

**Committee for Risk Assessment**  
**RAC**

**Opinion**  
proposing harmonised classification and labelling  
at EU level of

**Nicotine (ISO);**  
**3-[(2S)-1-methylpyrrolidin-2-yl]pyridine**

**EC Number: 200-193-3**  
**CAS Number: 54-11-5**

CLH-O-0000001412-86-68/F

**Adopted**  
**10 September 2015**



## **OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL**

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonized classification and labelling (CLH) of:

**Chemical name:**        **nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine**

**EC Number:**            **200-193-3**

**CAS Number:**         **54-11-5**

The proposal was submitted by the **Netherlands** and received by RAC on **17 April 2015**.

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

### **PROCESS FOR ADOPTION OF THE OPINION**

The Netherlands has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at <http://echa.europa.eu/harmonised-classification-and-labelling-consultation/> on **21 April 2015**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **5 June 2015**.

### **ADOPTION OF THE OPINION OF RAC**

Rapporteur, appointed by RAC: **Bogusław Barański**

The opinion takes into account the comments provided by MSCAs and parties concerned in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonized classification and labelling was adopted on **10 September 2015** by consensus.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	614-001-00-4	nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine	200-19 3-3	54-11-5	Acute Tox. 3* Acute Tox. 1 Aquatic Chronic 2	H301 H310 H411	GHS06 GHS09 Dgr	H301 H310 H411			
Dossier submitters proposal	614-001-00-4	nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine	200-19 3-3	54-11-5	<b>Retain</b> Acute Tox. 1 (dermal) Aquatic Chronic 2 <b>Add</b> Acute Tox. 2 (inhalation) <b>Modify</b> Acute Tox. 3* (oral) to Acute Tox. 1 (oral)	<b>Retain</b> H310 H411 <b>Add</b> H330 <b>Modify</b> H301 to H300	GHS06 GHS09 Dgr	H300 H310 H330 H411			
RAC opinion	614-001-00-4	nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine	200-19 3-3	54-11-5	Acute Tox. 2 Acute Tox. 2 Acute Tox. 2 Aquatic Chronic 2	H300 H310 H330 H411	GHS06 GHS09 Dgr	H300 H310 H330 H411			
Resulting Annex VI entry if agreed by COM	614-001-00-4	nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine	200-19 3-3	54-11-5	Acute Tox. 2 Acute Tox. 2 Acute Tox. 2 Aquatic Chronic 2	H300 H310 H330 H411	GHS06 GHS09 Dgr	H300 H310 H330 H411			

# GROUNDS FOR ADOPTION OF THE OPINION

## HUMAN HEALTH HAZARD ASSESSMENT

### RAC evaluation of acute toxicity

#### Summary of the Dossier Submitter's proposal

##### **Acute Oral Toxicity**

In the original CLH report, the Dossier Submitter (DS) proposed to classify nicotine as Acute Tox. 1; H300 by selecting the lowest LD<sub>50</sub> of 3.34 mg/kg (Lazutka *et al.*, 1969) out of various available LD<sub>50</sub> values from different species and strains. The lowest LD<sub>50</sub> of 3.34 mg/kg is below the limit value of  $\leq 5$ mg/kg bw (oral) for classifying a substance in category 1 for acute toxicity by the oral route. However, preliminary results from an ongoing acute oral toxicity study in mice were submitted during the public consultation. As a consequence, the DS revised his proposal in the response to comment document (see Annex 2) in category 2 (Acute Tox. 2; H300).

##### **Acute Inhalation Toxicity**

The DS summarised two acute toxicity inhalation studies but both had deficiencies in the study design, levels of concentrations tested or duration of exposure. However, the data from these two studies combined indicated an LC<sub>50</sub> in the range of doses between 0.1 and 0.5 mg/L (aerosol). According to the DS, the LC<sub>50</sub> is thus within the limits of 0.05 mg/L -  $\leq$  0.5mg/L (inhalation) justifying classification in category 2 for acute inhalation toxicity (Acute Tox. 2; H330).

##### **Acute Dermal Toxicity**

The current harmonised classification of nicotine by the dermal route as Acute Tox. 1 is based on a dermal study in rabbits with an LD<sub>50</sub> value of 50 mg/kg bw, but only a reference to this study (and not the study report) in secondary literature was available to the DS. The DS recognised that there were no reliable acute dermal toxicity studies in rabbits or rats with nicotine available at the time of CLH report submission. The only available acceptable study was performed in cats and indicated a dermal LD<sub>50</sub> in the range of 50 mg/kg bw. This value is deducted from a mortality of 81% at a dose of approximately 80 mg/kg bw. A LD<sub>50</sub> of 50 mg/kg bw or lower warrants classification in category 1 for acute dermal toxicity. Therefore, the DS considered to retain the current classification as Acute Tox. 1; H310. However, preliminary results from an ongoing acute oral toxicity study in rabbits were submitted during the public consultation of the CLH proposal for nicotine. As a consequence, the DS revised their classification in the response to comments document (see Annex 2) to category 2 (Acute Tox. 2; H310).

#### Comments received during public consultation

Twenty seven comments were provided during the public consultations from six MSCAs, nine industrial organisations, including the lead registrant for nicotine, four non-governmental organizations, one university and seven individuals.

The classification of acute toxicity proposed by the DS was commented on by six MSCAs. Some of them noted that the studies and data presented in the CLH report were not sufficiently detailed and that the justifications of the proposed classifications should be improved.

The proposed classification for acute oral toxicity was supported by four MSCAs. The other two MSCAs urged making a careful analysis of the data used for classification.

The proposed classification for acute inhalation toxicity was supported by four MSCAs. One MSCA proposed to use an extrapolation factor 12 when calculating LC<sub>50</sub> of nicotine. Two MSCAs questioned the proposed classification of acute inhalation toxicity due to the limitations in the design of the studies used for the classification proposal.

The proposed classification for acute dermal toxicity was supported by three MSCAs. One MSCA did not support the proposal.

Industrial organisations provided thorough literature data on metabolism and toxicokinetics of nicotine and preferred to retain the current classification for nicotine. They questioned the proposed classifications for acute toxicity of nicotine based on:

- the lack of sufficient justification for action at Community level,
- disagreement with the choice of the most relevant species for classification of acute oral toxicity,
- questioned the use of the oral LD<sub>50</sub> of 3.34mg/kg for mice as the basis for classification,
- disagreement with the classification for acute inhalation toxicity due to the lack of validity of extrapolations for exposure periods of less than 30 minutes, and
- uncertainty concerning acute dermal toxicity classification.

Industry also announced the initiation of two new tests for acute oral and dermal toxicity in mice and rabbit respectively. The motivation for conducting the new studies was that the quality of the information used by RIVM to prepare its CLH proposal was regarded as insufficient to justify the proposed harmonised classification.

Other comments received during the public consultation mainly concerned misunderstandings of the difference between hazard and risk assessment and the fact that CLP relies on hazard alone.

## **Assessment and comparison with the classification criteria**

### ***Acute Oral Toxicity***

The DS provided a number of different oral LD<sub>50</sub> values or estimates of the oral LD<sub>50</sub> for rats, mice or dogs based on data from studies or from other reference sources (see tables 11 and 12 of the Background Document, Annex 2). In some of these studies an aqueous solution of nicotine was used while in others an aqueous solution of nicotine sulfate was applied. Practically all studies have limitations, mainly in reporting. Other studies were not retrieved (Trochimowicz *et al.*, 1994 and DECOS, 2004) but cited and reviewed by others (Bibra, 2014). A detailed analysis of the available studies and LD<sub>50</sub> values is provided below per experimental species.

#### *Studies conducted in rats*

LD<sub>50</sub> values of nicotine for rats were derived from eight studies and ranged from 52.5 mg/kg to 188 mg/kg.

Three of these studies were considered as acceptable and yielded the following LD<sub>50</sub> values for nicotine: 52.5 mg/kg (Lazutka *et al.*, 1969); 70 mg/kg (Yam *et al.*, 1991); 70 mg/kg: (Van den Heuvel *et al.*, 1990). Only one study (Van den Heuvel *et al.*, 1990) followed OECD Test Guidelines (TG) 401 (1981).

Three studies in rats, deemed to be not acceptable due to deficiencies in study design including the number of animals used, provided the following LD<sub>50</sub> values: 70 mg/kg (Ben-Dyke *et al.*, 1970); 50-60 mg/kg (Farm Chemicals Handbook, 1991) and 188 mg/kg (Ambrose and DeEds, 1946). The studies of Trochimowicz *et al.* (1994) and of DECOS (2004) could not be retrieved. The LD<sub>50</sub> values (respectively 50-60 and 188 mg/kg) were derived from a report prepared by Bibra, (2014).

LD<sub>50</sub> values of nicotine sulfate in rats were derived from the following three studies: 56.7 mg/kg (Lazutka *et al.*, 1969); 75 mg/kg (Vernot *et al.*, 1977); 83 mg/kg (Gaines, 1960). Based on all these data, RAC is of the opinion that the oral LD<sub>50</sub> of nicotine in rats ranges from 52.5 to 70 mg/kg, while the LD<sub>50</sub> for nicotine sulphate in rats ranges from 56.7 to 83 mg/kg bw. RAC assumes that nicotine and nicotine sulphate have similar mechanisms of action and toxicity. However no comparison of these two substances is provided in the CLH report.

#### Studies conducted in mice

LD<sub>50</sub> values of nicotine in mice from available studies reviewed by the DS ranged from 3.34 mg/kg (Lazutka *et al.*, 1969) to 24 mg/kg (Heubner and Papierkowski, 1937). These studies are individually summarised and assessed below.

1. In the Lazutka *et al.* (1969) oral acute toxicity study, a single dose of nicotine or nicotine sulfate dissolved in an aqueous solution was given by gavage to mice or rats. Mice were given the test substance in a dose range of 0.25 – 16 mg/kg, and rats in a dose range of 1 – 90 mg/kg bw. 25 groups of animals were used in this oral acute toxicity study. Although the number of animals per group was not specified, it was reported that in total 332 animals were used in the various experiments described in this paper. RAC assumes that in the acute oral toxicity testing the number of animals was probably at least 5 per group, i.e. 125 animals. The other animals (of the reported total 332 animals) were used for testing dermal absorption (6 rats and 6 rabbits), for short-term oral toxicity testing (8 weeks) on four groups of rats and for a sub-chronic inhalation toxicity study (4-months) on three groups of animals.

RAC notes that the relatively scant description of the studies in Lazutka *et al.* (1969) is typical for this journal at the time the study was published. Bearing in mind that the aim of the study was to derive the occupational exposure limit for nicotine sulphate, used as a pesticide, these investigations were likely carried out in accordance with relevant national recommendations and cannot be dismissed. Oral LD<sub>50</sub> values of nicotine in mice obtained in this study was 3.34 mg/kg and oral LD<sub>50</sub> values of nicotine in rats was 52.5 mg/kg (Lazutka *et al.*, 1969). A LD<sub>50</sub> of nicotine for rabbits was not determined in this study. The oral LD<sub>50</sub> value of nicotine sulphate in mice was 8.55mg/kg and 56.7 mg/kg in rats (Lazutka *et al.*, 1969).

2. The study Contraft-Nicotex-Tabacco (2015a) evaluated the acute oral toxicity of nicotine in mice. As written above, the study was announced by the lead registrant for nicotine during the public consultation. Preliminary results were submitted but the full study report was made available in July 2015. The study has been performed in May – June 2015 according to the Up-and-Down-Procedure in line with OECD TG 425 (3<sup>rd</sup> October 2008), under GLP.

Altogether, 9 mice were treated with nicotine. Four animals died shortly after nicotine administration. Five animals survived until the end of the 14 days observation period. The stepwise oral dosing of nicotine with a dose progression factor of 3.2 and the lethality within 48 hours after dosing are given in the Table below:

<b>Sequential number of animal</b>	<b>Dose (mg/kg bw)</b>	<b>Lethality/Survival 48 hrs after dosing</b>
1	17.5	survived
2	55	survived
3	175	survived
4	550	died
5 ( first reversal of dose)	175	died
6	55	died
7	17.5	survived

8 (second reversal of dose)	55	died
9 (third reversal of dose)	17.5	survived
Dosing was stopped because according to the study report the likelihood-ratios (LR) calculated exceeded the critical likelihood-ratio, the LR stopping criterion was satisfied.		

In the Table below the survival time of female mice treated with different doses is summarised. All remaining animals survived until the end of the 14 days observation period:

<b>Dose (mg/kg bw)</b>	<b>Number of female mice</b>	<b>Number of dead animals and survival time after treatment</b>	<b>Clinical observations</b>
550	1	1 mouse died 10 seconds after treatment	10 sec post treatment: tonic and clonic convulsion.
175	2	1 mouse died 10 seconds after treatment	10 sec. post treatment: clonic convulsion. 30 min. to 4 hours post treatment: decreased activity, tremor, closed eyes, clonic convulsion, disturbance of autonomic functions (decreased respiration rate, dyspnoea)
55	3	1 death 20 seconds after treatment 1 death 30 minutes after treatment	Directly post treatment: clonic convulsion (in all mice) 30 min. to 4 hours post treatment: decreased activity, tremor, tonic convulsion, clonic convulsion, closed eyes, disturbance of the autonomic functions (dyspnoea)
17.5 mg/kg	3	none	30 min to 3 hours post treatment: 1 animal with decreased activity, tremor, clonic convulsion, disturbance of coordination (abnormal gait) and autonomic functions (dyspnoea)

The LD<sub>50</sub> calculated with Probit analysis by 'SPSS+' software results in 77.83 mg/kg bw . The approximate 95 % confidence limits were not calculated because their range was too wide. The second LD<sub>50</sub> calculated based on results of this study with the statistical program recommended in OECD TG 425 using maximum likelihood (AOT425StatPGM), is equal also to 77.83 mg/kg bw with a 95% confidence interval ranging from 0 mg/kg bw to 20 000mg/kg bw.

The study was claimed by the authors in compliance with OECD TG 425 in a laboratory having GLP certification. RAC notes however that only 9 animals were used and dosing of further animals was stopped "*because the stopping criteria according to the Guideline was met: LR criterion (if the likelihood-ratios calculated exceed the critical likelihood-ratio, the LR stopping criterion is satisfied and testing stops).*"



However, as noted in the OECD TG 425: "A combination of stopping criteria should be used to keep the number of animals low while adjusting the dosing pattern to reduce the effect of a poor starting value or low slope (see paragraphs 33 and 34). "Dosing continues depending on the fixed-time interval (e.g., 48-hour) outcomes of all the animals up to that time. The testing stops when one of the following stopping criteria first is met":

(a) 3 consecutive animals survive at the upper bound;

(b) 5 reversals occur in any 6 consecutive animals tested;

(c) at least 4 animals have followed the first reversal and the specified likelihood-ratios exceed the critical value. (See paragraph 44 and Annex 3. Calculations are made at each dosing, following the fourth animal after the first reversal).

"For a wide variety of combinations of  $LD_{50}$  and slopes, stopping rule (c) will be satisfied with 4 to 6 animals after the test reversal. In some cases for chemicals with shallow slope dose-response curves, additional animals (up to a total of fifteen tested) may be needed."

"Dosing is stopped when one of these criteria is satisfied, at which time an estimate of the  $LD_{50}$  and a confidence interval are calculated for the test based on the status of all the animals at termination."

After a careful comparison of the study report with OECD TG 425, RAC considers that these criteria have not been properly analysed and confirmed even though it was evident that the 95% confidence limits for  $LD_{50}$  could not be calculated by Probit analysis using the SPSS+software.

In this context it is important to note that according to paragraph 45 of OECD TG 425 "A wide confidence interval indicates that there is more uncertainty associated with the estimated  $LD_{50}$ . The reliability of the estimated  $LD_{50}$  is low and the usefulness of the estimated  $LD_{50}$  may be marginal." Therefore, RAC does not consider the calculated  $LD_{50}$  in that study as reliable.

3. In the study of Heubner and Papierkowski (1938), between 36 and 55 white mice (animal weight: 17-26 grams; strain not specified) were used to assess the acute oral toxicity of nicotine. Nicotine was administered in aqueous solution by gavage to 5 animals per group. The doses follow a geometric progression, with a range of 20 %, but the actual doses administered were not described. Mortality occurred within 25 minutes. The estimated oral  $LD_{50}$  of nicotine in mice is 24 mg/kg bw using the Spearman-Kärber method (Kärber, 1931).

The study was performed decades before OECD test guidelines and principles of GLP were established. However, RAC notes that the overall study design is similar to the OECD test guideline for acute oral toxicity and the number of animals per group allowed for a reliable calculation of the  $LD_{50}$ .

The two additional references providing oral  $LD_{50}$  values in mice, which were cited in Bibra (2014), provided the following  $LD_{50}$  values: 24 mg/kg (DECOS, 2004; Trochimowicz et al., 1994) and 50-60 mg/kg (Trochimowicz et al., 1994). The  $LD_{50}$  values of nicotine sulphate in mice ranged from 8.55 mg/kg (Lazutka et al., 1969) to 16 mg/kg (Vernot et al., 1977), while the  $LD_{50}$  of nicotine tartrate for mice amounted to 87 mg/kg (Heubner and Papierkowski, 1937).

Based on all these data, RAC is of the opinion that the oral  $LD_{50}$  of nicotine in mice is within a range of 3.34 - 24 mg/kg bw and for nicotine sulphate it is 8.55 mg/kg bw. RAC assumes that nicotine and nicotine sulphate have similar mechanisms of action and toxicity.

#### Study conducted in dogs

In the oral toxicity study of Franke and Thomas (1932) nicotine was dropped undiluted on the tongue or between the lips and gums of a total of 19 dogs.

The results of this study are presented in the Table below.

<b>Dose (mg/kg bw)</b>	<b>No. of dogs</b>	<b>Number of deaths</b>	<b>Number of surviving dogs</b>	<b>% mortality</b>	<b>Average time till death (minutes)</b>
20	2	2	0	100	2.5
12	1	1	0	100	3.5
9.2-10.3	14	8	6	57.1	3.77
4.6-5.0	2	0	2	0	-

After a single oral administration of undiluted nicotine alkaloid in the mouth, the LD<sub>50</sub> in dogs was 9.2 mg/kg (Franke and Thomas, 1932).

RAC recognises that the study was performed before OECD test guidelines and principles of GLP were available. However, while the study design is not in line with the OECD guidelines, the number of animals is sufficient, the dosing method seems relevant and the data and allows for an estimation of the LD50.

Discussion and conclusion on acute oral toxicity

Overall, RAC concludes from all relevant and acceptable studies that the LD<sub>50</sub>'s for nicotine are 9.2 mg/kg in dogs and in the range of 3.34 mg/kg to 24 mg/kg in mice. Both species seem to be more sensitive to nicotine than rats, which show an LD<sub>50</sub> in the range of 52.5 mg/kg to 70 mg/kg.

Differences in LD<sub>50</sub> between species can be explained by metabolic and toxicokinetics differences. The metabolism of nicotine is mostly mediated through the hepatic cytochrome P450 CYP2A6 with the C-oxidation of nicotine to cotinine as the major detoxication reaction. As outlined in the CLH report, the metabolism of nicotine is complex and differs between species. The available information indicates that the rat may be less relevant for extrapolation to humans due to hepatic cytochrome P450 differences. As commented by industry during the public consultation, there is a high similarity in human and mouse cytochrome P450 enzymes, which are the main enzymes in nicotine metabolism (CYP2A6 in human and CYP2A5 in mouse). In contrast, the enzyme metabolising nicotine in rats is a member of the CYP2B family (Mwenifumbo and Tyndele, 2009). However, the plasma half-life of nicotine in each species is more important than the type of P450 enzyme(s) responsible for the metabolism. The nicotine half-life in rats is within the range of 45 to 66 min (Kyerematen *et al.*, 1988) and closer to the half-life time in humans (120 min) (Benowitz *et al.*, 1982, 2009) than the very short half-life of 6 to 9 minutes in mice (Peterson *et al.*, 1984; Siu and Tyndale, 2007).

Other factors such as nicotine uptake and distribution in the body are also important, contributing to the toxicity profile. Additionally, differences between the different tests in different species may also be attributed to the method of administration. Gavage studies in the rat (resulting in uptake via the gastro-intestinal tract) caused lethality after a minimum of 50 minutes (Lazutka, 1969), whereas the studies by Franke and Thomas in dogs (1932) using drops into the mouth resulted in lethality already after a few minutes. This is probably due to the rapid absorption of nicotine via the gums. Absorption via the gums is considered as relevant exposure route of nicotine in humans. An estimate of the lethal dose in humans seems to be in the range of 6.5 to 13 mg/kg bw (Mayer, 2014), which is comparable to the value found in dogs.

RAC concludes that oral LD<sub>50</sub> values from rat studies using gavage exposure probably underestimate the human toxicity. Taking into account different variables which can influence acute toxicity in mammals, it is not possible to demonstrate that toxicity data generated in mice, dogs or rats are more relevant for human hazard assessment.

RAC also notes that the individual sensitivity of mice to acute oral toxicity of nicotine is highly variable, which is reflected by the wide range of estimated LD<sub>50</sub> (3.34 mg/kg and 24 mg/kg). High variability in individual sensitivity of mice to acute toxicity of nicotine is also confirmed in the recent study CONTRAFT-NICOTEX-TABACCO (2015) in which, one out of 2 treated mice died after single administration of nicotine at a dose of 175 mg/kg, while only 2 out of 3 mice treated died after a dose of 55 mg/kg.

The rat is considered being the least sensitive species to acute oral toxicity of nicotine among all species tested, with the lowest reported LD<sub>50</sub> of 52.5 mg/kg. For the reasons noted above, this was not taken forward for determining the LD50 of nicotine.

Overall, since the estimated oral LD<sub>50</sub> of nicotine in mice (3.34 mg/kg and 24 mg/kg) and LD<sub>50</sub> of nicotine in dogs (9.2 mg/kg) as the most sensitive species both fit best within the range of > 5 mg/kg and ≤ 50 mg/kg, RAC is of the opinion that nicotine warrants a classification as **Acute Tox. 2 (oral) with the hazard statement H300: Fatal if swallowed**.

#### Acute toxicity Estimate (ATE; oral)

RAC proposes an ATE of 5 mg/kg bw for the classification of mixtures containing nicotine. The oral ATE for nicotine is converted from the acute toxicity point estimate of acute toxicity hazard category 2 (see Table 3.1.2 in the CLP Regulation). However, RAC considers that the default ATE value of 5 mg/kg is justified because the classification for acute oral toxicity of nicotine is based by RAC on a weight of evidence analysis of all existing acute oral toxicity data instead of selecting an LD<sub>50</sub> value from one particular study.

#### **Acute Inhalation Toxicity**

The results of two acute inhalation toxicity studies conducted in rats were presented by the DS. One acute inhalation toxicity study was conducted to assess the toxicity of nicotine (Shao *et al.*, 2012) whereas another study was performed to evaluate the toxicity of a tobacco extract formulation (Werley *et al.*, 2014). Since the latter study was done on a mixture, RAC considers that it is not acceptable for the purpose of classification of acute inhalation toxicity of nicotine. However, the results are briefly presented below.

The study of Werley *et al.* (2014) evaluated the acute toxicity of a tobacco extract formulation containing 37.3% glycerol, 28.6% propylene glycol (PG), 19.2% ethanol, 4.1% nicotine, 8.8% water and 2% tobacco essential oils by weight. The extract was derived using a patented extraction process. The study was conducted in accordance with OECD TG 403. Two groups of six rats of each sex were used. Animals were exposed to the tobacco extract formulation for 4 hours at concentrations of 2.13 mg/L (Group 1) or 1.0mg/L (Group 2). The corresponding nicotine concentrations were 0.114 and 0.060 mg/L respectively. All animals, except one female exposed at 2.13 mg/L, survived and gained weight during the 14-day recovery period. At the end of the study, all animals appeared healthy and active. The LC<sub>50</sub> for the inhaled tobacco extract was considered to be greater than 2 mg/L. The study results suggest that the LC<sub>50</sub> of nicotine itself is above 0.114 mg/L.

In the acute inhalation toxicity study of Shao *et al.* (2012), the LC<sub>50</sub> of nicotine (water solution of nicotine at pH 6.8, 7.4 and 8.0) has been established using the up and down procedure (UDP) recommended by the US EPA Health Effects Test Guidelines (EPA, 2002). The MMAD of droplets produced in the nicotine mist was between 1.69 and 3.55 µm with a geometric standard deviation (GSD) of 1.8 to 2.48 depending on the nicotine concentration. Nicotine was dissolved in water or NaCl solution to achieve an osmolality of ~300 mOsm/kg (Shao *et al.*, 2012).

The results of Shao *et al.* (2012) revealed that the pH of an aqueous solution of nicotine affects the LC<sub>50</sub> value, which was the highest at pH 6.8 (> 4.1 mg/L, 20 min). At pH values of 7.4 and 8.0, a lower LC<sub>50</sub> (20 min) of 2.3 mg/L was determined.

According to point 3.1.2.1. (c) of the CLP Regulation, the conversion of existing inhalation toxicity data which was generated using a 1-hour exposure can be carried out by dividing by a factor of 4 for dusts and mists. In Shao *et al.* (2012), the duration of exposure to nicotine mist (20 minutes) was 12-fold shorter than 4 hours (240 minutes). Using the Haber's law formula ( $C^n \cdot t = k$ ) that allows a direct comparison with the criteria for classification (Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.7a: Endpoint specific guidance Version 3.0, August 2014), the converted LC<sub>50</sub> (4 hours) of nicotine from Shao *et al.* (2012) is 0.19 mg/L as follows:

$$C^n \times t = \text{constant}$$
$$C \times 240 \text{ min.} = 2.3 \text{ mg/L} \times 20 \text{ min.}$$
$$C = \frac{2.3 \frac{\text{mg}}{\text{L}} \times 20 \text{ min.}}{240 \text{ min.}} = 0,19 \text{ mg/L}$$

RAC considers that 20 minutes exposure is not substantially different from 30 minutes exposure and conversion can be made to 4 hour LC<sub>50</sub> value.

#### Discussion and conclusion on acute inhalation toxicity

Overall, taking into account that the LC<sub>50</sub> (4 hours) of nicotine in rats is 0.19 mg/L which is within the range 0.05 mg/L- ≤ 0.5mg/L, RAC is of the opinion that nicotine warrants a classification as **Acute Tox. 2 (inhalation) with the hazard statement H330: Fatal if inhaled.**

#### Acute inhalation toxicity estimate (ATE; inhalation)

RAC proposes an ATE of 0.19 mg/L for the classification of mixtures containing nicotine (see Table 3.1.2 in the CLP Regulation). RAC considers that this is justified because the LD<sub>50</sub> value is derived from a single reliable study.

### **Acute Dermal Toxicity**

#### Rats

In the acute dermal toxicity study (Gaines1960); considered as acceptable by the DS), nicotine sulphate was dissolved in a water solution of lead arsenate and calcium arsenate. The solution was applied on the skin of rats at a dose as high as 0.96 mL/kg. The concentrations of lead and calcium arsenates are not known, but have probably been very low, since according to the FAO/WHO Monograph "Evaluations of Some Pesticide Residues in Food" (1968), these salts are practically insoluble in water. The presence of arsenate could rather reduce the LD<sub>50</sub> value. The LD<sub>50</sub> of nicotine sulphate established in this study amounted to 285 mg/kg bw.

#### Rabbits

The results of three acute dermal toxicity studies (FDA, 1952; Trochimowicz et al., 1994; UK PSD, 2008) were presented by the DS in the CLH report. LD<sub>50</sub> values of nicotine in rabbits were in the range from 50 mg/kg to 140 mg/kg. However, none of these studies were considered as acceptable by the DS due to serious deficiencies in study design.

During the public consultation the preliminary results of an acute dermal rabbit toxicity study were submitted by the lead registrant. The study Contraft-Nicotex-Tabacco (2015a) has been performed in May – June 2015 according to Method B.3 described in Council Regulation (EC) No 440/2008 (equivalent to OECD TG 402), under GLP.

In the main study, mortality occurred only in female rabbits within the 14 day observational period as follows: at 50 mg/kg bw, 1/5 animals (20% mortality), at 100 mg/kg bw, 4/5 females (80% mortality) and at 200 mg/kg bw, 5/5 animals (100% mortality) died. None out

of 5 male rabbits treated at 50 mg/kg died after 24 h dermal exposure to nicotine, indicating that the sensitivity to dermally applied nicotine in female and male rabbits is similar.

The test item caused dermal irritation symptoms on the treatment site in both sexes. In the Table below the survival time and systemic clinical observations of male and female rabbits treated with different doses are summarised:

<b>Dose (mg/kg bw)</b>	<b>Number of male/female rabbits</b>	<b>Number of dead animals and survival time after treatment</b>	<b>Clinical observations</b>
50	5/5	1 female rabbit died 2 h after treatment	1 hour post treatment: CNS symptoms (decreased activity, tremor), disturbances of coordination (incoordination, lateral position), disturbance of autonomic functions (dyspnoea) in one female which died on same day. No systemic clinical signs in males.
100	0/5	4 female rabbits died within one day after treatment	30 min. to 5 hours post treatment: CNS symptoms (decreased activity, tremor, closed eyes, clonic convulsion), disturbance of coordination (abnormal gait), disturbances of the autonomic functions (increased respiration rate, dyspnoea)
200	0/5	All females died within 1 to 2 h after treatment	30 min. to 1 hour post treatment: CNS symptoms (decreased activity, tremor, closed eyes, clonic convulsion), disturbance of coordination (abnormal gait), disturbance of the autonomic functions (salivation)

The dermal LD<sub>50</sub> for female rabbits calculated by probit analysis (SPSS+software) amounted to 70.4 mg/kg bw, with 95% confidence limits of 28.3 mg/kg bw to 131.2 mg/kg bw.

### Cats

In the study of Travell (1960), 21 cats each received a single dermal dose (200 mg) nicotine or nicotine sulphate. The substances were applied on the skin after fur was clipped as a 40% aqueous solution with respect to the nicotine. The weight of the cats included in the study varied between 2 and 3 kg. The dermal doses of nicotine are estimated to range between 66 and 100 mg/kg. The frequency of symptoms and mortality is presented in the table below.

<b>Acute dermal toxicity, female and male cats</b>				
<b>Compound</b>	<b>Number of cats</b>	<b>Incidence of</b>		
		<b>Nausea (%)</b>	<b>Vomiting (%)</b>	<b>Death (%)</b>
Nicotine	21	100	100	81
Nicotine sulfate	21	52	19	0

When nicotine base was used, 81% of the animals died between 21 to 195 minutes after dermal application. When nicotine sulfate was used, none of the animals died.

Based on the results of this study, the dermal LD<sub>50</sub> of nicotine was in the range of 66 – 80 mg/kg, while the dermal LD<sub>50</sub> of nicotine sulfate is higher than 100mg/kg. By the dermal route, nicotine appears more toxic than nicotine sulphate. This study in cats may be considered as supportive for the assessment of acute dermal toxicity of nicotine, although due to specific experimental design deficiencies the calculation of an exact LD<sub>50</sub> value is not possible. The dose of 66 mg/kg is taken as LD<sub>50</sub> of nicotine for cats, because this dose is at the lower end of the range of doses (66 – 100 mg/kg) at which 81% mortality was observed.

#### Discussion and conclusion on acute dermal toxicity

RAC is of the opinion that there are no acceptable acute dermal toxicity studies for nicotine in rats; the cat study can be considered as supportive. Only the acute dermal toxicity study in rabbits recently submitted by the lead registrant is acceptable to be used for classification purposes.

Taking into account that dermal LD<sub>50</sub> of nicotine for rabbits in this study equals 70.4 mg/kg bw, which is within the range of 50 mg/kg - ≤ 200 mg/kg, RAC is of the opinion that nicotine warrants a classification as **Acute Tox. 2 (dermal) with the hazard statement H310: Fatal in contact with skin.**

#### Acute dermal toxicity estimate (ATE, dermal)

RAC proposes an ATE of 70.4 mg/kg for the classification of mixtures containing nicotine (see Table 3.1.2 in the CLP Regulation). RAC considers that the **ATE of 70 mg/kg** (rounded down from 70.4) is justified because the LD<sub>50</sub> by the dermal route is derived from a single reliable study.

### **Additional references (not included in the CLH report)**

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## **ANNEXES:**

- Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.
- Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).