MRLs for *lasalocid* are covered by Commission Regulation (EC) No 37/2010 [5]. However MRLs for *DNC* have been already established by Commission Regulation (EC)

No 875/2010 [6] and Regulation (EC) No 885/2010 [7] but are not covered by Commission Regulation (EC) No 37/2010 [5]. Therefore corresponding methods of analysis are evaluated by the EURL.

Note: The EURL previously evaluated the analytical methods for the determination of *nicarbazin* [8] and *lasalocid A sodium* [9] in the frame of several dossiers.

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 *Nilablend^{TM} 200G* 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)

Lasalocid A sodium

For the quantification of *lasalocid A sodium* in the *feed additive*, the Applicant submitted a single-laboratory validated [10] and further verified method [11] based on High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-DAD) [12].

Lasalocid A sodium is extracted by sonication in a dimethyl sulfoxide:acetonitrile:methanol (50:25:25 v:v:v) mixture, diluted and filtered through a Polyvinylidene Fluoride (PVDF)/Nylon filter before injection into the HPLC system. The target analyte is determined by reversed-phase HPLC using spectrophotometric detection at 305 nm. According to the Applicant potential interferences in the determination of *lasalocid A sodium* are not expected [10][12].

This method was single-laboratory validated and further verified for *Nilablend^{TM} 200G* leading to relative standard deviations for *repeatability* (RSD_r) of 0.4 % (validation) and 0.7 % (verification), relative standard deviations for *intermediate precision* (RSD_{ip}) of 0.7 % (validation and verification) and recoveries ranging from 99 % to 102 % [10][11].

The Applicant proposed for the quantification of *lasalocid A sodium* in *premixtures* and *feedingstuffs* the ring-trial validated AOAC method 2008.01 [13]. This method is slightly different than the European Union method described in Regulation (EC) No 152/2009 [14]. However, the Applicant has provided evidence of the equivalence of both standard methods in terms of precision and recoveries [15].

In the European Union method *lasalocid A sodium* is extracted from samples with acidified methanol in an ultrasonic bath during 20 min followed by dilution with acidified methanol. The mixture is then allowed to stand for 1 h until the suspended material has settled and then an aliquot of the supernatant is filtered through a 0.45 μm membrane filter. The clear filtrate is further diluted with acidified methanol to produce a final test solution containing about 4 μg/ml of *lasalocid A sodium* and analysed by reverse-phase HPLC using fluorescence detection (FLD) at an excitation wavelength of 310 nm and at an emission wavelength of 419 nm [14].

This method was ring-trial validated for *premixtures* and *feedingstuffs* and the performance characteristics reported are: RSD_r ranging from 2.1 to 5.4 %; a relative standard deviation for *reproducibility* (RSD_R) ranging from 5.7 and 10.7 % and *recovery* rates from 75 % to 101 %. Additionally limits of detection (LOD) and quantification (LOQ) of 5 and 10 mg/kg *feedingstuffs*, respectively, have been estimated [14].

Based on the provided performance characteristics the EURL recommends for official control the single-laboratory validated and further verified HPLC-DAD method for the quantification of *lasalocid A sodium* in *Nilablend^{TM} 200G* and the HPLC-FLD European Union method for the quantification of *lasalocid A sodium* in *premixtures* and *feedingstuffs.*

Nicarbazin

For the quantification of *nicarbazin* in the *feed additive,* the Applicant submitted a single-laboratory validated [10] and further verified method [16] based on HPLC coupled to spectrophotometric detection (HPLC-DAD) [17].

Nicarbazin is extracted by sonication in a dimethyl sulfoxide:acetonitrile:methanol (50:25:25 v:v:v) mixture, diluted and filtered through a Polyvinylidene Fluoride (PVDF)/Nylon filter before injection into the HPLC system. The target analyte is determined by reversed-phase HPLC using spectrophotometric detection at 290 nm (for *2-hydroxy-4,6-dimethyl pyridine (HDP)*) and 350 nm (for *4,4-dinitrocarbanilide (DNC)*). According to the Applicant potential interferences in the determination of *nicarbazin* are not expected [10][17].

For the quantification of *nicarbazin* in *premixtures* and *feedingstuffs* the Applicant proposed a single-laboratory validated and further verified method based on EN 15782 [18] using HPLC coupled to spectrophotometric detection (HPLC-UV).

In the EN 15782 method *nicarbazin* is extracted using acetonitrile: methanol (50:50 v:v) with manual shaking, heated in a water bath at 50 ^{o}C for 15 min, mixed, and sonicated for another 15 min. After appropriate dilution with the eluent, an aliquot is filtered and subjected to analysis without further clean-up. The target analyte is determined by reversed-phase HPLC and the *4,4'-dinitrocarbanilide (DNC)* moiety is detected at 350 nm. Potential interferences in the determination of *nicarbazin* are not expected [19]. This method was ring-trial validated for broiler *feedingstuffs* and *premixtures* at a mean *nicarbazin* content ranging from 22 to 7308 mg/kg leading to the following performance characteristics [18]: RSD_r ranging from 2.6 to 10.2 %; RSD_R ranging from 4.8 to 12.3 %; and LOD of 0.5 mg/kg *feedingstuffs.*

In order to obtain precision values below 10 % for *premixtures* and *feedingstuffs* the Applicant modified the EN method by using a different extraction solvent (dimethyformamide instead of acetonitrile:methanol (50:50 v:v)) [20].

In addition, the EURL has developed and fully validated a multi-analyte method based on liquid chromatography coupled to mass spectrometry for the determination in *feedingstuffs* of all coccidiostats currently authorised as *feed additives* within the EU, including the ones of the current application. In this method the coccidiostats are extracted with acetonitrile:methanol:water (80:10:10 v:v:v). The extracts are centrifuged and supernatants filtered. After a first screening analysis, the analytes are determined by reverse phase liquid chromatography using electrospray ionization with tandem mass spectrometry detection (LC-MS/MS). The quantification of the detected target analytes is performed using a multi-level standard addition approach. This method has been ring-trial validated and recently published as CEN standard (EN 17299) [21].

Based on the performance characteristics available the EURL recommends for official control the single-laboratory validated and further verified HPLC-DAD method [17] for the quantification of *nicarbazin* in the *feed additive*, the ring-trial validated HPLC-UV method EN 15782 for the quantification of *nicarbazin* in *premixtures* and *feedingstuffs* [18] and the ring-trial validated LC-MS/MS method EN 17299 [21] for the quantification of *nicarbazin* in *feedingstuffs*.

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)

<u>Nicarbazin</u>

For the quantification of *DNC* (marker residue for *nicarbazin*) in target *tissues* (skin/fat, muscle, liver and kidney) the Applicant submitted a single-laboratory validated [22] method based on LC-MS/MS with the electrospray ionisation (ESI) mode [23].

However the method proposed by the Applicant does not fully comply with the confirmatory requirements set by Commission Decision 2002/657/EC [24]. Therefore it is not suitable to enforce the MRLs for *nicarbazin* (as *DNC*) in the target *tissues* in the frame of official control.

The EURL is aware of another multi-residue method also based on LC-MS/MS and previously validated by the European Union Reference Laboratory “Pharmacologically Active Substances” for muscle and liver *tissues*. Additionally, in the frame of a previous *nicarbazin* dossier [8], the EURL already evaluated and recommended a similar method (AOAC 2013.07) [25] based on LC-MS/MS with the electrospray ionisation (ESI) mode validated for muscle, kidney, skin/fat and liver according to the Commission Decision 2002/657/EC [24].

The method performance characteristics of the AOAC method together with those reported for the European Union Reference Laboratory “Pharmacologically Active Substances” (BVL) method are presented in Table 3. Furthermore a LOQ of 20 µg/kg was reported for muscle, liver, kidney and skin/fat *tissues* [25].

The satisfactory performance characteristics provided by the AOAC method for muscle and liver *tissues* demonstrate that the EURL method was equivalent to the AOAC method. Additionally the results provided by the AOAC method for kidney and skin/fat further demonstrate the applicability - and therefore the extension of scope - of the AOAC method to these two additional *tissues.*

Based on the performance characteristics presented, the EURL recommends for official control the LC-MS/MS AOAC method or any equivalent analytical method complying with the requirements set by Commission Decision 2002/657/EC to enforce the MRLs for *nicarbazin* (as *DNC*) in the target *tissues*.

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.

Table 3. Performance characteristics for the quantification of *DNC* residues in chicken tissues obtained with the AOAC 2013.07 method, compared to those reported for the European Union Reference Laboratory "Pharmacologically Active Substances" (BVL) method.

Tissue		Content (µg /kg)	RSD_r (%)	RSD_{ip} (%)
Muscle	BVL	0.75-2.75	3.4-8.7	8.0-11.6
	AOAC	100	0.81-5.3	10.0
		200	1.6-7.0	11.3
		400	1.9-4.9	4.9
		2000	1.8-5.0	4.5
		4000	1.4-4.5	5.7
		8000	1.6-2.2	3.0
Liver	BVL	0.75-2.75	3.4-8.7	8.0-11.6
	AOAC	100	2.2-10.4	8.2
		200	3.6-7.2	8.6
		400	2.2-4.8	4.8
		2000	3.2-5.4	4.8
		4000	3.2-6.8	5.5
		8000	2.1-2.6	2.5
Kidney	AOAC	100	1.3-10.8	6.6
		200	1.1-4.7	8.3
		400	1.2-1.9	6.9
		2000	1.9-4.0	5.4
		4000	0.7-5.7	4.4
		8000	1.2-6.2	8.4
Skin/Fat	AOAC	100	2.1-5.6	6.8
		200	2.0-11.6	8.2
		400	1.7-11.5	7.8
		2000	2.0-10.2	6.9
		4000	1.5-8.3	5.8
		8000	1.6-8.1	6.1

RSD_r; RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

Evaluation of corresponding methods of analysis is not considered necessary by the EURL.

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 methods based on HPLC-DAD for the quantification of *lasalocid A sodium* and *nicarbazin* in the *feed additive*; ii) the European Union method described in the Regulation (EC) No 152/2009 for the quantification of *lasalocid A sodium* in *premixtures* and *feedingstuffs;* iii) the ring-trial validated EN 15782 for the quantification of *nicarbazin* in *premixtures* and *feedingstuffs;* iv) the ring-trial validated EN 17299 method for the quantification of *lasalocid A sodium* and *nicarbazin* in *feedingstuffs*; and v) the AOAC 2013.07 method based on LC-MS/MS - or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC - for the quantification of *nicarbazin* (as *DNC*) in chicken *tissues*.

Recommended text for the register entry (analytical method)

For the quantification of *lasalocid A sodium* in the *feed additive*:

- High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-DAD)

For the quantification of *lasalocid A sodium* in *premixtures and feedingstuffs*:

- High Performance Liquid Chromatography coupled to fluorescence detection (HPLC-FLD) – Commission Regulation (EC) No 152/2009

For the quantification of *nicarbazin* in the *feed additive*:

- High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV)

For the quantification of *nicarbazin* in *premixtures*:

- High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV) - EN ISO 15782

For the quantification of *nicarbazin* in *feedingstuffs*:

- High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV) - EN ISO 15782 and
- Liquid Chromatography coupled to a triple quadrupole mass spectrometer (LC-MS/MS) – EN 17299

For the quantification of *nicarbazin* (as *4,4-dinitrocarbanilide (DNC))* in chicken *tissues*:

- Liquid Chromatography coupled to a triple quadrupole mass spectrometer (LC-MS/MS) - AOAC 2013-07 or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *NILANBLEND™ 200G* 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 SANTE/E5: FORW. APPL. 1831-0059-2019

[2] *Application. Application Form - Annex I. Submission number 1567142845734-2442

[3] *Technical dossier, Section II: 2.1 Identity of the additive

[4] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[5] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

[6] Commission Regulation (EU) No 875/2010 of 5 October 2010 concerning the authorisation for 10 years of an additive in feedingstuffs.

[7] Commission Regulation (EU) No 885/2010 of 7 October 2010 concerning the authorisation of the preparation of narasin and nicarbazin as a feed additive for chickens for fattening (holder of authorisation Eli Lilly and Company Ltd) and amending regulation (EC) No 2430/1999.

[8] EURL Evaluation Reports (Nicarbazin):
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2007-0016.pdf
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2008-0037.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2012-0027-monimax.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2014-0036_0045_maxiban160.pdf

[9] EURL Evaluation Reports (Lasalocid):
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2008-0050.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2008-0001.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2008-0001.pdf

[10] *Technical dossier, Section II: Annex II.26

[11] *Technical dossier, Section II: Annex II.27

[12] *Technical dossier, Section II: Annex II.25

[13] AOAC Official method 2008.01. Lasalocid Sodium in Animal Feeds and Premixes

[14] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed

[15] *Technical dossier, Section II: Annex II.28

[16] *Technical dossier, Section II: Annex II.30

[17] *Technical dossier, Section II: Annex II.29

[18] EN ISO 15782:2009 Animal feedingstuffs – Determination of nicarbazin – High performance liquid chromatography method

[19] Jacob de Jong et al. Liquid Chromatographic Method for Nicarbazin in Broiler Feeds and Premixtures: Development, Validation, and Interlaboratory Study, J. of AOAC Int., 87, 6, 1269 – 1277, 2004

[20] *Technical dossier, Section II: Annex II.31

[21] EN 17299:2019 Animal feedingstuffs: Methods of sampling and analysis – Screening and determination of authorised coccidiostats at additive and 1 % and 3 % cross-contamination level, and of non-registered coccidiostats and of one antibiotic at sub-additive levels, in compound feed with High Performance Liquid Chromatography – Tandem Mass Spectrometry detection (LC-MS/MS)

[22] *Technical dossier, Section II: Annex II.40

[23] *Technical dossier, Section II: Annex II.38

[24] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

[25] AOAC Official method 2013.07. Determination and Confirmation of Nicarbazin in chicken Tissues. Liquid Chromatography with Tandem Mass Spectrometry.

*Refers to Dossier no: FAD-2019-0056

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:

- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Istituto Superiore di Sanità. Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Roma (IT)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft, Nossen (DE)

- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Wageningen Food Safety Research1 (WFSR) (NL)

[1] Name and address according to according Commission Implementing Regulation (EU) 2015/1761: RIKILT Wageningen UR, Wageningen