Safety and efficacy of pyridine and pyrrole derivatives belonging to chemical group 28 when used as flavourings for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of nine compounds belonging to chemical group 28 (pyridine, pyrrole and quinoline derivatives). They are currently authorised as flavours in food. The FEEDAP Panel concludes that piperine, 3-methylindole, indole, 2-acetylpyridine and 2-acetylpyrrole are safe at the proposed maximum use level of 0.5 mg/kg complete feed for all animal species; trimethyloxazole, 3-ethylpyridine, pyrrolidine and 2,6-dimethylpyridine are safe at the proposed use level of 0.5 mg/kg complete feed for cattle, salmonids and non-food-producing animals, and at the use level of 0.3 mg/kg complete feed for pigs and poultry. No safety concern would arise for the consumer from the use of these compounds up to the highest safe level in feeds. Hazards for skin and eye contact, and respiratory exposure are recognised for the majority of the compounds under application. Most are classified as irritating to the respiratory system. The concentrations considered safe for the target species are unlikely to have detrimental effects on the terrestrial and fresh water environments. As all the compounds under assessment are used in food as flavourings, and their function in feed is essentially the same as that in food, no further demonstration of efficacy is necessary. In the absence of data on the stability in water for drinking, the FEEDAP Panel is unable to conclude on the safety or efficacy of the substances under this mode of delivery.

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Keywords: sensory additives, pyridine, pyrrole, quinoline derivatives, safety, chemical group 28

Requestor: European Commission

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003\(^1\) establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7, in addition, Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of 7 years after the entry into force of this Regulation.

The European Commission (EC) received a request from Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)\(^2\) for authorisation of nine substances belonging to chemical group (CG) 28, when used as feed additives for all animal species (category: sensory additives; functional group: flavouring compounds). CG 28 for flavouring substances is defined in Commission Regulation (EC) No 1565/2000\(^3\) as ‘pyridine, pyrrole and quinoline derivatives’.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive) and under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 9 June 2010.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5.

EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of trimethyloxazole, piperine, 3-methylindole, indole, 2-acetylpyridine, 2-acetylpyrrole, 3-ethylpyridine, pyrrolidine and 2,6-dimethylpyridine, when used under the proposed conditions of use (see Section 0).

1.2. Additional information

All nine substances have been assessed by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA; WHO 2005, 2006 and 2008) and were considered safe for use in food. No acceptable daily intake values were specified. Subsequently the EFSA Panel on Food Additive, Flavourings, Processing Aids and Materials in Contact with Food (CEF) considered the same compounds for use as food flavourings (EFSA, 2008a,b, 2009; EFSA CEF Panel, 2015a,b) reaching the same conclusions.

The compounds are all currently listed in the European Union (EU) database of flavouring substances\(^4\) and in the EU Register of Feed Additives, respectively, and thus authorised for use in food and feed in the EU. They have not been previously assessed by EFSA as feed additives.

Regulation (EC) No 429/2008\(^5\) allows substances already approved for use in human food to be assessed with a more limited procedure than for other feed additives. However, the use of this

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\(^2\) On 13/03/2013, EFSA was informed by the applicant that FFAC EEIG was liquidated on 19/12/2012 and their rights as applicant were transferred to FEFANA asbl (EU Association of Specialty Feed Ingredients and their Mixtures). Avenue Louise 130A, Box 1, 1050 Brussels, Belgium.


Pyridine, pyrrole and quinoline derivatives (CG 28) for all animal species

procedure is always subject to the condition that food safety assessment is relevant to the use in feed.

2. Data and Methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier\(^6\) in support of the authorisation request for the use of the pyridine, pyrrole and quinoline derivatives as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008 and the applicable EFSA guidance documents.

The FEEDAP Panel has sought to use the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers and experts' knowledge, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of flavourings from CG 28 – pyridine, pyrrole and quinoline derivatives – in animal feed. The Executive Summary of the EURL report can be found in Annex A.\(^7\)

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the pyridine, pyrrole and quinoline derivatives is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012a), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008c), Guidance for the preparation of dossiers for additives already authorised for use in food (EFSA FEEDAP Panel, 2012b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012c) and Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012d).

3. Assessment

3.1. Characterisation

3.1.1. Characterisation of the flavouring additives

The molecular structures of the nine additives under application are shown in Figure 1 and their physico-chemical characteristics in Table 1.

\(^{6}\) FEED dossier reference: FAD-2010-0117.

\(^{7}\) The full report is available on the EURL website https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2010-0117.pdf
Pyridine, pyrrole and quinoline derivatives (CG 28) for all animal species


Figure 1: Molecular structures and [EU Flavour Information System (FLAVIS) numbers] of the nine flavouring compounds under assessment

Table 1: Chemical Abstracts Service (CAS) and FLAVIS numbers and some characteristics of the chemically defined flavourings under assessment

<table>
<thead>
<tr>
<th>EU Register name</th>
<th>CAS No</th>
<th>Flavis No</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Physical state</th>
<th>Log Kow(^{(a)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethyloxazole</td>
<td>20662-84-4</td>
<td>13.169</td>
<td>C(_6)H(_9)ON</td>
<td>111.14</td>
<td>Liquid</td>
<td>1.09</td>
</tr>
<tr>
<td>Piperine</td>
<td>94-62-2</td>
<td>14.003</td>
<td>C(<em>{17})H(</em>{18})O(_3)N</td>
<td>285.34</td>
<td>Solid</td>
<td>3.69</td>
</tr>
<tr>
<td>3-Methylindole</td>
<td>83-34-1</td>
<td>14.004</td>
<td>C(_8)H(_7)N</td>
<td>131.18</td>
<td>Solid</td>
<td>2.6</td>
</tr>
<tr>
<td>Indole</td>
<td>120-72-9</td>
<td>14.007</td>
<td>C(_8)H(_7)N</td>
<td>115.15</td>
<td>Solid</td>
<td>2.14</td>
</tr>
<tr>
<td>2-Acetylpyridine</td>
<td>1122-62-9</td>
<td>14.038</td>
<td>C(_7)H(_7)ON</td>
<td>121.14</td>
<td>Liquid</td>
<td>0.85</td>
</tr>
<tr>
<td>2-Acetylpyrrole</td>
<td>1072-83-9</td>
<td>14.047</td>
<td>C(_6)H(_7)ON</td>
<td>109.13</td>
<td>Solid</td>
<td>0.93</td>
</tr>
<tr>
<td>3-Ethylpyridine</td>
<td>536-78-7</td>
<td>14.061</td>
<td>C(_8)H(_7)N</td>
<td>107.06</td>
<td>Liquid</td>
<td>1.66</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>123-75-1</td>
<td>14.064</td>
<td>C(_4)H(_9)N</td>
<td>71.12</td>
<td>Liquid</td>
<td>0.46</td>
</tr>
<tr>
<td>2,6-Dimethylpyridine</td>
<td>108-48-5</td>
<td>14.065</td>
<td>C(_8)H(_7)N</td>
<td>107.16</td>
<td>Liquid</td>
<td>1.68</td>
</tr>
</tbody>
</table>

EU: European Union; CAS No: Chemical Abstract Service No.; Flavis number: EU Flavour Information System numbers; (a): Logarithm of octanol–water partition coefficient.

These substances are produced by chemical synthesis. Typically several routes of synthesis are available and described in the dossier.\(^5\)

Batch-to-batch variation data were provided for five batches of each additive. The mean content of the active substance for all compounds except piperine exceeded the JECFA specifications (Table 2).

\(5\) Technical dossier/Section II.
Table 2: Identity of the substances and data on purity

<table>
<thead>
<tr>
<th>EU Register name</th>
<th>Flavis No</th>
<th>JECFA specification minimum % (a)</th>
<th>Assay %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>Trimethyloxazole</td>
<td>13.169</td>
<td>&gt; 95</td>
<td>99.7</td>
</tr>
<tr>
<td>Piperine</td>
<td>14.003</td>
<td>&gt; 97</td>
<td>95.3</td>
</tr>
<tr>
<td>3-Methylindole</td>
<td>14.004</td>
<td>&gt; 97</td>
<td>97.7</td>
</tr>
<tr>
<td>Indole</td>
<td>14.007</td>
<td>&gt; 97</td>
<td>99.7</td>
</tr>
<tr>
<td>2-Acetylpyridine</td>
<td>14.038</td>
<td>&gt; 97</td>
<td>97.9</td>
</tr>
<tr>
<td>2-Acetylpyrrole</td>
<td>14.047</td>
<td>&gt; 97</td>
<td>99.9</td>
</tr>
<tr>
<td>3-Ethylpyridine</td>
<td>14.061</td>
<td>&gt; 98</td>
<td>99.8</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>14.064</td>
<td>&gt; 95</td>
<td>99.7</td>
</tr>
<tr>
<td>2,6-Dimethylpyridine</td>
<td>14.065</td>
<td>&gt; 99</td>
<td>99.6</td>
</tr>
</tbody>
</table>

EU: European Union; Flavis number: EU Flavour Information System numbers; JECFA: The Joint FAO/WHO Expert Committee on Food Additives

Potential contaminants are considered as part of the product specification and are monitored as part of the Hazard Analysis and Critical Control Point procedure applied by all consortium members. The parameters considered include residual solvents, heavy metals and other undesirable substances. However, no evidence of compliance was provided for these parameters.

3.1.2. Stability and homogeneity

A minimum shelf-life for the compounds under assessment range from 6 to 24 months when stored in closed containers under recommended conditions. This assessment is made on the basis of compliance with the original specification over this storage period.

Although no data are required for the stability of flavours in premixtures and feed, their use in water for drinking introduces other issues relating to product stability, such as degradation due to microbial activity. The FEEDAP Panel notes that three out of the nine compounds in CG 28 have low water solubility (log $K_{ow} > 2$). Considering this, and in the absence of data on the short-term stability and homogeneity of the all additives in water for drinking, the FEEDAP Panel is not in the position to conclude on the use of the additives in water for drinking.

3.1.3. Conditions of use

The applicant proposes the use of the nine additives in feed or water for drinking for all animal species without withdrawal period. The normal use level is 0.1 mg/kg feed and a high use level of 0.5 mg/kg feed. No specific proposals are made for doses to be used in water for drinking.

3.2. Safety

The assessment of safety is based on the highest use level proposed by the applicant (0.5 mg/kg complete feed).

3.2.1. Absorption, distribution, metabolism and excretion (ADME) and residue studies

Absorption, distribution and excretion

In its evaluation of pyridine and pyrrole derivatives, JECFA reported that flavourings from this group are expected to be rapidly absorbed from the gastrointestinal tract (WHO, 2005). JECFA also reported that oxazoles, such as trimethyloxazole [13.169] are rapidly absorbed. Pyrroles and indoles are weak bases with pKa values of > 3 and are readily absorbed from the intestine by passive diffusion because the base will not be ionised and pass through intestinal membranes with ease. Pyridines are weak bases and undergo rapid absorption in the gastrointestinal tract (WHO, 2006).

The excretion of indole in rats is fairly rapid with 75% of the dosage excreted after 24 h, and more than 80% of the radioactivity recovered from the pooled 48-h urine of female albino Wistar rats given
a single oral dose of 64 to 80 mg/kg [2-14C]-indole. The radioactivity measured in urine, faeces and expired air after 48 h was 80.6, 11.1 and 2.4% of the dose (King et al., 1966).

Twenty-four male Holtzman rats were fed diets supplemented with 0, 0.25, 0.50 or 0.75% (corresponding to 0, 150, 300 and 450 mg/kg body weight (bw) per day) of indole for 3 weeks. During week 3, all groups (including controls) received a single dose of [2-14C]-indole via stomach tube. Urine collected for 72 h after the single dose administration revealed that 32, 47, 49 and 51% of the radioactive dose were recovered from the 0 (control), 0.25, 0.50 and 0.75% indole groups (Martinez and Roe, 1972).

Mice and rats given single intraperitoneal injections of 400 mg/kg of [14C]-3-methylindole (skatole) excreted 69.4% and 66.2% of the total radioactivity, respectively, after 48 h (Skiles and Yost, 1992).

When piperine was administered to male albino rats by gavage (170 mg/kg bw per day), about 97% was absorbed, the remaining 3% of the administered dose was excreted as piperine in the faeces. Piperine was not detectable in urine (the limit of detection was not known), indicating a complete metabolic conversion of this compound. Examination of the passage of piperine through the gut indicated that the highest concentration in the stomach and small intestine was attained at about 6 h. Only traces (less than 0.15%) of piperine were detected in serum, kidney and spleen from 30 minutes to 24 h. For intraperitoneally administered piperine (85 mg/kg bw per day), about 1–2.5% of the dose was detected in the liver during 0.5–6 h after administration as contrasted with 0.1–0.25% of the orally administered dose (Bhat and Chandrasekhara, 1986).

When everted sacs of rat intestines were incubated with 200–1,000 µg piperine, about 47–64% of the added piperine disappeared from the mucosal side. Only piperine was present in the serosal fluid and also in the intestinal tissue, indicating that piperine did not undergo any metabolic change during absorption (Bhat and Chandrasekhara, 1986).

**Metabolism**

Once absorbed, pyridine and pyrrole derivatives are oxidised to polar metabolites, and eliminated primarily in the urine and, to a minor extent, in the faeces. Alkyl-substituted pyroles and indoles may undergo cytochrome P450-mediated side-chain oxidation to yield the corresponding alcohol. Unsubstituted indole is metabolised primarily by ring hydroxylation at the C-3 position. The resulting hydroxyl derivatives are conjugated with glucuronic acid or sulfate and excreted in the urine. To a lesser extent, the double bond of the pyrrole ring may undergo epoxidation and subsequent glutathione conjugation. Alkyl-substituted pyridines are principally subject to side-chain oxidation, primarily at the C-1 position. N-Oxide formation has also been reported (WHO, 2005, 2006). Alicyclic secondary amines such as pyrrolidine undergo C-oxidation at the α-carbon, but oxidation can also occur at other carbons on the ring (WHO, 2006). The metabolism of oxazoles, such as trimethyloxazole, involves oxazole ring cleavage, and depending on the degree of ring substitution, ring hydroxylation. The presence of a substituent at the 2-position tends to stabilise the oxazole ring.

Metabolism studies in laboratory animals are available for a number of compounds belonging to CG 28 and have been extensively reviewed by the CEF Panel (EFSA CEF Panel, 2013, 2014).

Hydroxylation of both rings of indole derivatives has been well documented. 3-Hydroxyindole (indoxyl), resulting from electrophilic attack at C-3, is the main metabolite of indole in various species. Minor metabolites are oxindole and 5-hydroxyoxindole, resulting from hydroxylation at C-2 and subsequent C-5 hydroxylation (Damani and Crooks, 1982). The metabolism of [2-14C]-indole was investigated by King et al. (1966). When single oral doses of 64–74 mg/kg bw of [2-14C]-indole were administered to three albino Wistar rats, 63% of the dose was detected in 48-h urine pools as 3-hydroxyindole-sulfate (49.6%) and 3-hydroxyindole-glucuronide (13.2%). Minor metabolites identified in the urine were 5-hydroxyoxindole (3.5%), indole-2-one (1.4%) and indole-2,3-dione (or isatin, 5.8%). In faeces, indole, 3-hydroxyindole-sulfate and total 3-hydroxyindole metabolites represented 0.14, 0.40 and 0.64% of the dose, respectively; in the bile, 5-hydroxyoxindole, 3-hydroxyindole-sulfate and total 3-hydroxyindole metabolites, accounted for 0.56, 0.80 and 0.82% of the dose, respectively. Similar findings were reported by Martinez and Roe (1972). Twenty-four male Holtzman rats were administered diets supplemented with 0, 0.25, 0.50 or 0.75% (corresponding to 0, 150, 300 and 450 mg/kg bw per day) indole for 3 weeks. After a single dose administration of [2-14C]-indole by gavage (week 3), sulfate and glucuronide conjugates of hydroxylated indole were the two main
radioactive metabolites detected in the urine collected for 72 h. The excretion of glucuronide was increased with increasing doses of administered indole.

The major pathways of metabolism of 3-methylindole in mice and goat (Skiles et al., 1991; Smith et al., 1993) are oxygenation of the double bond in position 2 and 3 (epoxidation product), and stepwise oxidation of the methyl group and hydroxylation of the ring structure, leading to metabolites mainly conjugated with glucuronic acid, sulfate and glutathione. Mice and rats given single intraperitoneal injections of 400 mg/kg of [methyl-\(^{14}\)C]-3-methylindole excreted 2.6 and 7.3%, respectively, as the mercapturic acid conjugate of 3-methylindole, 3-[(\(N\)-acyctelycystein-S-yl)methyl]indole (Skiles et al., 1991). Three primary pathways were characterised and at least six metabolites isolated from the urine after 36 h of male Swiss-Webster mice receiving 400 mg/kg of ring labelled [\(\text{ring-\(UL\)-}\]C]-3-methylindole by intraperitoneal injection. In the first pathway, side-chain oxidation yields indole-3-carbinol, which is further oxidised to the corresponding carboxylic acid. Alternatively, 3-methylindole can be converted to the reactive substance 3-methyleneindolene, which subsequently is conjugated with glutathione to yield 3-[(\(N\)-acyctelycystein-S-yl)methyl]indole. In the third pathway, the 2,3-alkene is epoxidised to yield 3-methylxindole or 3-hydroxy-3-methylindolene intermediates. These metabolites are conjugated with glucuronic acid or sulfate, followed by excretion in the urine or are further oxidised to yield a series of dihydroxy-3-methylxindole metabolites that are also conjugated and excreted. The second pathway through epoxidation of 3-methylindole predominates (Smith et al., 1993).

No unchanged piperine was found in the urine of male albino rats administered piperine either by gavage (170 mg/kg bw per day) or intraperitoneal (85 mg/kg bw per day). Cleavage of the methylenedioxy group of piperine followed by glucuronidation and sulfation appear to be the major steps in the metabolism of piperine in the rat (Bhat and Chandrasekhar, 1986). After oral administration of piperine (170 mg/kg bw per day) to rats, bile and urine were examined for metabolites. Four metabolites, piperonylic acid, piperonyl alcohol, piperonal and vanillic acid were identified in the free form in 0–96 h urine whereas only piperic acid was detected in 0–6 hours bile (Bhat and Chandrasekhar, 1987). This metabolic profile could not be confirmed in a more recent study (Bajad et al., 2003), where a new major metabolite was identified in the urine of rats and characterised as 5-(3,4-methylenedioxy-phenyl)-2E,4E-pentadienoic acid-\(\text{-\(N\)}\)-\(\text{(3-yl-propionic acid)}\)amide. This metabolite is proposed to be formed by the oxidation of the piperidine ring at position 4 followed by loss of the ethylene group by \(\alpha\)-carbon cleavage and further oxidation.

Mendes (2012) detected three major compounds in the urine of male and female rats dosed with 2-acetylpyrrole [14.047] by oral gavage. Unchanged 2-acetylpyrrole and pyrrrol-2,5-dione were identified by gas chromatography–mass spectrometry (GC–MS); the third compound was thought to be 1,5-dihydropyrrrol-2-one, but its identity was not confirmed (EFSA CEF Panel, 2015a).

3-Ethylpyridine was converted to the corresponding N-oxide by fortified hepatic microsomal preparations from hamster, guinea-pig, rabbit, rat and mouse, and by pulmonary microsomal preparations from guinea-pig and rabbit (Cowan et al., 1978 as quoted by EFSA CEF Panel 2013). \textit{In vivo}, no urinary N-oxidation products were found for dimethylpyridines, the major metabolism being the result of the oxidation of one of the methyl groups. When 2,6-dimethylpyridine (100 mg/kg bw) was administered via gavage to male Wistar rats, 90% was excreted as the glycine conjugate of the 6-methyl-2-carboxylic acid (Hawksworth and Scheline, 1975).

Studies of metabolism of compounds belonging to CG 28 in animals, other than rats, are lacking in the scientific literature. Oxidation is ubiquitous and phase II conjugation via glucuronidation or sulfation occurs in mammals, although the predominance of one pathway over another varies among animal species (Gupta, 2007). Data collected in a review by Ioannides (2006) show that the cytochrome P450 enzymes responsible for the majority of oxidation reactions are expressed in the liver of the main food-producing animals (cattle, pig, sheep, goat) as well as in the rabbit and chicken (Nebbia et al., 2003). Biotransformation through oxidation followed by conjugation with glucuronid acid and sulfate has also been reported for birds (Pan and Fouts, 1978). A recent study showed that the principal cytochrome P450 enzymes responsible for oxidation of xenobiotics, as well as glutathione transferases, are present in the liver of chickens (Blevins et al., 2012). Fish have analogous mechanisms for handling xenobiotic compounds, including both phase I and phase II biotransformation reactions, and many of the same microsomal and cytosolic enzymes as mammals (Wolf and Wolfe, 2005). Thus, fish can transform endobiotic and xenobiotic compounds through
oxidation or hydroxylation, conjugate the metabolites to polar substrates through sulfate, and glucuronide conjugation (James and Pritchard, 1987) with further elimination via bile or urine (Di Giulio and Hinton, 2008). Therefore, food-producing animals, including fish and birds, can also be assumed to have the ability to metabolise and excrete the flavouring substances from CG 28 and there is no evidence that they or their metabolites would accumulate in tissues.

3.2.2. Toxicological studies

Toxicological data (subchronic, repeated-dose studies, with multiple doses tested) could be found for piperine [14.003], 2-acetylpyridine [14.038] and 2-acetylpyrrole [14.047]. A no observed adverse effect level (NOAEL) of 37 mg 2-acetylpyridine/kg bw per day was identified in a 90-day study in rats (doses: 0, 37, 110, 330 and 1,000 mg/kg bw per day, 6 days a week; M/F, 20 animals each group, administration route: gavage). Slight anaemia was observed in females from 110 mg/kg bw per day and in males from 330 mg/kg bw per day. Liver enlargement was observed at 330 and 1,000 mg/kg bw per day (Til and van der Meulen, 1971, unpublished).

A 90-day study was performed with piperine [14.003] (Bauter, 2013). The study was performed according to The Organisation for Economic Co-operation and Development (OECD) Guideline 408. Four groups of adult Crl: Sprague–Dawley® CD® IGS rats (10/sex and group) were maintained on diets, calculated to provide piperine intake levels of 4.8, 14.5 and 47.8 mg/kg bw per day in males, and 4.8, 14.6 and 48.4 mg/kg bw per day in females, giving an average daily intake of 0 (vehicle), 5, 15 or 50 mg/kg bw per day for males and females.

There were no mortalities, clinical or ophthalmological changes, attributable to piperine administration. Decreased male body weight gain (20% reduction) and reduced male (15% reduction) and female (12% reduction) food consumption in the top dose group were attributed to the possible decrease in palatability caused by the test substance at high dietary levels. No effect was observed on the final body weights.

There were no gross and microscopic changes or clinical pathology or organ weight changes attributed to the administration of piperine. A statistical significant and dose-dependent increase in plasma cholesterol in males was observed of approximately 30% in the 15 mg/kg bw per day and 55% in the 50 mg/kg bw/day groups. No effect on cholesterol was observed in females. Based on the dose-dependent increase in plasma cholesterol levels in males at the mid and high dose, the CEF Panel decided that the lowest dose level of 5 mg/kg bw per day should be considered as the NOAEL. This conclusion is supported by the FEEDAP Panel.

In a 90-day study compliant with OECD guideline 408, groups of rats (10/sex and dietary intake level) of male and female Sprague–Dawley CD rats were fed a diet designed to provide 0 (dietary control), 1050, 2100 and 4200 mg 2-acetylpyrrole [14.047]/kg feed daily (Bauter, 2012). These dietary levels correspond to the calculated average daily intakes of 0, 68, 133 and 263 mg/kg bw per day for males and 0, 79, 155 and 298 mg/kg bw per day for males, respectively.

The test material was not stable in the diet, and in the report (Bauter, 2012) it is suggested that part of it was probably not detected by the extraction method employed due to formation of complexes with metal ions in the feed. It is calculated that over the course of the study the animals received concentrations of 35–40% of the target intake level on average. Therefore, values for exposure levels based on the measured intake are proportionally lower. Based on this analysis of the test diets, the mean daily intakes were calculated to 367, 754 and 1705 mg/kg feed. Assuming that possible toxicity is only related to the free 2-acetylpyrrole, these dietary concentrations correspond to average daily intakes of 24, 48 and 107 mg/kg bw per day for males, and 28, 56 and 121 mg/kg bw per day for females, respectively, over 90 days.

Statistically significant dose-dependent reductions in body weight, body weight gain, feed consumption (males and females) and feed to gain ratio (females) at the highest dietary level (1705 mg/kg feed measured concentration) during the study were attributed to the possible decrease in palatability caused by the test substance at high dietary levels.

Female rats of the high intake groups displayed minimal to moderate dark bilateral thyroid glands. Microscopic changes were slight thyroid hypertrophy/hyperplasia among 4/10 and 10/10 high intake group males and females, respectively. This was characterised by enlarged subgross tall columnar
appearance of the follicular epithelial cells which appeared with fine cytoplasmic vacuolation with intermittent focally piled papillary projections into the follicular lumen.

The thyroid effects at the high exposure level are considered adverse. Therefore, a NOAEL for 2-acetylpyrrole is derived from the dose of 48 mg/kg bw per day in males.

### 3.2.3. Safety for the target species

The first approach to the safety assessment for target species takes account of the applied use levels in animal feed relative to the maximum reported exposure of humans on the basis of the metabolic body weight. Human exposure in the EU ranges from 0.12 to 50 µg/person per day (EFSA, 2008a,b, 2009; EFSA CEF Panel 2015a,b). This corresponds from 0.006 to 2.31 µg/kg bw^0.75^ per day. Table 3 summarises the result of the comparison with human exposure for representative target animals. The body weight of target animals is taken from the default values shown in Table 4.

**Table 3:** Comparison of exposure of humans and target animals (calculated from the proposed maximum feed concentrations, see 3.1.3) to the flavourings under application

<table>
<thead>
<tr>
<th>Flavouring</th>
<th>Use level in feed (mg/kg)</th>
<th>Human exposure (µg/kg bw^0.75^ per day)^a^</th>
<th>Target animal exposure (µg/kg bw^0.75^ per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Salmon</td>
<td>Piglet</td>
</tr>
<tr>
<td>Trimethyloxazole</td>
<td>0.5</td>
<td>0.17</td>
<td>11.0</td>
</tr>
<tr>
<td>Piperine</td>
<td>0.5</td>
<td>0.93</td>
<td>11.0</td>
</tr>
<tr>
<td>3-Methylindole</td>
<td>0.5</td>
<td>0.11</td>
<td>11.0</td>
</tr>
<tr>
<td>Indole</td>
<td>0.5</td>
<td>1.21</td>
<td>11.0</td>
</tr>
<tr>
<td>2-Acetylpyridine</td>
<td>0.5</td>
<td>2.32</td>
<td>11.0</td>
</tr>
<tr>
<td>2-Acetylpyrrole</td>
<td>0.5</td>
<td>0.15</td>
<td>11.0</td>
</tr>
<tr>
<td>3-Ethylpyridine</td>
<td>0.5</td>
<td>0.43</td>
<td>11.0</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>0.5</td>
<td>0.006</td>
<td>11.0</td>
</tr>
<tr>
<td>2,6-Dimethylpyridine</td>
<td>0.5</td>
<td>0.012</td>
<td>11.0</td>
</tr>
</tbody>
</table>

(a): Metabolic body weight (kg bw^0.75^) for a 60-kg person = 21.6.

Table 3 shows that for all nine compounds the intake by the target animals greatly exceeds that of humans resulting from use in food. As a consequence, safety for the target species at the feed concentration applied cannot be derived from the risk assessment for food use.

As an alternative, the maximum feed concentration considered as safe for the target animals can be derived from the lowest NOAEL available. Toxicological data could only be found for piperine [14.003], 2-acetylpyridine [14.038] and acetylpyrrole [14.047]. The FEEDAP Panel notes that the purity of piperine for feed use is lower than in the JECFA specification (95 vs 97%). However, considering the intended use level and the availability of relevant toxicological studies, this difference is not considered of concern. Applying an uncertainty factor (UF) of 100 to the NOAELs, the maximum safe intake for the target species was derived for the three compounds following the EFSA guidance for sensory additives (EFSA FEEDAP Panel, 2012a), and thus the maximum safe feed concentration was calculated. The results are summarised in Table 4.

**Table 4:** Maximum safe concentration in feed for different target animals for piperine (A), 2-acetylpyridine (B) and 2-acetylpyrrole (C)

<table>
<thead>
<tr>
<th>Target animal</th>
<th>Default values</th>
<th>Maximum safe intake/feed concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (kg)</td>
<td>Intake (mg/day)</td>
</tr>
<tr>
<td></td>
<td>Feed intake (g/day)^a^</td>
<td>A</td>
</tr>
<tr>
<td>Salmonids</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>Veal calves (milk replacer)</td>
<td>100</td>
<td>2,000</td>
</tr>
<tr>
<td>Cattle for fattening</td>
<td>400</td>
<td>8,000</td>
</tr>
<tr>
<td>Pigs for fattening</td>
<td>100</td>
<td>3,000</td>
</tr>
<tr>
<td>Sows</td>
<td>200</td>
<td>6,000</td>
</tr>
<tr>
<td>Dairy Cows</td>
<td>650</td>
<td>20,000</td>
</tr>
<tr>
<td>Turkeys for fattening</td>
<td>12</td>
<td>400</td>
</tr>
<tr>
<td>Piglets</td>
<td>20</td>
<td>1,000</td>
</tr>
</tbody>
</table>
Pyridine, pyrrole and quinoline derivatives (CG 28) for all animal species

<table>
<thead>
<tr>
<th>Target animal</th>
<th>Default values</th>
<th>Maximum safe intake/feed concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (kg)</td>
<td>Feed intake (g/day)⁹ (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chickens for fattening</td>
<td>2</td>
<td>120</td>
</tr>
<tr>
<td>Laying hens</td>
<td>2</td>
<td>120</td>
</tr>
<tr>
<td>Dogs</td>
<td>15</td>
<td>250</td>
</tr>
<tr>
<td>Cats</td>
<td>3</td>
<td>60</td>
</tr>
</tbody>
</table>

(a): Complete feed with 88% dry matter (DM), except milk replacer for veal calves (94.5% DM), and for cattle for fattening, dairy cows, dogs and cats for which the values are DM intake.
(b): Complete feed containing 88% DM, milk replacer 94.5% DM.

For the six remaining compounds, adequate subchronic, repeated-dose studies performed with the additive under assessment were not available. Therefore, the threshold of toxicological concern (TTC) approach was followed to derive the maximum safe feed concentration (EFSA FEEDAP Panel, 2012a).

For the Cramer class I compounds, 3-methylindole [14.004] and indole [14.007], the calculated safe use level is 1.5 mg/kg complete feed for cattle, salmonids and non-food-producing animals, and 1.0 mg/kg complete feed for pigs and poultry.

For the Cramer Class II compounds, trimethyloxazole [13.169], 3-ethylpyridine [14.061], pyrrolidine [14.064] and 2,6-dimethylpyridine [14.065], the calculated safe use level is 0.5 mg/kg complete feed for cattle, salmonids and non-food-producing animals, and 0.3 mg/kg complete feed for pigs and poultry.

Conclusions on safety for the target species

The FEEDAP Panel concludes that:
- piperine [14.003], 3-methylindole [14.004], indole [14.007], 2-acetylpyridine [14.038] and 2-acetylpyrrole [14.047] are safe at the proposed maximum dose level (0.5 mg/kg complete feed) for all animal species;
- trimethyloxazole [13.169], 3-ethylpyridine [14.061], pyrrolidine [14.064] and 2,6-dimethylpyridine [14.065] are safe at the proposed use level of 0.5 mg/kg complete feed for cattle, salmonids and non-food-producing animals, and at the use level of 0.3 mg/kg complete feed for pigs and poultry.

3.2.4. Safety for the consumer

The safety for the consumer of the compounds in CG 28, used as food flavours, has already been assessed by JECFA (WHO, 2005, 2006 and 2008) and EFSA (EFSA, 2008a,b, 2009; EFSA CEF Panel 2015a,b). All these compounds are presently authorised as food flavourings without limitations.

Given the low use levels of CG 28 compounds to be applied in feed, and the expected extensive metabolism and excretion in target animals (see Section 3.2.1), the FEEDAP Panel considers that the possible residues in food derived from animals fed with these flavourings would not appreciably increase the human intake levels of these compounds from natural sources and food use. Therefore, the FEEDAP Panel concludes that the use of the nine additives under assessment in animal nutrition at the proposed use levels is safe for the consumer.

3.2.5. Safety for the user

No specific data on the safety for the user were provided. In the material safety data sheets, hazards for skin and eye contact, and respiratory exposure are recognised for the majority of the compounds under application. Most are classified as irritating to the respiratory system.

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⁹ Technical dossier/Section II/Annex II.3
3.2.6. Safety for the environment

The additions of naturally occurring substances that will not result in a substantial increase of the concentration in the environment are exempt from further assessment (EFSA, 2008c). Examination of the published literature shows that this applies to piperine [14.003], 3-methylindole [14.004], indole [14.007], 2-acetylpyridine [14.038], 2-acetylpyrrole [14.047] and pyrrolidine [14.064], which occur in the environment at levels above the application rate of 0.5 mg/kg feed [data taken from the Netherlands Organisation for Applied Scientific Research (TNO) database Volatile Compounds in Food ver. 14.1; Burdock, 2003].

The applicant did not demonstrate that the other three compounds trimethyloxazole [13.169], 3-ethylpyridine [14.061] and 2,6-dimethylpyridine [14.065] occur in the environment at levels above the application rate of 0.5 mg/kg feed. These substances are therefore assessed in a predicted environmental concentration (PEC) calculation for soil (PEC\textsubscript{soil}) arising from the application rate. When the calculations are performed according to the EFSA guidance (EFSA, 2008c) with a fixed concentration in feed, there is a fixed order of PEC\textsubscript{soil} from each species, with the lamb being the most critical.

Table 5: PEC values for lambs of specific flavourings of CG 28 under assessment

<table>
<thead>
<tr>
<th>EU Register name</th>
<th>CAS No</th>
<th>Dose mg/kg</th>
<th>PEC soil (µg/kg)</th>
<th>PEC pore water (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethyloxazole</td>
<td>20662-84-4</td>
<td>0.5</td>
<td>11</td>
<td>1.8</td>
</tr>
<tr>
<td>3-Ethylpyridine</td>
<td>536-78-7</td>
<td>0.5</td>
<td>11</td>
<td>2.7</td>
</tr>
<tr>
<td>2,6-Dimethylpyridine</td>
<td>108-48-5</td>
<td>0.5</td>
<td>11</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Table 5 shows the PEC\textsubscript{soil} for lambs. The values are slightly above the threshold of 10 µg/kg (EFSA, 2008c). The PEC for pore water, however, is dependent on the sorption, which is different for each compound. For these calculations, the substance-dependent constants organic carbon sorption constant ($K\textsubscript{oc}$), molecular weight, vapour pressure and solubility are needed. These were estimated from the Simplified Molecular Input Line Entry Specification (SMILES) notation of the chemical structure using EPIWEB 4.1 (Table 6). This program was also used to derive the SMILES notation from the CAS numbers. The $K\textsubscript{oc}$ value derived from the first-order molecular connectivity index was used, as recommended by the EPIWEB program.

Table 6: Physico-chemical properties predicted by EPIWEB 4.1

<table>
<thead>
<tr>
<th>EU Register name</th>
<th>CAS No</th>
<th>DT\textsubscript{50} (days)</th>
<th>Predicted by EPIWEB 4.1</th>
<th>Solubility (mg/L)</th>
<th>$K\textsubscript{oc}$ (L/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethyloxazole</td>
<td>20662-84-4</td>
<td>16</td>
<td>111.14</td>
<td>762</td>
<td>2,801</td>
</tr>
<tr>
<td>3-Ethylpyridine</td>
<td>536-78-7</td>
<td>18</td>
<td>107.16</td>
<td>264</td>
<td>84,780</td>
</tr>
<tr>
<td>2,6-Dimethylpyridine</td>
<td>108-48-5</td>
<td>22</td>
<td>107.16</td>
<td>702</td>
<td>81,500</td>
</tr>
</tbody>
</table>

The half-life (DT\textsubscript{50}) was calculated using BioWin3 (Ultimate Survey Model), which gives a rating number. This rating number r was translated into a half-life using the formula by Arnot et al. (2005):

$$DT_{50} = 10^{(-r \times 1.07 + 4.12)}$$

This is the general regression used to derive estimates of aerobic environmental biodegradation half-lives from BioWin3 model output.

All three substances in Table 5 have PEC\textsubscript{pore water} above 0.1 µg/L. Therefore, they are subjected to phase II risk assessment.

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10 Technical dossier/Supplementary information June 2011.
11 Available online: http://www.epa.gov/opptintr/exposure/pubs/episuiteld.htm
In the absence of experimental data, the phase II risk assessment was performed using ECOSAR v1.11, which estimates the half-maximal effective concentration (EC$_{50}$) for earthworms, fish, algae and Daphnia from the SMILES notation of the substance.

**Table 7:** The Predicted Environmental Concentration for surface water for lambs compared with the EC$_{50}$ values in mg/L predicted by ECOSAR 1.11

<table>
<thead>
<tr>
<th>EU Register name</th>
<th>LC$_{50}$(a) Earthworm (mg/kg)</th>
<th>LC$_{50}$(a) Fish (mg/L)</th>
<th>LC$_{50}$(a) Daphnia (mg/L)</th>
<th>EC$_{50}$(b) Algae (mg/L)</th>
<th>PEC surface water (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethyloxazole</td>
<td>200</td>
<td>123</td>
<td>69</td>
<td>49</td>
<td>0.57</td>
</tr>
<tr>
<td>3-Ethylpyridine</td>
<td>193</td>
<td>122</td>
<td>68</td>
<td>48</td>
<td>0.89</td>
</tr>
<tr>
<td>2,6-Dimethylpyridine</td>
<td>191</td>
<td>108</td>
<td>61</td>
<td>44</td>
<td>1.05</td>
</tr>
</tbody>
</table>

EU: European Union; EC$_{50}$: half-maximal effective concentration; LC$_{50}$: lethal concentration 50; PEC: predicted environmental concentration.

(a): LC$_{50}$, the concentration of a test substance which results in a 50% mortality of the test species.

(b): EC$_{50}$, the concentration of a test substance which results in 50% of the test animals being adversely affected (i.e. both mortality and sublethal effects).

The LC$_{50}$ and EC$_{50}$ values (Table 7) divided by a UF of 1,000 were much higher than the PEC values for soil and surface water, for all compounds indicating that, according to the guidance (EFSA, 2008c), there is no risk to the environment at the doses mentioned in Table 5.

The use of all additives in fish feed in land-based aquaculture systems does not give a predicted environmental concentration of the additive (parent compound) in surface water (PEC$_{swaq}$) above the trigger value of 0.1 µg/L when calculated according to the guidance. For sea cages, a safe dose of 0.047 mg/kg feed was calculated according to the EFSA guidance (EFSA, 2008c). This dose would give a sediment concentration of 10 µg/kg which is the threshold level of no concern.

**Conclusions on safety for the environment**

The concentrations considered safe for the target species (see Section 3.2.3) are unlikely to have detrimental effects on the terrestrial and fresh water environments. For the marine environment, the safe use level is estimated to be 0.05 mg/kg feed.

**3.3. Efficacy**

Since all nine compounds are used in food as flavourings, and their function in feed is essentially the same as that in food, no further demonstration of efficacy is necessary.

**4. Conclusions**

The FEEDAP Panel concludes that piperine [14.003], 3-methylindole [14.004], indole [14.007], 2-acetylpyridine [14.038] and 2-acetylpyrrole [14.047] are safe at the proposed maximum use level of 0.5 mg/kg complete feed for all animal species; trimethyloxazole [13.169], 3-ethylpyridine [14.061], pyrrolidine [14.064] and 2,6-dimethylpyridine [14.065] are safe at the proposed use level of 0.5 mg/kg complete feed for cattle, salmonids and non-food-producing animals, and at the use level of 0.3 mg/kg complete feed for pigs and poultry.

No safety concern would arise for the consumer from the use of these compounds up to the highest proposed level in feeds.

Hazard for skin and eye contact, and respiratory exposure are recognised for the majority of the compounds under application. Most are classified as irritating to the respiratory system.

The concentrations considered safe for the target species are unlikely to have detrimental effects on the terrestrial and fresh water environments.

As all of the compounds under assessment are used in food as flavourings and their function in feed is essentially the same as that in food, no further demonstration of efficacy is necessary.

In the absence of data on the stability in water for drinking, the FEEDAP Panel is unable to conclude on the safety or efficacy of the substances under this mode of delivery.
Pyridine, pyrrole and quinoline derivatives (CG 28) for all animal species

Documentation provided to EFSA


5. Evaluation report of the European Union Reference Laboratory for Feed Additives on the methods(s) of analysis for CDG 28 Pyridine, pyrrole, and quinoline derivatives.

6. Comments from Member States.

References


EFSA (European Food Safety Authority), 2008b. Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on Flavouring Group Evaluation 85
Pyridine, pyrrole and quinoline derivatives (CG 28) for all animal species

EFSA (European Food Safety Authority), 2008c. Technical Guidance of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) for assessing the safety of feed additives for the environment. The EFSA Journal, 842, 1–28.


Pyridine, pyrrole and quinoline derivatives (CG 28) for all animal species


Abbreviations

ADME absorption, distribution, metabolism and excretion
bw body weight
CAS Chemical Abstracts Service
CD Commission Decision
CEF EFSA Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CG chemical group
CDG chemically defined group
DM dry matter
DT$_{50}$ degradation half-time
EC European Commission
EC$_{50}$ half-maximal effective concentration
ECOSAR component program of EPI suite™
EPI suite Estimation Programs Interface (EPI) Suite™
EURL European Union Reference Laboratory
FAO Food and Agriculture Organization
FEEDAP EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
FFAC Feed Flavourings authorisation Consortium of (FEFANA) the EU Association of Specialty Feed Ingredients and their Mixtures
FGE Flavouring Group Evaluation
FID flame ionisation detector
FLAVIS the EU Flavour Information System
FL-No FLAVIS number
GC–MS gas chromatography–mass spectrometry
JECFA The Joint FAO/WHO Expert Committee on Food Additives
$K_{OC}$ organic carbon sorption constant
$K_{OW}$ octanol–water partition coefficient
LC$_{50}$ lethal concentration 50
log $K_{OW}$ logarithm of octanol–water partition coefficient
NOAEL no observed adverse effect level
OECD Organisation for Economic Co-operation and Development
PEC predicted environmental concentration
PEC$_{swaq}$ predicted environmental concentration of the additive (parent compound) in surface water
RTL retention time locking
SMILES Simplified Molecular Input Line Entry Specification
TNO Netherlands Organisation for Applied Scientific Research
TTC threshold of toxicological concern
UF uncertainty factor
WHO World Health Organization
Annex – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Pyridine, pyrrole, and quinoline derivative

The Chemically Defined Flavourings – Group 28 (Pyridine, pyrrole and quinoline derivatives), in this application comprises nine substances, for which authorisation as feed additives is sought under the category ‘sensory additives’, functional group 2(b) ‘flavouring compounds’, according to the classification system of Annex I of Regulation (EC) No 1831/2003.

In the current application submitted according to Article 4(1) and Article 10(2) of Regulation (EC) No 1831/2003, the authorisation for all species and categories is requested. The flavouring compounds of interest have a purity ranging from 95% to 99%.

Mixtures of flavouring compounds are intended to be incorporated only into feedingstuffs or drinking water. The Applicant suggested no minimum or maximum levels for the different flavouring compounds in feedingstuffs or in water.

For the identification of volatile chemically defined flavouring compounds chemically defined group (CDG) 28 in the feed additive, the Applicant submitted a qualitative multianalyte gas chromatography–mass spectrometry (GC–MS) method, using retention time locking (RTL), which allows a close match of retention times on GC–MS. By making an adjustment to the inlet pressure, the retention times can be closely matched to those of a reference chromatogram. It is then possible to screen samples for the presence of target compounds using a mass spectral database of RTL spectra. The Applicant maintained two FLAVOR2 databases/libraries (for retention times and for MS spectra) containing data for more than 409 flavouring compounds. These libraries were provided to the European Union Reference Laboratory (EURL). The Applicant provided the typical chromatogram for the CDG 28 of interest.

In order to demonstrate the transferability of the proposed analytical method (relevant for the method verification), the Applicant prepared a model mixture of flavouring compounds on a solid carrier to be identified by two independent expert laboratories. This mixture contained twenty chemically defined flavourings belonging to twenty different chemical groups to represent the whole spectrum of compounds in use as feed flavourings with respect to their volatility and polarity. Both laboratories properly identified all the flavouring compounds in all the formulations. As the substances of CDG 28 are within the volatility and polarity range of the model mixture tested, the Applicant concluded that the proposed analytical method is suitable to determine qualitatively the presence of the substances from CDG 28 in the mixture of flavouring compounds.

Based on the satisfactory experimental evidence provided, the EURL recommends for official control for the qualitative identification in the feed additive of the individual (or mixture of) flavouring compounds of interest listed in Table 1 (*) the GC–MS–RTL (Agilent specific) method submitted by the Applicant.

For the identification of piperine in the feed additive, the Applicant proposed a single laboratory validated and further verified method based on GC coupled to a flame ionisation detector (GC-FID). Based on the satisfactory performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified GC-FID method, submitted by the Applicant, for the qualitative identification of piperine in the feed additive.

As no experimental data were provided by the Applicant for the identification of the active substance(s) in feedingstuffs and water, no methods could be evaluated. Therefore, the EURL is unable to recommend a method for the official control to identify the active substance(s) of interest listed in Table 1 (*) in feedingstuffs or 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) is not considered necessary.

(*) Full list provided in EURL evaluation report, available from the EURL website.