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Committee for Risk Assessment
RAC

Annex 1
Background document
to the Opinion proposing harmonised classification and labelling at Community 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

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

10 September 2015

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Substance Name: nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine

EC Number: 200-193-3

CAS Number: 54-11-5

Index Number: 614-001-00-4

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Part A.

Part A.

1   PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1   Substance

Table 1:   Substance identity

Substance name:	nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine
EC number:	*200-193-3*
CAS number:	*54-11-5*
Annex VI Index number:	*614-001-00-4*
Degree of purity:	*Minimum purity >99%*
Impurities:	*cotinine <= 0.15%,* *myosmine <= 0.15%,* *FAB (N-(4-oxo-4-pyridin-3-yl-butyl)-formamide) <= 0.10%,* *nicotine N-oxide <= 0.15%,* *nornicotine <= 0.15%,* *anatabine <= 0.15%,* *beta-nicotyrine <= 0.10%,* *anabasine <= 0.10%.*

1.2   Harmonised classification and labelling proposal

Table 2:   The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	Acute Tox. 3* (H301) Acute Tox. 1 (H310) Aquatic Chronic 2 (H411)
Current proposal for consideration by RAC	Changing Acute Tox. 3* (oral) into Acute Tox. 1 (oral)

	Adding Acute Tox 2 (inhalation)
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Acute Tox. 1 (H300) Acute Tox. 1 (H310) Acute Tox. 2 (H330) Aquatic Chronic 2 (H411)

*Minimum classification

1.3 **Proposed harmonised classification and labelling based on CLP Regulation**

Table 3: **Proposed classification according to the CLP Regulation**

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1]	Reason for no classification [2]
2.1.	Explosives				Not assessed
2.2.	Flammable gases				Not assessed
2.3.	Flammable aerosols				Not assessed
2.4.	Oxidising gases				Not assessed
2.5.	Gases under pressure				Not assessed
2.6.	Flammable liquids				Not assessed
2.7.	Flammable solids				Not assessed
2.8.	Self-reactive substances and mixtures				Not assessed
2.9.	Pyrophoric liquids				Not assessed
2.10.	Pyrophoric solids				Not assessed
2.11.	Self-heating substances and mixtures				Not assessed
2.12.	Substances and mixtures which in contact with water emit flammable gases				Not assessed
2.13.	Oxidising liquids				Not assessed
2.14.	Oxidising solids				Not assessed
2.15.	Organic peroxides				Not assessed
2.16.	Substance and mixtures corrosive to metals				Not assessed
3.1.	Acute toxicity - oral	Acute Tox. 1 (H300)		Acute Tox. 3 (H301)	
	Acute toxicity - dermal			Acute Tox. 1 (H310)	
	Acute toxicity - inhalation	Acute Tox. 2 (H330)			
3.2.	Skin corrosion / irritation				Not assessed
3.3.	Serious eye damage / eye irritation				Not assessed
3.4.	Respiratory sensitisation				Not assessed
3.4.	Skin sensitisation				Not assessed
3.5.	Germ cell mutagenicity				Not assessed
3.6.	Carcinogenicity				Not assessed
3.7.	Reproductive toxicity				Not assessed
3.8.	Specific target organ toxicity –single exposure				Not assessed
3.9.	Specific target organ toxicity – repeated exposure				Not assessed

3.10.	Aspiration hazard				Not assessed
4.1.	Hazardous to the aquatic environment			Aquatic Chronic 2 (H411)	
5.1.	Hazardous to the ozone layer				Not assessed

[1] Including specific concentration limits (SCLs) and M-factors
[2] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Pictogram: GHS06, GHS09

Signal word: Danger

Hazard statements: H300 "Fatal if swallowed",
H310 "Fatal in contact with skin",
H330 " Fatal if inhaled",
H411 "Toxic to aquatic life with long lasting effects"

Precautionary statements: No precautionary statements are proposed since precautionary statements are not included in Annex VI of Regulation EC no. 1272/2008.

Proposed notes assigned to an entry:

: none

2   BACKGROUND TO THE CLH PROPOSAL

2.1   History of the previous classification and labelling

The current harmonised classification of nicotine for acute toxicity is the translation of the DSD classification with T+; R27 and T; R25. The old proposal for harmonised classification (available in the IUCLID file) shows that the proposal for acute oral toxicity was based on a list of LD50 values with references. The list for acute oral toxicity included several species including rat, mouse and dog. However, the DSD criteria were based on rats. The rat LD50 values in the range of 50-80 mg/kg bw resulted in a classification with R25 (criterion: LD50 oral, rat: $25 < LD50 \geq 200$ mg/kg). Translation of R25 resulted in Acute Tox. 3*; H310 because the DSD and the CLP criteria differ and a lower classification could not be excluded without going back to the original proposal. For acute dermal toxicity, LD50 values were available for rat (140-280 mg/kg bw) and rabbit (50 mg/kg bw/day). The classification with R27 was based on the rabbit LD50 (criterion: LD50 dermal, rat or rabbit: ≤ 50 mg/kg) using the lower value of both species. Translation of R27 resulted in Acute Tox. 1; H310 because the DSD and the CLP criteria both relate to an LD50 below 50 mg/kg bw.

2.2   Short summary of the scientific justification for the CLH proposal

This proposal is based on the information available in the REACH-registration (accessed January 2015), the DAR of nicotine (1), EFSA 2009 (2) and other information available in literature.

The proposed classification for acute oral toxicity with Acute Tox. 1; H300 is based on the lowest LD50 of 3.34 mg/kg bw for the available LD50 values for different species and strains. This LD50 value fulfils the requirement for Acute Tox. 1; H300 being an ATE (LD50) below 5 mg/kg bw.

The available acute dermal toxicity studies are very limitedly described. The current classification is based on a study in rabbits with an LD_{50} of 50 mg/kg bw. This LD_{50} value fulfils the requirement for Acute Tox. 1; H301 although there is no access to the original study. However, one acceptable study with nicotine in cats is available that supports the current harmonised classification. As such, it is considered justified to keep the current classification.

There are two acute toxicity inhalation studies with limitations in tested concentration or exposure duration. However, combined these two studies indicate an LC50 in the range between 0.1 and 0.5 mg/L (aerosol), justifying classification in category 2.

2.3   Current harmonised classification and labelling

2.3.1   Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

Acute Tox. 3* (H301), Acute Tox. 1 (H310), Aquatic Chronic 2 (H411).

2.3.2   Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

Table 4. Self-classification by the registrant (on 23 December 2014)

Hazard Class	Statement Code	# of notifiers
Acute Tox. 3	H301	174
Acute Tox. 1	H310	177
Aquatic Chronic 2	H411	178
Acute Tox. 2	H300	4
Aquatic Acute 1	H400	4

Total number of notifiers; 178. Number of aggregated notifications; 6.

2.4.2 Current self-classification and labelling based on DSD criteria

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

The current classification of nicotine for acute oral toxcitiy, Acute Tox. 3*, has led to confusion in several European countries, including the Netherlands. The * indicates that this is a minimum classification and requires manufacturers and importers of nicotine to investigate whether he has access to data or other information that lead to a more severe category and apply this more severe category (CLP Annex VI, 1.2.1). Whereas industry bases its classification on an $LD50_{oral}$ of 50 mg/kg bw in rats, the RIVM came to an $LD50_{oral}$ of 5 mg/kg bw based on the possible translations of R25 into Cat 2 or Cat 3 and the much lower LD50 values for mouse and dogs compared to rats. The lowest available LD_{50} value of 3.34 mg/kg bw (for mice) warrants a harmonized classification of Acute Tox. 1, instead of 3.

In addition for the classification of mixtures containing nicotine for acute toxicity, the determination of the ATE of nicotine for the calculation of the ATE of the mixture is very relevant as there is a difference in opinion between inspectorates and industry. An advice of RAC on the LD50 value that was determinative for the classification and that should be used in the ATE calculation of the mixture would therefore be very helpful.

Given the current policy discussions on the use of the e-cigarette, the increase in accidents with e-cigarette refills and its increasing popularity, the Netherlands deems it important to submit a CLH dossier on nicotine to propose a classification change from Acute Tox. 3 to Acute Tox. 1 and if possible have an advice on the ATE for acute oral toxicity.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

1   IDENTITY OF THE SUBSTANCE

1.1   Name and other identifiers of the substance

Table 5:   Substance identity

EC number:	200-193-3
EC name:	nicotine (ISO); 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine
CAS number (EC inventory):	54-11-5
CAS number:	54-11-5
CAS name:	Pyridine, 3-[(2S)-1-methyl-2-pyrrolidinyl]-
IUPAC name:	3-[(2S)-1-methylpyrrolidin-2-yl]pyridine
CLP Annex VI Index number:	614-001-00-4
Molecular formula:	$C_{10}H_{14}N_2$
Molecular weight range:	162.23

Structural formula:
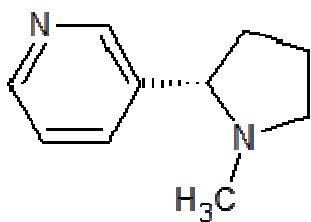
1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Nicotine	99%	99-100%	According to European Pharmacopoeia 8.0

Current Annex VI entry:

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
cotinine	<= 0.15%		Impurity profile derived from European Pharmacopoeia 8.0
myosmine	<= 0.15%		
FAB (N-(4-oxo-4-pyridin-3-yl-butyl)-formamide)	<= 0.10%		
nicotine N-oxide	<= 0.15%,		
nornicotine	<= 0.15%		
anatabine	<= 0.15%		
beta-nicotyrine	<= 0.10%		
anabasine	<= 0.10%		

Current Annex VI entry: Not relevant

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks

Current Annex VI entry: Not relevant

1.2.1   Composition of test material

1.3   <u>Physico-chemical properties</u>

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Colourless liquid with brown tint and fishy smell	DAR	Visual and olfactory assessment Print; DAR3 B1-B5
Melting/freezing point	-79 °C	DAR	
Boiling point	247 °C	DAR	
Relative density	1.010	DAR	
Vapour pressure	5.62 Pa at 25 °C	DAR	
Surface tension	No data provided	DAR	
Water solubility	1000 g/L at unknown temperature and pH	DAR	
Partition coefficient n-octanol/water	Log K_{ow} = 1.17 at unknown temperature and pH, Log K_{ow} = 0.93	DAR	
Flash point	Not relevant	DAR	
Flammability	101 °C, auto flammability 243 °C, auto-ignition 244 °C	DAR	
Explosive properties	Based on molecular structure, nicotine is unlikely to be explosive as it does not possess any of the chemical groups expected to impart explosive properties on a molecule, with only carbon, hydrogen and nitrogen present.	DAR	
Self-ignition temperature			
Oxidising properties	No data or case provided, stated by notifier as non-oxidising	DAR	
Granulometry			
Stability in organic solvents and identity of relevant degradation products	Soluble in chloroform, diethyl ether, ethanol and petroleum ether.	DAR	
Dissociation constant	pKa1= 3.1; pKa2 = 8.2	DAR	
Viscosity			

2   MANUFACTURE AND USES

2.1   Manufacture

Nicotine is a naturally occurring alkaloid obtained from the leaves of the tobacco plant.

2.2   Identified uses

Nicotine is the main constituent in tobacco smoke. In recent years there has been an increased interest in the development of nicotine replacement therapies based on alternative exposure routes. As such, the primary therapeutic use of nicotine is in treating nicotine dependence in order to eliminate smoking. Controlled levels of nicotine are given to patients through gums, dermal patches, lozenges, electronic/substitute cigarettes or nasal sprays in an effort to wean them off their dependence. Nicotine is also used in e-cigarettes. Nicotine is also present in mushrooms (2) possibly due to the use as insecticide. Nicotine was not included in Annex I of 91/414 because the existing evidence did not demonstrate safe use.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not relevant as this proposal is limited to classification for acute toxicity.

Table 10:   Summary table for relevant physico-chemical studies

Method	Results	Remarks	Reference

3.1   *[Insert hazard class when relevant and repeat section if needed]*

3.1.1   Summary and discussion of

3.1.2   Comparison with criteria

3.1.3   Conclusions on classification and labelling

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1   Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1   Non-human information

4.1.2   Human information

4.1.3   Summary and discussion on toxicokinetics

As summarized by EFSA, 2009 (2):

"Nicotine is rapidly absorbed through the oral cavity, lung, and gastrointestinal tract. Absorption of nicotine across biological membranes depends on pH. In its ionised state, such as in acidic environments, nicotine does not rapidly cross membranes. The respiratory absorption of nicotine was found to be 60% to 80%. Nicotine base can be absorbed through the skin, and there have been cases of poisoning after skin contact with pesticides containing nicotine. Nicotine is poorly absorbed from the stomach because it is protonated (ionized) in the acidic gastric fluid, but is well absorbed in the small intestine, which has a more alkaline pH and a large surface area. Following the administration of nicotine capsules or nicotine in solution, peak concentrations in blood are reached in about 1 h (Benowitz et al., 1991; Zins et al., 1997; Dempsey et al., 2004). The oral bioavailability of nicotine is incomplete because of the hepatic first-pass metabolism and ranges between 20% to about 45% (Andersson et al., 2003; Benowitz et al., 1991; Compton et al., 1997; Zins et al., 1997; Hukkanen et al., 2005). After intravenous administration, the highest levels of nicotine were found in spleen, liver, lungs and brain (UK DAR, 2007).

The metabolism of nicotine is mediated mostly through the hepatic cytochrome P450 CYP2A6 with the C-oxidation of nicotine to cotinine as the major detoxication reaction, followed by the hydroxylation of cotinine to 3-hydroxycotinine (Dorne et al., 2004; Hukkanen et al., 2005). The lungs and the kidneys are also partially involved in the metabolism of nicotine. Variants in the CYP2A6 gene have been associated with altered nicotine metabolism and with effects on smoking behaviour. A number of genotypes of CYP2A6 have been determined and a recent intravenous study (Benowitz et al, 2006b) classified subjects in three phenotypes according to CYP2A6 activity (fractional clearance of nicotine to cotinine and on plasma ratio of 3-hydroxycotinine to cotinine) with respective CYP2A6 activities and mean total plasma clearances of 100%, 80% and 50%, and 18.5, 15.5 and 11.7 ml/min/kg. Elimination half-lives ranged from 1.8 to 2.9 hours between the three phenotypes (Benowitz et al., 2006b). Considering the short biological half-live of nicotine in humans, no accumulation of nicotine is foreseen.
Nicotine readily crosses the placenta. Nicotine is mainly excreted through urine, and faeces. The rate of nicotine excretion is influenced by the pH of the urine. When the pH of the urine is made alkaline, the proportion of uncharged nicotine increases and re-absorption of nicotine and as a result, less nicotine is excreted (UK DAR, 2007).

Recently, a mechanistic population model for the pharmacokinetics of nicotine, its primary (CYP2A6-generated) metabolite cotinine and 3-hydroxycotinine has been developed from sixty-six subjects receiving orally 2 mg of deuterium-labelled nicotine and 10 mg deuterium-labelled cotinine simultaneously. The model showed high correlation between nicotine clearance to cotinine and the 3-hydroxycotinine to cotinine concentration ratio in saliva supporting the idea that the 3-hydroxycotinine: cotinine ratio can be used as a predictor of CYP2A6 activity and nicotine clearance. The model-based analysis extends and further justifies this conclusion (Levi et al., 2007a). This model has been applied to predict nicotine clearance using cotinine and 3-hydroxy-cotinine spot saliva samples (Levi et al., 2007b).
A recent study (Yun et al., 2008) in subjects exposed to transdermal nicotine patches administered as single and multiple doses, demonstrated that nicotine clearance in smokers is slower than in non-smokers: in smoking individuals nicotine induces glucuronidation, and higher plasma concentrations are thus maintained."

Species differences in nicotine metabolism as summarised by Hukkanen, 2005 (25):

"Nicotine metabolism in various species has been reviewed previously (Gorrod and Jenner, 1975; Scheline, 1978; Seaton and Vesell, 1993). Cotinine and 3-hydroxycotinine are major urinary nicotine metabolites in all mammalian species studied (Jenner et al., 1973; Nwosu and Crooks, 1988; Kyerematen et al., 1990a); however, about as much nicotine *N*-oxide as cotinine and 3-hydroxycotinine is formed by guinea pigs and rats. Guinea pig and hamster hepatocytes show the highest total metabolism of nicotine, followed by mouse, rat, and human hepatocytes (Kyerematen et al., 1990a). In general, there is considerable variation between rodent species in the activity of nicotine metabolism, as well as in the stereospecificity and relative amounts of nicotine metabolites produced. Also, P450 enzymes responsible for nicotine metabolism vary in species. For example, CYP2B1/2 is the P450 enzyme metabolizing nicotine in rats, whereas rat CYP2A is inactive in nicotine metabolism (Hammond et al., 1991; Nakayama et al., 1993).
Nicotine metabolism in nonhuman primates resembles human metabolism. In macaque monkeys, nicotine and cotinine half-lives are similar to humans (Seaton et al., 1991). Like humans, African green monkeys metabolize 80 to 90% of nicotine via a CYP2A6-like enzyme, but hepatic protein levels are about 4 times higher in green monkeys than humans resulting in 2-fold higher *V*max for cotinine formation (Schoedel et al., 2003). Rhesus monkey hepatocytes metabolize about 80% of nicotine to cotinine (Poole and Urwin, 1976).

Nicotine *N*-glucuronidation activity is highest in human liver microsomes followed by rhesus and cynomolgus monkey microsomes, although the activity in monkey microsomes is only about 7 to 11% of human glucuronidation activity (Ghosheh and Hawes, 2002a). Low-level nicotine glucuronidation activity was also detected in minipig and guinea pig microsomes, whereas activity was not measurable in rats, mice, dogs, and rabbits. Cotinine glucuronidation was below limit of quantification for all the animal species, including rhesus, cynomolgus, and marmoset monkeys (Tsai and Gorrod, 1999; Ghosheh and Hawes, 2002a)."

In addition, according to Tutka, 2005 (26) this indicates that the rat may not be the most relevant species for humans:
"In a recent study of Tutka et al. [unpublished data], the significant differences in NIC metabolism were found among human, rabbit, and rat, confirming species variability in NIC metabolism. The study showed that a profile of NIC metabolism in rabbit was different from that of the rat. In contrast to rats, rabbits seem to be a good model for studying human NIC metabolism."

4.2   Acute toxicity

4.2.1   Non-human information

4.2.1.1 Acute toxicity: oral

Table 11:   Summary of acute oral toxicity studies using nicotine

Method	Dilution	LD$_{50}$ (mg/kg bw)	Animal	Remarks	Acceptability	Reference
Single dose administration, peroral administration	0.15-0.3% aqueous solutions	52.5	Rat	Strain, sex and number not specified, nicotine base	Acceptable	Lazutka *et al.*, 1969
Single dose administration, oral	Not described	70	Rat	Strain, sex and number not specified	Not acceptable	Ben-Dyke *et al.*, 1970
Not described, oral	Not described	50 - 60	Rat	Strain, sex and number not specified	Not acceptable	Farm Chemicals Handbook, 1991
Up and down method; gavage	Not described	70	Rat	Sprague-Dawley, 5 male & 5 female	Acceptable	Yam *et al.*, 1991
OECD 1981, acute oral toxicity; gavage Fixed dose procedure	Not described	70 LD50 between 25 and 200 mg/kg bw	Rat	Sprague-Dawley, 15 male, 15 female On average 14 rats per test	Acceptable	Van den Heuvel *et al.*, 1990
Unkown, oral	Not described	50 – 60	Rat	Strain, sex and number not specified	Not acceptable	Trochimowicz et al., 1994*
Unkown, oral	Not described	188	Rat	Strain, sex and number not specified	Not acceptable	DECOS, 2004 (cited as Ray91)*
Unknown, oral	Alkaloid dissolved in water, pH adjusted to 7.0, dilution not described	188	Rat	35 animals, strain and sex not described	Acceptable	Ambrose and DeEds, 1946
Unknown, oral	Not described	24	Mouse	Strain, sex and number not specified	Not acceptable	DECOS 2004; Trochimowicz et al., 1994*
Unknown, oral	Not described	50 – 60	Mouse	Strain, sex and number not specified	Not acceptable	Trochimowicz et al., 1994*
Single dose administration, oral, peroral administration	0.15-0.3% aqueous solutions	3.34	Mouse	Strain, sex and number not specified, nicotine base	Acceptable	Lazutka et al., 1969
Single dose, gavage	Aqueous solution,	24	White	36-55 mice; 5 mice/group. Strain and	Acceptable	Heubner and

	dilution unknown, nicotine base		mouse	sex not specified.		Papierkowski, 1938
Single dose administration, oral; dropped on tongue or between lips and gums	Undiluted alkaloid	9.2	Dog	# of animals; 19, strain and sex not specified	Acceptable	Franke and Thomas, 1932

*These studies could not be retrieved. The above description was derived from the Bibra proposal, 2014 (3). They will not be further described below. As other studies showed the same LD50 values as for some studies which could not be retrieved, it is considered likely that these are the same studies.

Table 12   Summary of oral toxicity studies using nicotine salts

Method	Dilution	LD$_{50}$ (mg/kg bw)	Animal	Remarks	Acceptability	Reference
Single dose, oral, gavage	Not described	75	Rat	Sprague-Dawley, male, # not specified (5/dose), nicotine sulfate	Acceptable	Vernot et al., 1977
Single dose administration, oral, gavage	Suspension in water-lead arsenate and calcium arsenate	83	Rat	Sherman, female, 80/group, nicotine sulfate	Acceptable	Gaines, 1960
Single dose administration, oral, peroral administration	0.15-0.3% aqueous solutions	56.7	Rat	Strain, sex and number not specified, nicotine sulfate*	Acceptable	Lazutka *et al.*, 1969
Single dose administration, oral, peroral administration	0.15-0.3% aqueous solutions	8.55	Mouse	Strain, sex and number not specified, nicotine sulfate*	Acceptable	Lazutka *et al.*, 1969
Single dose, oral, gavage	Not described	16	Mouse	CF-1, male, # not specified (5/dose), nicotine sulfate	Acceptable	Vernot et al., 1977
Single dose, gavage	Aqueous solution, dilution unknown,	87	White mouse	36-55 mice; 5 mice/group. Strain and sex not specified. Nicotine tartrate	Acceptable	Heubner and Papierkowski, 1938

*Nicotine sulfate is an aqueous solution containing 40% nicotine equivalent.

Reference: Lazutka et al., 1969 (4)
Study design:
Short-term toxicity studies included 25 series of experiments using a single peroral administration (gavage) of aqueous solution of nicotine base and nicotine sulfate in doses of 1 -90 mg/kg for albino rats and 0.25 – 16 mg/kg for white mice. Mouse and rat strains, sex and number are not specified. Rats and rabbit were used for skin absorption and conjunctiva studies. Only the total number of animals used is mentioned, which is 332.
Results:
The peroral administration of lethal doses caused irritation of the respiratory tract and motor restlessness, followed by marked hyperemia of the ears and extremities. After 30-40 min, there

were tonic contractions of various groups of muscles, often with transition to clonic spasms. Traube's symptom was positive in the majority of cases. After 40-50 min the spasms were superseded by relaxation of the muscles, and the animals assumed a one-sided position. There was marked dyspnea and tremor of the entire body. In animals surviving the lethal dose the symptoms of poisoning gradually disappeared after 3-7 hr from the beginning. In other animals, their condition became worse, and they ceased to react to outside stimuli. They developed asphyxia and died within 1-3 days. The severity of poisoning and the rate of its development, as well as the interval before death, were directly related to the dose.

Table 13

Parameter	Mice		Rats	
	dose, mg/kg			
	Nicotine	Nicotine sulfate*	Nicotine	Nicotine sulfate
LD_{16}	0.25	1	20	30
LD_{50}	3.34	8.55	52.5	56.7
LD_{100}	10	16	80	90

*Nicotine sulfate is an aqueous solution containing 40% nicotine equivalent.

Acceptability:
Limited description but acceptable given the period in which it was performed.
Conclusions:
The mice and rats differed in their susceptibility, the mice proving more sensitive to nicotine than rats, the LD_{50} for mice being 3.34 mg/kg, and for rats 52.5 mg/kg.

Reference: Ben-Dyke et al., 1970 (5)
This paper lists acute toxicity data for a number of pesticides, including nicotine. This data has been prepared from experimental results of the Toxicology Laboratory, Chesterford Park Research Station, or from published literature and manufacturer's bulletins. However, there are no actual references, the study design is not described, nor the number of animals used; only the oral LD_{50} of 70 mg/kg bw is mentioned.
Acceptability:
Not acceptable

Reference: Farm Chemicals Handbook, 1991 (6)
Only the value for the rat oral LD_{50} is listed; 50-60 mg/kg bw. No mention of study design, number of animals used, etc.
Acceptability:
Not acceptable

Reference: Ambrose and DeEds, 1946 (27)
The acute toxicity of nicotine was determined orally in 35 rats. The alkaloid was dissolved in distilled water and the pH was adjusted to 7.0 with concentrated hydrochloric acid. There is no further mention of study design, strain or sex of rats used.
In this study nicotine was also intraperitoneally injected into 12 rats.

Results: Oral LD_{50} rat 188 mg/kg bw, LD_{50} after intraperitoneal injection rats 30 mg/kg bw. Convulsions were observed after administration.

Acceptability: Acceptable

Reference: Heubner and Papierkowski, 1938 (28).

Between 36 and 55 white mice (17-26 grams) whose strain was not specified were used to assess the acute oral toxicity of nicotine. Nicotine was administered in aqueous solution by gavage at 5 animals per group. The total number of animals are not mentioned. The doses follow a geometric progression , with a range of 20 %, but the actual dosaging is not described. The LD50 was estimated by the method of Spearman_Kärber (Kärber, 1931).

A comparable study was performed using nicotine tartrate.

Results: Oral LD_{50} mouse nicotine base 24 mg/kg bw. Mortality occurred within 25 minutes.

Oral LD_{50} mouse nicotine salt 87 mg/kg bw equivalent with 28 mg/kg bw based on nicotine fraction.

Acceptability: Acceptable

Reference: Franke and Thomas, 1932 (7)

Nicotine was administered orally to 19 dogs. Nicotine was dropped on the tongue or between the lips and gums in the form of the undiluted alkaloid.

Table 14   Results:

Dose (mg/kg bw)	# of dogs	# fatal	# non-fatal	% fatal	Average time till death (minutes)
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	-

Acceptability:

Limited description but acceptable given the period in which it was performed.

Conclusions:

Oral LD_{50} dogs 9.2 mg/kg bw.

Reference: Yam et al., 1991 (8)

Study design:

Two different methods were used, the fixed-dose procedure and the up-and-down method, which were compared to the classical method of obtaining an LD_{50}. The fixed-dose procedure was conducted according to the method described by van den Heuvel *et al.*, 1990 (9). It involves dosing 10 rats (5 males and 5 females) with one of four predetermined dose levels, selected on the basis of a sighting study (3-4 animals) so that only evident toxicity and no deaths were observed. Depending on the outcome of the first dose, a second dose group was used. As the fixed-dose procedure does not use death as an endpoint, no LD_{50} can be determined.

The up-and-down method was conducted according to the method described by Bruce (1985 and 1987). Female rats were dosed, one at a time, starting the first animal at the best estimate of the

LD_{50}. If the first animal was alive at the end of 24hr, the next animal was given a higher dose. If the first animal died, the next received a lower dose. The dose was either increased or decreased by a factor of 1.3. The dosing options were repeated until 4 animals had been treated after reversal of the initial outcome.

Classical LD_{50} data were generated by another laboratory (van den Heuvel *et al.*, 1990), described below.

Results:

In all 3 methods, nicotine produced the first sign of toxicity within 1 day. The duration of signs of toxicity was 3 days in the classical LD_{50} and fixed-dose study, but 5 days in the up-and-down study. There were no autopsy findings in the classical LD_{50} and up-and-down-method, but in the fixed-dose method lungs appeared red and slightly congested.

Table 15   Rat LD_{50} values

LD50 values (mg/kg bw)		
Classical method		Up-and-down method
Females only	Combined sex	Females only
71 (42-128)	70 (49-109)	70 (51-96)

Acceptability:

Acceptable

Conclusions:

For the fixed-dose procedure, death is not an endpoint and thus an LD_{50} can't be determined. The conclusion based on the results of the classic method was classification as toxic meaning an expected LD50 between 50 and 500 mg/kg bw. The up-and-down method resulted in a similar LD_{50} as when using the classical LD_{50} method (70-71 mg/kg bw).

Reference: van den Heuvel et al., 1990 (9)

Study design:

The classical LD_{50} method is being compared to the fixed-dose procedure (described above). The classical LD_{50} study is performed according to OECD 1981, using 15 male and 15 female rats. For the fixed-dose procedure, nicotine is tested in 26 different laboratories. In total 355 rats are used, half of which male and the other half female. In total, 31 labs are involved, 21 of those used Sprague-Dawley rats, 9 used Wistar and 1 used Fischer 344 rats; this is not further specified. Mortality occurred in both methods within a day. Observed effects were none for the classical method and oedema of the stomach and pale kidney for the fixed dose procedure.

Table 16   Results:

LD50 values (mg/kg bw)		
Classical method, OECD 1981		
Males only	Females only	Combined sex
68 (41-129)	71 (42-128)	70 (49-109)

Table 17

Classification*

Classical LD$_{50}$	Fixed-dose tests - #of labs classifying compound as:			
	Very toxic	**Toxic**	**Harmful**	**Classified**
Toxic	-	23	3	-

*(see van den Heuvel et al., 1990 for criteria) (toxic relates to the DSD criteria meaning an LD50 between 25 and 200 mg/kg bw)

Acceptability:
Acceptable
Conclusions:
Oral LD$_{50}$ rat: 70 mg/kg bw

Reference: Vernot et al., 1977 (10)
Study design:
The single oral LD$_{50}$ of nicotine sulfate in mouse and rat was determined by the method of Smyth et al. 1962 (24), which is not further specified in this paper. The paper from Smyth et al (24) was retrieved for the method use, summarized here: single oral toxicity is estimated by gastric intubation of groups of 5 non-fasted male rats. The dosages are arranged in a logarithmic series differing by a factor of 2. Whenever possible, the chemical is administered undiluted. Based upon mortalities during a 14-day observation period, the most probable LD50 value and its fiducial range are estimated by the method of Thompson (1947) using the Tables of Weil (1952).

Table 18.   Results:

LD$_{50}$ values (mg/kg bw)	
Sprague-Dawley rat, male	**CF-1 mouse, male**
75 (44-127)	16 (12-21)

Acceptability:
Acceptable

Reference: Gaines, 1960 (11)
Study design:
Eighty female rats, at least 90 days old were used. They were not fasted prior to dosage. The survivors were held for daily observation until they appeared to have recovered completely or for a minimum of 14 days. The poisoned rats were observed at least once each hour during the first day after dosage, and twice a day thereafter, for symptoms of poisoning and time of death. The compounds were given orally by means of a stomach tube. Dosing was done with a syringe with 0.1-cc graduations and a blunt-pointed 17-gauge spinal needle which served as the stomach tube. The tube did not actually reach the stomach of the rats, but extended far enough into the esophagus to prevent regurgitation. The poison formulations were given at the rate of 0.005 ml per gram of body weight. Nicotine sulphate was suspended in water-lead arsenate and calcium arsenate (concentrations unknown) at dosage rates as high as 0.00096 ml/g. The LD$_{50}$ values were determined by the method of Litchfield and Wilcoxon (1949).The oral LD$_{50}$ values for lead arsenate and calcium arsenate were determined to be 1050 mg/kg bw and 298 mg/kg bw, respectively.

Table 19   Results:

Acute oral toxicity, female rats

Compound	Survival time		LD 50 mg/kg bw
Nicotine sulphate	Min. (hr)	Max. (days)	
	1	4	83 (75-91)

Acceptability:
Limited description but acceptable given the period in which it was performed.
Conclusion:
Oral LD_{50} rat 83 mg/kg bw

4.2.1.2 Acute toxicity: inhalation

Table 20   Summary of acute inhalation studies

Method	Dilution	LC_{50} (mg/L)	Animal	Remarks	Acceptability	Reference
Long term exposure (4 months)	Liquid nicotine sulfate aerosol	> 0.33 mg/m^3	Not described	Type and # of animals not specified	Not acceptable	Lazutka et al., 1969
Up and down method	Nicotine (freebase), in water or NaCl solution	2.3 (20 minutes)	Rat	Sprague-Dawley, 7 males	Acceptable	Shao et al., 2012
OECD 403, 1981	Tobacco extract with 4.1% nicotine	> 2	Rat	Sprague-Dawley, 6 male, 6 female	Acceptable	Werley et al., 2014

Reference: Lazutka et al., 1969 (4)
Study design:
Only long-term (4-months) exposure to liquid nicotine sulfate aerosol was investigated, in concentrations of 0.33 and 0.2 mg/m^3. These are the maximum and minimum concentrations determined under industrial conditions in the respiration zone of personnel working with nicotine sulphate. Type and number of animals are not specified. No information is available on the duration of the exposure per day and the particle size.

Results:
The animals did not exhibit any visible phenomena after long-term (4-month) exposure at either concentration. Exposure to 0.33 mg/m^3 inhibited the inculcation of the conditioned reflex to bell with alimentary reinforcement, throughout the entire four-month period of poisoning, whereas there was no difference with controls in the lower concentration.
Acceptability:
Unacceptable due to absence of information on many essential parameters.
Conclusion:
Due to the limitations of the reporting no conclusion can be drawn.

Reference: Shao *et al.*, 2012 (12)

Nicotine in water can be in three forms: freebase (Nic), monoprotonated (NicH+), and diprotonated (NicH2 2+). Nic and NicH+ are predominant, with pKa = 8.06 at 20 °C (Pankow, Tavakoli, Luo, & Isabelle, 2003). Therefore, ~50% of nicotine is as Nic at pH 8.0. For inhalation route, the pH of the particles of tobacco smoke or testing aerosol affects nicotine absorption in the lung and its bioavailability (Burch et al., 1993; Pankow et al., 2003).

Study design:

Male Sprague-Dawley rats of 8–11-week-old (body weight 250–400 g) were used in this study, and nicotine used was (s)-(-)-nicotine freebase (liquid, 99%) ordered from Alfa Aesar Co. The rats were housed in the vivarium under a 12-hr light/dark cycle and had *ad libitum* access to food and water. Rats were exposed to nicotine aerosol by inserting rat holders into a nose-only chamber. The MMAD was between 1.69 and 3.55 um with a GSD of 1.8 to 2.48 depending on the nicotine concentration. Nicotine (freebase) was dissolved in water or NaCl solution for an osmolality ~300 mOsm/kg. pH was adjusted with HCl to pH 8.0 except when indicated otherwise.

Nicotine LC50 in rats was examined using the up and down procedure (UDP) recommended by EPA Health Effects Test Guidelines (EPA, 2002). With this method, 6–9 animals could be used to obtain LC50 and its confidence interval (*CI*). Rats were exposed to nicotine aerosol for a fixed time (20 min) and with a fixed air pressure (40 psi) to the nebulizer. To determine the inhalation LC50 of nicotine for rats using the UDP, the nicotine concentrations in the nebulizer solution container were varied. An ordered concentration progression in a range of 5%–56% nicotine was defined. Since the nicotine dose–response curve is quite steep, a concentration progression factor of antilog 0.25 = 1.78 was chosen. pH was 8.0 in the first experiment. Starting with a nicotine concentration of 10% in the nebulizer container, the first rat survived. A concentration of 18% (increase of one progression factor) was used for the next rat. According to the UDP, if the animal survives, the concentration for the next animal is increased by one step. The post exposure observation period was limited to 24 hours.

Results:

Table 21

pH	Nicotine concentration in nebulizer (%)	95% CI	LC$_{50}$ in air (20 min) (mg/L)[a]	95% CI of LC$_{50}$ (mg/L)[a]	n
6.8	>56[b]		>4.1		6
7.4	32	20.4-69.2	2.3	1.46-4.96	7
8.0	32	12.3-56.7	2.3	1.24-4.07	7

Note. *CI* represents confidence interval. Air pressure for generating nicotine aerosol was 40 psi in LC$_{50}$ experiments.

[a]LC$_{50}$ in air and its *CI* are calculated as nicotine concentration in solution × aerosol mass concentration in air.

[b]The value is at least 56%. Only one rat died at 56% nicotine solution during the up and down procedure. No other rats died at 32%, 56%, or 68% in multiple trials.

LC50 values were not significantly different between experimental groups of nicotine solutions at pH 7.4 and at pH 8 (Table 21). Note that the *CI* values of LC50 at pH 8 were slightly lower than those at pH 7.4. However, the LC50 of nicotine solution at pH 6.8 was >4 mg/L (>56% nicotine concentration in the nebulizer). Higher nicotine concentrations could not be used, since pure nicotine freebase is liquid and very alkaline (pH ~10). The amount of HCl required to adjust pH to 6.8 significantly diluted the solution; therefore, 68% was the maximum concentration we could achieve. Although the exact value of LC50 cannot be determined, the (see legend of Table 21) experiment with pH 6.8 suggests that the LC50 at pH 6.8 is much higher than those at pH 7.4 and pH 8. These results suggest that the method of delivering nicotine through aerosol inhalation is very efficient. Exposure to 2.3 mg/L nicotine in air for 20 min causes death in 50% of rats. In addition,

we showed that pH affects nicotine actions. Acidification, but not basification, of the nicotine solution in the nebulizer minimizes the effects of nicotine, probably due to a reduction in nicotine absorption and/or bioavailability in the lungs.

Acceptability:
Acceptable with limitations (20 minutes exposure only)

Conclusion:
The acute inhalation toxicity of nicotine: LC_{50} (20 minutes) = 2.3 mg/L.

Reference: Werley *et al*., 2014 (13)
Acute inhalation exposure effects to increasing concentrations of propylene glycol and glycerol aerosols containing tobacco extract and nicotine in rats was studied. Tobacco extract formulation was composed of the USP grade ingredients in the following proportions: 37.3% glycerol, 28.6% propylene glycol (PG), 19.2% ethanol, 4.1% nicotine, 8.8% water and 2% tobacco essential oils by weight, derived using a patented extraction process. The nicotine formulation was 38.4% glycerol, 28.8% PG, 19.2% ethanol, 4% nicotine and 9.6% water by weight. A nose-only exposure chamber was used. A single capillary tube CAG (capillary aerosol generator) was used to attain the targeted exposure concentrations up to approximately 2 mg/L.

Study design:
The acute inhalation study was conducted in accordance with OECD Guideline for testing of Chemicals (OECD 403, 1981) entitled Acute Inhalation Toxicity. Twelve male and 12 female Sprague Dawley_ rats (Crl:CD(SD)IGS BR) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). They were 7–8 weeks of age and weighed 168–237 g and 135–193 g, males and females, respectively. The rats were acclimated for approximately 2–3 weeks, double-housed in stainless steel hanging cages to determine suitability for use before assignment to the study. Two groups of six rats of each sex were used in the study; each group (Group 1 and Group 2) was exposed to different concentrations of tobacco extract formulation test material for four hours to estimate the LC50 (the inhaled concentration of test material which produces 50% mortality in the test animals). Group 1 was exposed to a target concentration of 2 mg/L and group 2 was exposed to a target concentration of 1 mg/L. Animals were observed for signs of toxicity during exposure and then daily for 14 d post-exposure. Body weights were determined immediately before exposure, and weekly thereafter. At necropsy on Day 14, the rats were euthanized using an overdose of sodium pentobarbital, and all tissues and organs were examined for signs of gross pathology.

Results:
The mean exposure concentrations in the LC50 determinations for Group 1 and Group 2 were 2.13 and 1.00 mg/L, respectively, and corresponding nicotine concentrations were 0.114 and 0.060 mg/L, respectively. Particle size distribution (MMAD and GSD) from the aerosol in Group 1 and Group 2 were 0.40 (2.61) mm and 0.81 (2.72) mm, respectively. One female in Group 1 died on Day 1. The remaining females in this group had hypoactivity, wet and discolored inguinal fur, weight loss, redness around eyes and nose, convulsions, lethargy, hunched posture, severe tremors, reduced body temperature and salivation over Days 1–4. Males in Group 1 had wet inguinal fur, redness around the eyes, slight tremors, reduced body temperature, and salivation, which resolved by Day 2. Males and females in Group 2 had wet inguinal fur, redness around the eyes and nose, and salivation which resolved by Day 2. Necropsy showed no abnormal gross observations except for darkened spleen and mottled lungs in the female from Group 1 that died. All animals, except one female, survived and gained weight during the 14-day recovery period, at which time all animals appeared healthy and active. The LC50 for the inhaled tobacco extract was considered to be greater than 2 mg/L and 1 mg/L was determined as the maximum exposure concentration for repeated inhalation exposure.

Acceptability:
Acceptable with limitations (mixture tested, testing not up to the limit dose)

Conclusions:
The rat LC$_{50}$ for inhaled tobacco extract (containing 4.1% nicotine) is considered to be > 2mg/L corresponding to > 0.114 mg/L for nicotine.

4.2.1.3 Acute toxicity: dermal

Table 22:   Summary table of relevant acute dermal toxicity studies

Method	Dilution	LD$_{50}$ (mg/kg bw)	Animal	Remarks	Acceptability	Reference
Single dose application	Suspension in water-lead arsenate and calcium arsenate	285	Rat	Sherman, 70 females, nicotine sulfate	Acceptable	Gaines, 1960
Single dose application	Not described	140	Rat	Strain, sex and number not specified	Not acceptable	Ben-Dyke *et al.*, 1970
Not specified		140	Rat	Strain, sex and number not specified	Not acceptable	Trochimo wicz *et al.*, 1994*
OECD 402		>360 (no deaths were seen	Rat	Sprague-Dawley, 5 male, 5 female. A mixture of 18% nicotine and 82% of an ion-exchange resin applied at 2 g/kg to the covered skin, followed by rinsing with water	Not acceptable	Guerriero et al., 2001*
Not described, repeated exposure	0.15-0.3% aqueous solutions	-	Rabbit & Rat	Not described, 6 of each, nicotine sulfate	Not acceptable	Lazutka et al., 1969
Single dose application	Not described	50	Rabbit	Strain, sex and number not specified	Not acceptable	FDA, 1952
Not described		50	Rabbit	Strain, sex and number not specified	Not acceptable	Trochimo wicz *et al.*, 1994*
Not described		140	Rabbit	Strain, sex and number not specified	Not acceptable	UK PSD, 2008*
Single dose application	40% aqueous solution	66-100	Cat	21 cat received nicotine base, and 21 cats received nicotine sulfate.	Acceptable	Travell, 1960

*These studies could not be retrieved. The above description was derived from the Bibra proposal, 2014. They will not be further described below.

Reference: Gaines, 1960 (11)
Study design:
Seventy female rats, at least 90 days old, were used. They were not fasted prior to dosage. The survivors were held for daily observation until they appeared to have recovered completely or for a

minimum of 14 days. The poisoned rats were observed at least once each hour during the first day after dosage, and twice a day thereafter, for symptoms of poisoning and time of death. Nicotine was dissolved in water-lead arsenate and calcium arsenate at dosage rates as high as 0.00096 ml/g. It is unclear how the presence of arsenate affected the study but it could only reduce the LD_{50} value.

Table 23   Results:

Acute dermal toxicity, female rats		
Compound	**Survival time**		**LD_{50} mg/kg bw**
Nicotine sulphate	**Min. (hr)**	**Max. (days)**	
	7	5	285 (228-356)

Acceptability:
Limited description but acceptable given the period in which it was performed.
Conclusion:
Dermal LD_{50} rat 285 mg/kg bw for nicotine sulfate.

Reference: Lazutka et al., 1969 (4)
Study design:
The general effect of absorption of nicotine sulphate was studied by application of 1/5 LD_{50} to the skin of 6 rabbits and 6 rats. The experiment lasted 2 months. Further details are not described.
Results:
The experimental animals' behaviour did not differ from that of the controls after the application of nicotine sulphate. There were no local reactions or clinical manifestations of poisoning, with the exception of a lag in weight-growth. The reflex to bell was completely inhibited and was not recovered by the experimental rats throughout the poisoning period. Cutaneous application of nicotine sulphate increased the amount of potassium ions in blood serum by 41% and diminished that in the erythrocytes by 30%; ATP decreased by 80%.
Acceptability:
Unacceptable as no dose levels in mg/kg bw are stated.

Reference: Ben-Dyke et al., 1970 (5)
This paper lists acute toxicity data for a number of pesticides, including nicotine. This data has been prepared from experimental results of the Toxicology Laboratory, Chesterford Park Research Station, or from published literature and manufacturer's bulletins. However, there are no actual references, the study design is not described, nor the number of animals used; only the dermal LD_{50} of 140 mg/kg bw is mentioned.
Acceptability:
Not acceptable

Reference: FDA, 1952 (14)
No access to the original study.
LD_{50} rabbit, dermal: 50 mg/kg bw
Acceptability:
Not acceptable

Reference: Trochimowicz *et al.*, 1994 (15)

No access to the original study (but mentioned in the bibra report and the report of the Health Council of the Netherlands, 2004 (17)).
LD_{50} rat, dermal: 140 mg/kg bw.
Acceptability:
Not acceptable

Reference: Travell, 1960 (16)
Study design:
21 cats received a single dose (200 mg) dermal application of nicotine base, and 21 cats received nicotine sulfate; the concentration of nicotine in each instance was 40% with respect to the base. Solutions were prepared by rapidly weighing the fluid nicotine oil and diluting it to volume with either distilled water or a solution of sulphuric acid to provide a slight excess of acid above the theoretical neutralization equivalent. Application of 0.5 cc. was done after fur was clipped from about a 5x6 cm. area of skin over the groin. Weights of the cats were about 2 to 3 kg, and the percutaneous dose of nicotine was thus about 66 – 100 mg/kg.
Results:
When nicotine base was used, 81% of the animals died between 21 to 195 minutes. When nicotine sulfate was used, none of the animals died. The dermal LD50 of nicotine is probably below 80 mg/kg bw in cats.

Table 24   Results

Acute dermal toxicity, female & male cats				
Compound	**# of cats**	**Incidence of**		
		Nausea (%)	**Vomiting (%)**	**Death (%)**
Nicotine base	21	100	100	81
Nicotine sulfate	21	52	19	0

Acceptability:
Limited description but acceptable given the period in which it was performed.

4.2.1.4 Acute toxicity: other routes

4.2.2   Human information

Nicotine poisoning produces nausea, vomiting, abdominal pain, diarrhea, headaches, sweating, and pallor. More severe poisoning results in dizziness, weakness, and confusion, progressing to convulsions, hypotension, and coma. Death is usually due to paralysis of respiratory muscles and/or central respiratory failure (Health council of the Netherlands (17), Karaconji, (18)).

Dermal exposure can also lead to poisoning. Such exposure has been reported after spilling or applying nicotine-containing insecticides on the skin or clothes and as a consequence of occupational contact with tobacco leaves (Health council of the Netherlands (17), Benowitz, 1987 (19)). Acute intoxication of children has been reported after ingestion of tobacco materials. Ingestions of tobacco are rather common, but deaths as a result are extremely rare, due to early vomiting and first pass metabolism of the nicotine that is absorbed (18 and ref therein).

Reviews of nicotine contain estimates of the lethal dose in human mostly in the range of 30-60 mg/person indicating a dose in the range of 1 mg/kg bw. However, most reviews refer to secondary

literature which does not contain actual case descriptions. A review of the available human data and a search to the origin of the value of 60 mg/person as performed by Mayer (20) shows that this value cannot be scientifically justified. Mayer estimates a lower limit value for fatal outcomes of 6.5 – 13 mg/kg bw. The often stated value of 1 mg/kg bw in humans is not reliable and cannot be used without additional justification.

In 1991, a report was published of a fatal nicotine ingestion. A 17-year-old male smoker had ingested an unknown amount of liquid nicotine base. The container was later assayed to contain 870 mg/ml of nicotine. Serum nicotine levels were shown to be 13,600 ng/ml, and he died 64 hours post ingestion. As such, this person was estimated to have ingested in excess of 5000 mg (or 71 mg/kg bw). (21). A more recent report describes a non-fatal nicotine poisoning of a 27-year-old man after ingestion of potentially 420 mg (i.e. 6 mg/kg bw) (22). Finally, another paper reports that nicotine exposure through e-cigarettes is increasing. They report 35 cases – 4 in 2010, 12 in 2011 and 19 in 2012. Age range 8 months to 60 years. Reported symptoms were mild and transient. Product concentrations ranged from 4 to 30 mg of nicotine per ml (23).

As summarised by EFSA (2):

A report (Woolf et al., 1997) on a postmarketing surveillance study over a 24-month period, involving 34 United States poison centres, was published in 1997. Patients were represented by 36 children aged 0 to 15 years (mean: 3 years) exposed to a Transdermal Nicotine Patch (TNP). Eighteen exposures were dermal; 18 additional children had bitten, chewed, or swallowed part of a patch. Exposures were unintentional and transient (<20 minutes duration). Twenty-two children (64%) suffered no toxic effects from the TNP exposure: 13 of the 18 children (72%) with oral exposures and 9 of the 18 (50%) with dermal exposures remained asymptomatic. The 5 children who became symptomatic after an oral exposure to a TNP had only transient and local signs of toxicity; children with dermal exposures more often had systemic complaints. Seven of the nine children who were symptomatic after a dermal TNP exposure had nausea and/or vomiting. Five of the nine children were triaged to the emergency department and two were admitted. Fourteen children (39%) developed symptoms, including gastrointestinal distress (nausea, vomiting, diarrhea, abdominal pain), weakness, dizziness, or localized rashes. Occurrence of symptoms after a dermal exposure of children to a TNP was associated with an estimated nicotine dose of 100 μg (10 μg/kg b.w.). All children recovered fully (Woolf et al, 1997).

Lindgren et al. (1999) investigated the dose-response relationship for electroencephalographic parameters (EEG) and heart rate frequency over a wide range of intravenously infused nicotine doses in human volunteers. Fourteen regular smokers who had abstained from nicotine for at least 12 h were given intravenous infusions of 0, 3.5, 7, 14 and 28 μg/kg b.w. nicotine over 10 min in a single-blind randomised cross-over design and they were monitored for 120 minutes. Findings showed linear dose-related changes in EEG measures indicative of arousal, i.e., decrease in EEG delta and theta power, and increase in the alpha2 power, at all doses tested, markedly at 14 and 28 μg/kg b.w. Nicotine infusion caused heart rate acceleration (ranging from 8% to 20% of the baseline), with a highly significant linear trend contrast. The nicotine X time interaction was significant, with pronounced heart rate acceleration after infusion of the 14 and 28 μg/kg nicotine dose. Heart rate frequency returned back to a level comparable to the baseline within 2 hours from the end of the intravenous infusion. It is noted that changes in the heart rate frequency in the order of up to 50% of the baseline heart frequency are considered in a light physical exercise.
In a semi-blinded, within-subject, crossover study with inhaled nicotine, Benowitz et al. (2006a) examined plasma nicotine and cardiovascular responses in 12 healthy smokers receiving cigarettes with 5 graded nicotine contents (between 0.6 and 10.1 mg/cigarette). Non-abstinent smokers were asked to smoke on five subsequent occasions a research cigarette, each with a different nicotine

content. Systemic nicotine exposure (0.26-1.47 mg per cigarette) varied linearly with the nicotine content of the cigarette (average intake of 13- 43% of the cigarette's nicotine content). Cigarette smoking increased heart rate and decreased skin temperature, but the nicotine dose-response curve showed a flattening at higher doses, with a maximal response being observed from 8 mg of nicotine per cigarette. An increase in the heart rate was observed after a systemic dose of approximately 0.004 mg/kg b.w. equal to 0.26 mg in a 60 kg b.w. person (BfR, 2009). The effects on the blood pressure were not significant. The flat nicotine dose–cardiovascular response curve may be consistent with the tolerance of smokers to the cardiovascular effects of nicotine. In non-smokers stronger effects would possibly be observed (Benowitz et al., 2006a).

4.2.3 Summary and discussion of acute toxicity

Overall, no reliable human data are available.

For acute oral toxicity, the quality of the available studies and their reports vary significantly. Of all the available oral studies only the rat studies by Van den Heuvel et al (1990) and Yam et al (1991) would probably fulfil the OECD TG requirements although the reporting is incomplete. All the acute oral rat studies show a comparable range of LD50 values between 50 and 70 mg/kg bw/day with one exception. However, the available acute oral data for mouse and dog show a much lower LD50 value for nicotine. This is specifically shown in the study by Lazutka (1969) which used both rats and mice within the same experimental conditions. Although the quality of the reporting of these studies is limited some studies are considered acceptable seen the period (pre OECD and GLP) in which they were performed and seen the absence of more recent data from the same species. For these species the LD50 values are dog: 9.2 mg/kg and mouse: 3.34 and 24 mg/kg, respectively. The species differences may be due to toxicokinetic and or toxicodynamic differences. Limited information is available on toxicodynamic differences but some information is available for species differences in toxicokinetics. The metabolism of nicotine is complex and differs between species. The available information indicates that the rat may be less relevant to humans due to differences in the main type of P450 responsible for metabolism between rats and humans. The differences between the different tests in different species may also be caused by the method of oral administration. The gavage studies in the rat resulting in uptake via the gastro-intestinal tract resulted in lethalities after at least 50 minutes (Lazutka, 1969) whereas the studies by Franke and Thomas in dogs (1932) using drops into the mouth resulted in lethalities within a few minutes. This is probably caused by direct uptake via the gums. This route is not possible when animals are exposed via gavage treatment. However, this route is considered relevant for human exposure to nicotine. Also, an estimate of the minimal lethal dose in humans seems to be in the range of 6.5 – 13 mg/kg bw/day (Mayer, 2014). Therefore, the oral LD50 values in the rat using gavage exposure seem to be less relevant to humans and may underestimate the human toxicity. The acceptable studies in other species than the rat are limited to mouse and dogs. As it is unknown which of these two species is more relevant to humans, it is suggested to take the lowest value in the most sensitive species in line with the CLP guidance. Therefore, it is proposed to use the acute oral LD50 in the mouse of 3.3 mg/kg bw as determined by Lazutka et al (1969) as the key study. Although this is also a gavage study, the LD50 after uptake via the gums is expected to be even lower. The value of this study is increased by the fact that in the same study rats were tested and showed an LD50 value in line with most other LD50 values in the rat. It is also proposed to assign this value of 3.3 mg/kg bw as the best ATE for calculation of the ATE of mixtures containing nicotine.

For acute dermal toxicity, also most studies are old and the reports are limited. The only acceptable study was in rats and performed using nicotine sulfate and in cats using both nicotine and nicotine sulfate. It shows a higher LD50 than the study in rabbits. For dermal toxicity, the study with nicotine sulphate is considered less relevant because dermal transport over the skin strongly

depends on the presence of nicotine as a neutral molecule as in nicotine or as an ion as in nicotine sulphate. The transport of neutral molecules over the skin is much better than for ions. This is confirmed in the cat study which at equal dose of approximately 80 mg/kg bw showed no mortalities for the sulfate and 81% mortalities for the base. The currently applied classification as Acute dermal 1; H310 was based on the acute dermal LD50 of 50 mg/kg bw in rabbits (FDA, 1952). However, this study is not acceptable according to the current requirements. However, the current classification is supported by the results of the cat study with nicotine which showed a dermal LD50 below approximately 80 mg/kg bw.

There are two acceptable acute inhalation studies available in which nicotine was tested as an aerosol. However, both have limitations. In the first study using the up and down method, the exposure duration was limited to 20 minutes and the post exposure observation period to 24 hours. The observed LC50 (20 minutes) was approximately 2.3 mg/L. In the second study a specified mixture was tested for four hours up to a limited concentration corresponding to 0.114 mg nicotine/L. A single lethality was observed and severe transient clinical effects. Overall this indicates that the tested concentration was close to the LC50 of nicotine.

4.2.4 Comparison with criteria

oral

Lazutka et al (1969) is selected as the key study, this study determined an acute oral LD50 in the mouse of 3.3 mg/kg bw. An acute oral LD50 of 3.34 mg/kg bw fulfils the requirement for classification in category 1 (LD50 below 5 mg/kg bw). An LD50 value of 3.3 mg/kg bw is suggested as ATE.

dermal

The current harmonised classification with Acute Tox. 1 is based on a dermal study in rabbits with an LD50 value of 50 mg/kg bw which is only available to us as a reference. As such this study would not be acceptable to propose a new harmonised classification. However, there are no acceptable acute dermal studies in rabbits or rats using nicotine and the only available acceptable study in cats indicates also that the dermal LD50 is in the range of 50 mg/kg bw as 81% mortality was observed at a dose of approximately 80 mg/kg bw. An dermal LD50 value of 50 mg/kg bw or lower warrants classification in category 1. Therefore, it is considered justified to keep the current classification as Acute Tox. 1 H310. The proposed ATE value is 50 mg/kg bw.

inhalation

The available acute inhalation data do not allow determination of an LC50 value. Based on the available data it can be estimated that the 4-hour LC50 is between 0.1 and 2.3 mg/L as an aerosol. According to the CLP criteria (footnote C to table 3.1.1), conversion of a one hour exposure to dusts and mists to a four hour exposure should be done by dividing with a factor of 4. At least this factor should be applied when extrapolating from 20 minutes to 4 hours. The use of a factor of 4 results in a LC50 value of 0.58 mg/L but probably even lower. Also the effects observed at 0.1 mg/L indicate that this exposure level is close to the LC50. Therefore, classification in category 2 (LC50 between 0.05 and 0.5 mg/L) seems justified. An LC50 value of 0.25 mg/L is suggested as ATE as this is in the middle between 0.1 and 0.5 mg/L.

4.2.5 Conclusions on classification and labelling

According to Regulation (EC) No. 1272/2008 on Classification, Labelling and Packaging, nicotine should be classified as Acute Tox. 1, H300, thereby replacing the current classification of Acute Tox. 3, H301. It is proposed to assign an ATE of 3.3 mg/kg bw for acute oral toxicity.

The available data do not warrant a change in the current classification for acute dermal toxicity (Acute Tox. 1, H310). It is proposed to assign an ATE of 50 mg/kg bw for acute dermal toxicity.

According to Regulation (EC) No. 1272/2008 on Classification, Labelling and Packaging, nicotine should be classified as Acute Tox. 2, H330. It is proposed to assign an ATE of 0.25 mg/L for acute inhalation toxicity.

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_{50} of 3.34 mg/kg (Lazutka *et al*., 1969) out of various available LD_{50} values from different species and strains. The lowest LD_{50} of 3.34 mg/kg is below the limit value of $\leqslant$ 5mg/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_{50} in the range of doses between 0.1 and 0.5 mg/L (aerosol). According to the DS, the LC_{50} is thus within the limits of 0.05 mg/L - $\leqslant$ 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_{50} 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_{50} 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_{50} 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_{50} 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 justificationfor 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_{50} 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_{50} values or estimates of the oral LD_{50} for rats, mice or dogs based on data from studies or from other reference sources (see tables 11 and 12 of the Backgound 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_{50} values is provided below per experimental species.

Studies conducted in rats
LD_{50} 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_{50} 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_{50} 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_{50} values (respectively 50-60 and 188 mg/kg) were derived from a report prepared by Bibra, (2014).

LD_{50} 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_{50} of nicotine in rats ranges from 52.5 to 70 mg/kg, while the LD_{50} 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_{50} 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_{50} values of nicotine in mice obtained in this study was 3.34 mg/kg and oral LD_{50} values of nicotine in rats was 52.5 mg/kg (Lazutka *et al.*, 1969). A LD_{50} of nicotine for rabbits was not determined in this study. The oral LD_{50} 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 (3rd 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:

Sequential number of animal	Dose (mg/kg bw)	Lethality/Survival 48 hrs after dosing
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:

Dose (mg/kg bw)	Number of female mice	Number of dead animals and survival time after treament	Clinical observations
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_{50} 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_{50} 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 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 administred 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.

Dose (mg/kg bw	No. of dogs	Number of deaths	Number of surviving dogs	% mortality	Average time till death (minutes)
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_{50} 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_{50}'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_{50} in the range of 52.5 mg/kg to 70 mg/kg.

Differences in LD_{50} 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_{50} 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_{50} (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_{50} 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_{50} of nicotine in mice (3.34 mg/kg and 24 mg/kg) and LD_{50} of nicotine in dogs (9.2 mg/kg) as the most sensitive speciesboth 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 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_{50} 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_{50} for the inhaled tobacco extract was considered to be greater than 2 mg/L. The study results suggest that the LC_{50} of nicotine itself is above 0.114 mg/L.

In the acute inhalation toxicity study of Shao *et al*. (2012), the LC_{50} 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_{50} 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_{50} (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 ($C^n.t = k$) 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_{50} (4 hours) of nicotine from Shao *et al*. (2012) is 0.19 mg/L as follows:

$$C^n \times t = \text{constant}$$

$$C \times 240 \text{ min.} = 2.3 \text{ mg/L} \times 20 \text{ min.}$$

$$C = \frac{2.3 \frac{mg}{L} \times 933 \text{ mg/}}{240 \text{ min.}} = 0.19 \text{ mg/L}$$

RAC considers that 20 minutes exposure is not substantially different from 30 minutes exposure and conversion can be made to 4 hour LC_{50} value.

Discussion and conclusion on acute inhalation toxicity

Overall, taking into account that the LC_{50} (4 hours) of nicotine in rats is 0.19 mg/L which is within the range 0.05 mg/L- $\leqslant$ 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_{50} 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_{50} value. The LD_{50} 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_{50} 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:

Dose (mg/kg bw)	Number of male/female rabbits	Number of dead animals and survival time after treament	Clinical observations
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	30 min. to 5 hours post treatment: CNS symptoms (decreased activity,

		after treatment	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_{50} 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.

Acute dermal toxicity, female and male cats				
Compound	**Number of cats**	**Incidence of**		
		Nausea (%)	**Vomiting (%)**	**Death (%)**
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_{50} of nicotine was in the range of 66 – 80 mg/kg, while the dermal LD_{50} 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_{50} value is not possible. The dose of 66 mg/kg is taken as LD_{50} 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_{50} of nicotine for rabbits in this study equals 70.4

> mg/kg bw, which is within the range of 50 mg/kg - $\leqslant$ 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 mk/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_{50} by the dermal route is derived from a single reliable study.

4.3   Specific target organ toxicity – single exposure (STOT SE)

Not assessed in this dossier.

4.4   Irritation

Not assessed in this dossier.

4.5   Corrosivity

Not assessed in this dossier.

4.6   Sensitisation

Not ass**essed in this dossier.**

4.7   Repeated dose toxicity

Not assessed in this dossier.

4.8   Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

Not assessed in this dossier.

4.9   Germ cell mutagenicity (Mutagenicity)

Not assessed in this dossier.

4.10   Carcinogenicity

Not assessed in this dossier.

4.11   Toxicity for reproduction

Not assessed in this dossier.

4.12 Other effects

Not assessed in this dossier.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not assessed in this dossier.

6 OTHER INFORMATION

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