COLLECTIVE EXPERT APPRAISAL:
SUMMARY AND CONCLUSIONS

Related to the establishment of chronic Toxicity Reference Values (TRVs) by oral route based on the carcinogenic and non-carcinogenic effects of 1-chloro-2-nitrobenzene (ortho-chloronitrobenzene) (CAS No. 88-73-3)

AFSSET Solicited Request No. 070057

Only the French language version of this document shall prevail.

Overview of the question

On 12 November 2007 the Directorate General for Health (DGS) requested that AFSSET establish toxicity reference values (TRVs) for ortho-, meta- and para- isomers of chloronitrobenzene (CNB), following the contamination of groundwater in Alsace, north of Mulhouse, by organic products containing chloronitrobenzene coming from two industrial sites.

This document describes the process for establisheing the TRV and the results obtained for one of the isomers, 1-chloro-2-nitrobenzene.

Organisation of the expert appraisal

A preliminary review of this request was conducted by the CES for "Assessment of risks linked to chemical agents" at its meeting on 24 January 2008 and by the Working Group (WG) for "Cancer TRVs" on 31 January 2008. At the end of this review, the CES confirmed the importance of prioritising establishment of TRVs for the ortho- and para- isomers of CNB by oral route; the relevance of deriving TRVs for the other routes had to be evaluated.

Three expert members of the WG for "Carcinogenic TRVs" and two expert members of the CES for "Assessment of risks linked to chemical agents" were appointed as *rapporteurs,* on 22 February 2008 and 26 March 2009 respectively, to review the mechanism of carcinogenic action of CNB isomers so as to determine the nature of the dose-effect relationship (threshold dose or no threshold dose).
AFSSET conducted a detailed analysis of the literature currently available on CNBs and sent it to the *rapporteurs*.

This work was presented for comments to the Working Group for "Toxicity Reference Values", on several occasions: 19 December 2008, 13 February 2009 and 10 April 2009.

The available toxicity data were presented to the CES for "Chemistry" on 26 February 2009 and were the subject of questions and requests for clarification. Based on these observations, a meeting took place on 10 April 2009 with Afsset and the *rapporteurs* to exchange ideas.
The expert appraisal work was submitted to the CES on 20 March 2009 for comments and on 28 May 2009 for adoption.

This expert appraisal was therefore done by a group of experts with complementary expertise. It was carried out in accordance with the French Standard NF X 50-110 "Quality in Expertise

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Activities - General Requirements of Competence for Expert Appraisals" to ensure compliance with the following points: competence, independence, transparency and traceability.

Description of the working method for establishing a TRV

The development of TRVs differs depending on the assumption made or data acquired on the substance's mechanisms of toxic action.

In 2007 AFSSET published a methodology report[1] describing the process for establishing TRVs based on reprotoxic effects, which can be considered as a model of threshold effects. The method of establishing TRVs for substances that have no-threshold dose effects is being established by a working group dedicated to carcinogenic effects. This method is detailed in the "*Document de référence pour la construction d'une VTR fondée sur des effets cancérogènes*" [Reference document for the development of a TRV based on carcinogenic effects]. A preliminary version of this document served as a guide for the establishment of the TRVs for CNB.

In the case of a no-threshold TRV, the effect may arise irrespective of the dose received, and the probability of occurrence increases with the dose. The TRV is then expressed as an ERU (excess unit risk), and is defined as the additional probability, compared to an unexposed subject, that an individual will develop a disease (in this instance, cancer), if he is exposed over his entire lifetime to a unit dose of the substance.

In the case of a threshold TRV, the assumption for establishing a TRV based on given effects follows a relationship to the threshold dose. The effect is likely to appear only after a certain received dose, which corresponds to the TRV.

In practice, establishment of the TRV involves the following four steps:

- choice of the critical effect;
- choice of a good quality scientific study enabling establishment of a dose-response (or dose-effect) relationship;
- choice or establishment of a critical dose from experimental doses and/or epidemiological data;
- application of uncertainty factors to the critical dose to account for uncertainties (in the case of a threshold TRV) or extrapolation to the origin (in the case of a no-threshold TRV).

Results of the collective expert appraisal

Summary **of toxicity data**

- General toxicity

1-chloro-2-nitrobenzene causes hepatic and haematological toxicity during sub-chronic exposure by oral and respiratory routes, in rats and mice (Matsumoto *et al.* 2006a; NTP 1993; Matsumoto *et al.* 2006b). The following histological lesions were observed: liver necrosis and degeneration in rats, and atypical hepatocytes with an enlarged nucleus in mice. In addition, from a biochemical standpoint, the enzymatic activity of alanine aminotransferase (ALT) was increased. This increase is regarded as an indicator of cytolysis of hepatocytes, which causes a release of hepatic transaminases into the blood.

[1] Reference document for the establishment of a TRV based on reprotoxic effects
http://www.afsset.fr/upload/bibliotheque/210401124823782117964751534907/VTR_rapport_afsset.pdf

Furthermore, 1-chloro-2-nitrobenzene is haematotoxic. The changes observed were haematological, biochemical and anatomopathological, i.e. animals presented with anaemia, higher bilirubin counts, and methaemoglobinaemia; the spleen was congested and had haemosiderin deposition; and lastly, splenic extramedullary haematopoiesis was observed, indicating a compensatory phenomenon in response to the anaemia (Matsumoto *et al.* 2006a; Matsumoto *et al.* 2006b; NTP 1993).

It is also worth noting that most of the rats treated with 1-chloro-2-nitrobenzene – including female rats – were suffering from renal disorders and had chronic progressive nephropathy, in particular (Matsumoto *et al.* 2006b).

- Genotoxicity

The results of various genotoxicity tests conducted *in vitro* and *in vivo* show that 1-chloro-2-nitrobenzene is a genotoxic substance. This genotoxic potential seems rather low, however. In particular, during an Ames test (TA 98, TA 1538, WP*2uvrA*), only high concentrations (but not bacteriotoxic) in 1-chloro-2-nitrobenzene induced genotoxic effects (IARC 1996; Organisation for Economic Cooperation and Development [OECD] 2001). Furthermore, 1-chloro-2-nitrobenzene led to the formation of haemoglobin adducts in both rats and humans (Jones *et al.* 2006; Sabbioni 1994), providing additional evidence of its genotoxic potential.

- Carcinogenicity

In 1996 the International Agency for Research on Cancer (IARC) classified 1-chloro-2-nitrobenzene in Category 3 (non classifiable as to its carcinogenicity to humans). The European Union has not classified 1-chloro-2-nitrobenzene. Since 1996, new sub-chronic, chronic and carcinogenicity studies have been conducted, clearly showing evidence of the carcinogenic effects of 1-chloro-2-nitrobenzene (Matsumoto *et al.* 2006b). According to the experts, these data would be likely to change the classification of this compound.

Oral exposure to 1-chloro-2-nitrobenzene is responsible for the appearance of liver tumours, such as hepatocellular adenomas and carcinomas in male and female rats and mice, and hepatoblastomas in male and female mice. In mice, metastases occur mainly in the lungs (Matsumoto *et al.* 2006b).

An earlier study (Weisburger *et al.* 1978) had shown an increased frequency of liver cancers in male and female mice and an increased frequency of several cancers in rats after oral exposure to 1-chloro-2-nitrobenzene. However, the results of this study should be interpreted with caution because of its inadequate methodology (short duration of exposure, high 1-chloro-2-nitrobenzene dosages, cursory description of the method itself, limited histopathology data, etc.) (IARC 1996; OECD 2001).

Analysis and assessment of the choices for establishment **of the TRV**

Pivotal study

One study on carcinogenicity meets the scientific criteria defined by AFSSET in the method for establishing TRVs and enables a TRV to be derived. This was research conducted by Matsumoto *et al.* (2006b) in a two-year study of carcinogenicity and chronic toxicity in F344 rats and BDF_1 mice, which was given a Klimisch rating of 1a. In this study, groups of 50 animals of the same sex were exposed to various doses of 1-chloro-2-nitrobenzene added to their diet, 7 days a week for two years (three doses were tested in addition to the control).

In particular, this study shows that 1-chloro-2-nitrobenzene is a liver carcinogen that is responsible for the formation of hepatocellular adenomas and carcinomas in both male and female rats and mice. Hepatoblastomas were also observed in mice (aged mice only).

The carcinogenic potential is much more pronounced in mice than in rats (as evidenced by a higher incidence of liver tumours and development of metastases, especially pulmonary metastases). Rats were more sensitive than mice to nephrotoxicity (e.g. elevated creatinaemia and uraemia), resulting in the death of all the male rats at the highest dose.

Finally, biochemical disturbances were observed, particularly an increase in liver enzymes (ALT, AST, γ-GT and LDH) with a significant increase from 54 $mg.kg^{-1}.day^{-1}$ in male mice, and from 69 $mg.kg^{-1}.d^{-1}$ in female mice.

Establishment of a TRV for carcinogenic effects

Mechanism of carcinogenic action of 1-chloro-2-nitrobenzene

It is assumed that the genotoxic mechanism of 1-chloro-2-nitrobenzene in the appearance of liver tumours works through the production of reactive and mutagenic metabolites, including 2-chloroaniline (Matsumoto *et al.* 2006b).

Choice of the critical effect

Oral exposure to 1-chloro-2-nitrobenzene induces the formation of liver tumours: hepatocellular adenomas and carcinomas in rats and mice and hepatoblastomas in mice only.

Because of the significance of the dose-response relationship and the biological relevance of taking both types of tumours into account, the combined incidences of hepatoblastoma and hepatocellular carcinoma tumours in female mice was chosen as the critical effect.

Incidences of tumours from exposure to 1-chloro-2-nitrobenzene in BDF_1 female mice

	Daily exposure doses ($mg.kg^{-1}.d^{-1}$)			
	0*	14*	69*	396*
Hepatocellular carcinomas	0	3	14	48
Hepatoblastomas	0	0	9	28
Hepatocellular carcinomas and hepatoblastomas (combined)**	0	3	20	48

*50 animals per dose group

**Number of animals with at least one tumour

Calculation of the TRV

In the case of establishing a no-threshold dose TRV, the choice of a 'Point of departure' (POD) is determined by modelling the experimental data corresponding to a benchmark dose (BMD). Thus, the data were adjusted according to the models developed by the Dutch National Institute for Public Health and the Environment (RIVM) (PROAST software version 18.2) for dichotomous data (gamma, logistic, multi-stage, probit, and Weibull models).

The model chosen was the one best suited to the data by the method of maximum likelihood ($p > 0.1$). Ultimately, the gamma model was chosen for estimating the lower limit of the confidence interval at 95% of a dose corresponding to a 10% increase in response compared to the unexposed group ($BMD_{10}L_{95}$).

An allometric adjustment was then made according to the recommendations of the United States Environmental Protection Agency (US EPA 2006):

$$\text{Human dose equivalent} = \text{A}nimal\ dose \times \left(\frac{\text{Animal weight}}{\text{Human weight}}\right)^{1/4}$$

The average weight of a mouse was estimated at 30g, and that of a human at 70kg. The doses are expressed in $mg.kg^{-1}.d^{-1}$.

Critical dose in mice: $BMD_{10}L_{95} = 11.7\ mg.kg^{-1}.d^{-1}$
Adjusted dose in humans: $BMD_{10}L_{95} = 1.7\ mg.kg^{-1}.d^{-1}$

The linear extrapolation performed subsequently consisted of a straight line plotted from the POD to the origin representing the risk (slope) of developing cancer according to the dose.

After linear extrapolation to low doses:

$TRV = 6.10^{-5}\ (\mu g.kg^{-1}.d^{-1})^{-1}$

Thus, an excess risk of 10^{-6} for developing cancer would correspond to an exposure by oral route of 0.017 $\mu g.kg^{-1}.d^{-1}$.

Establishment of a chronic TRV for non-carcinogenic effects

Mechanism of hepatotoxic action of 1-chloro-2-nitrobenzene

1-chloro-2-nitrobenzene – and/or its metabolites – has a cytotoxic action on hepatocytes, leading to cell lysis with a release of liver enzymes in the blood. Hepatic cytotoxicity is mainly exhibited by increased hepatic enzyme levels (ALT, AST, γ-GT).

Choice of the critical effect

Use of the NOAEL as the point of departure has been proposed for elevated ALTs, which are the enzymes most specific to liver damage. The benchmark dose was not calculated because the experimental data could not be modelled.

ALT elevation in female mice

DED ($mg.kg^{-1}.d^{-1}$)	0	14	69	396
Number of animals examined	29	34	26	4
ALT (IU/L) (average + standard deviation)	36 ± 27	51 ± 62	480 ± 816*	2115 ± 779*

DED: Daily Exposure Dose, * significance p<0.01 (Dunnett test)

Calculation of the TRV

To be consistent with the no-threshold TRV calculated in female mice, the threshold dose TRV calculation was made only for female mice.

An allometric adjustment was applied to the selected NOAEL as recommended by the EPA (US EPA 2006).

The following uncertainty factors were applied:

- UF_A of 2.5 for inter-species variability (toxicodynamic variability and residual uncertainties (AFSSET 2007),
- UF_H of 10 for inter-individual variability (AFSSET 2007).

The TRV was calculated as follows:

$$TRV = \frac{NOAEL}{UFA \times UFH}$$ for hepatotoxic effects

Where UF_A = 2.5; UF_H = 10; adjusted NOAEL = 2 $mg.kg^{-1}.d^{-1}$

therefore, an oral TRV based on hepatotoxic effects = 80 $\mu g.kg^{-1}.d^{-1}$

Conclusions and recommendations of the collective expert appraisal

The study by Matsumoto *et al.* (2006b) showed that exposure to 1-chloro-2-nitrobenzene by ingestion was responsible for the growth of liver tumours in rats and mice. The mechanism underlying these liver tumours may be based on a mechanism of genotoxic action.

Similarly, 1-chloro-2-nitrobenzene has a hepatotoxic action, with cytolysis of hepatocytes indicated by an increase in hepatic enzymes and ALT in particular.

Two oral TRVs were Ved for the effects observed in animals and mechanisms of action:

- A no-threshold TRV based on hepatic carcinogenic effects (genotoxic mechanism of action),
- A chronic threshold TRV based on hepatotoxic effects.

The confidence level of the no-threshold TRV for 1-chloro-2-nitrobenzene was considered low due to uncertainties about the mechanism of carcinogenic action.

Type of TRV	Critical effect	Critical dose	UF**	TRV
Threshold, oral route	Hepatotoxicity in female BDF1 rats Matsumoto *et al.* (2006)[1]	NOAEL = 14 $mg.kg^{-1}.d^{-1}$ $NOAEL_{ADJ}$* = 2 $mg.kg^{-1}.d^{-1}$	25 UF_A = 2.5 UF_H = 10	**TRV = 80 $\mu g.kg^{-1}.d^{-1}$**
No-threshold, oral route	Hepatocellular carcinomas and hepatoblastomas in female BDF1 mice Matsumoto *et al.* (2006)[1]	$BMD_{10}L_{95}$ = 11.7 $mg.kg^{-1}.d^{-1}$ $BMD_{10}L_{95ADJ}$* = 1.7 $mg.kg^{-1}.d^{-1}$	-	After linear extrapolation to the origin: **TRV = 6.10^{-5} ($\mu g.kg^{-1}.d^{-1}$)$^{-1}$** 0.017 $\mu g.kg^{-1}.d^{-1}$ for a risk of 10^{-6} 0.17 $\mu g.kg^{-1}.d^{-1}$ for a risk of 10^{-5} 1.7 $\mu g.kg^{-1}.d^{-1}$ for a risk of 10^{-4}

*$NOAEL_{ADJ}$ = NOAEL adjusted in humans, $BMD_{10}L_{95ADJ}$ = $BMD_{10}L_{95}$ adjusted in humans
**UF = uncertainty factors. UF: overall uncertainty factor (applied), UF_A: inter-species variability, UF_H: inter-individual variability

The Expert Committee (CES) for "Assessment of risks linked to chemical agents" accepted the results of the collective expert appraisal at its meeting on 23 April 2009 and informed the Directorate General of AFSSET.

Maisons-Alfort, 28 May 2009

On behalf of the Expert Committee (CES) for
"Assessment of risks linked to chemical agents",

Chairman of the CES

M. Michel Guerbet

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