Danish Ministry of the Environment
Environmental Protection Agency

Evaluation of health hazards by exposure to

d-Limonene

and proposal of a health-based quality criterion for ambient air

Environmental Project No. 1496, 2013

Title:

Evaluation of health hazards by exposure to d-Limonene and proposal of a health-based quality criterion for ambient air

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Published by:

The Danish Environmental Protection Agency
Strandgade 29
1401 Copenhagen K
Denmark
www.mst.dk/english

Year:

2013

ISBN no.

978-87-93026-33-9

Content

Preface

The Danish Environmental Protection Agency has requested an evaluation of health hazards by exposure to *d*-limonene and proposal of a health based quality criterion for ambient air. This resulted in 2006 in the present report, which was prepared by Elsa Nielsen, Ole Ladefoged and Inge Søborg, Department of Toxicology and Risk Assessment, Danish Institute for Food and Veterinary Research, i.e. the present Division of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark.

The report has been subjected to review and discussion and has been endorsed by a steering committee consisting of representatives from the following Danish authorities:

The Danish Working Environment Authority,
The Danish Ministry of Food, Agriculture and Fisheries (The Faculty of Agricultural Sciences),
The Danish Veterinary and Food Administration,
The National Board of Health, Denmark,
The Danish Environmental Protection Agency

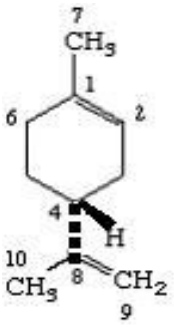
The Danish Environmental Protection Agency
Copenhagen, September 2013.

1 General description

1.1 Identity

Molecular formula: $C_{10}H_{16}$

Structural formula:



Molecular weight: 136.23

CAS-no.: 5989-27-5

Synonyms: Carvene;
Cyclohexene, 4-isopropenyl-1-methyl-;
(+)-4-Isopropenyl-1-methylcyclohexene;
d-Limonene;
D-(+)-Limonene;
(+)-R-Limonene;
d-p-Mentha-1,8-diene;
p-Mentha-1,8-diene, (R)-(+);
(R)-1-Methyl-4-(1-methyl-ethenyl)cyclohexene

1.2 Physical / chemical properties

Description: Colourless liquid with characteristic citrus odour.

Purity: *d*-Limonene is available commercially in technical grade (purity 95%) as a clear liquid, which is variably colourless to yellow cast with a strong citrus odour; as a food grade (purity 97%), a clear water-white liquid with a mild orange odour; and as a lemon-lime grade (purity 70%), a clear water-white liquid with a lemon-lime odour.

Melting point: –74.35°C or –75°C

Boiling point: 178°C (760 mmHg)

Density: 0.8411 g/ml (at 20°C)
0.8402 g/ml (at 25°C)

Vapour pressure:	1.43 mmHg (190 Pa) (at 20°C)
Concentration of saturated vapours:	1875 ppm (10600 mg/m^3) (at 20°C and 760 mmHg)
Vapour density:	4.7 (air = 1)
Conversion factor:	1 ppm = 5.66 mg/m^3 (at 20°C and 760 mmHg) 1 mg/m^3 = 0.177 ppm
Explosion limits:	-
Flash point:	48°C
Flammable limits:	0.7 – 6.1 %
Autoignition temp.:	-
Solubility:	Slightly soluble in water (13.8 mg/l at 25°C) (CICAD 1998); insoluble or practically insoluble in water (HSDB 2001). Soluble in acetone, dimethyl sulfoxide and ethanol. (HSDB 2001).
$logP_{octanol/water}$:	4.2
Henry's constant:	0.380 (atm x m^3)/mol = 50.7 Pa m^3/mol (at 25°C) (calculated). (HSDB 2001/01) 34.8 kPa m^3/mol (at 25°C) (calculated). (CICAD 1998)
pK_a-value:	-
Stability:	When heated to decomposition it emits acrid smokes and fumes.
Incompatibilities:	*d*-Limonene is easily oxidised by the oxygen of the air to form various oxidation products (carvone, limonene oxide, carveol and limonene hydroperoxides). Autooxidation gives the liquid a yellowish colour. If the oxidation process is continued polymers may be formed, which will make the liquid viscous. Reacts violently with a mixture of iodine pentafluoride and tetrafluoroethylene, causing fire and explosion hazard.
Odour threshold, air:	2.5 mg/m^3 (VOCBASE 1996) Based on recognition: 2.2 – 2.3 mg/m^3 (Christoph 1983 – quoted from BACIS 1999); 0.0059 mg/m^3 (Randebrock 1986 – quoted from BACIS 1999). Based on detection: 0.056-5.6 mg/m^3 (Stevens & Cain 1987a – quoted from BACIS 1999); 4.7 mg/m^3 (De Wijk 1989 – quoted from

BACIS 1999); 0.7 mg/m^3 (Blank & Grosch 1991 – quoted from BACIS 1999).

References: RTECS (2001), CICAD (1998), CEC (1999), HSDB (2001), BACIS (1999), VOCBASE (1996).

1.3 Production and use

d-Limonene has been used for many years as a flavour in foods and beverages. It is increasingly used as a solvent as a substitute for chlorinated hydrocarbons, chlorine fluorine hydrocarbons (CFCs) and other organic solvents. It is also used in the manufacture of resins, as a wetting and dispersing agent and in insect control (NTP 1991, IARC 1993 - quoted from IARC 1999 and from Karlberg & Lindell 1993). *d*-Limonene is also used in perfumes in concentrations between 0.005% and 1% and often used as fragrance in consumer household products. It is reported to be used as an additive to perfumes due to a putative effect as inhibitor of the allergic capacity of closely related chemical substances. (Karlberg & Lindell 1993).
In medical treatment, *d*-limonene has been used to dissolve retained cholesterol gallstones postoperatively (Igimi et al. 1991 - quoted from IARC 1993).

d- Limonene and its metabolite perillyl alcohol are currently undergoing clinical trials for use in treatment of breast cancer and other tumours, and chemoprevention trials are under consideration (Gould 1995, O'Shaugnessy 1996, Vigushin et al. 1998 – all three quoted from IARC 1999). *d*-Limonene is used as an additive to systems for transdermal application of medicines to increase the penetration of the active substance. (Karlberg & Lindell 1993).

Limonene (no specification of enantiomer) is one of the cyclic terpenes forming oil of turpentine (alpha- and beta-pinene together accounting for about 95%). (Emea 2002). Oulu 1A turpentine was found to contain around 2 % *d*-limonene and 4 % *l*-limonene (Larsen et al. (2000).

Technically *d*-limonene is much used as a degreasing agent (limonene content about 30%) prior to lacquering of various industrial products, for cleaning printed circuits in the electronic industry (limonene content 50 - 100%), for cleaning print cylinders in printing works (limonene content 30 - 100%), and as a solvent in dyes. (Karlberg & Lindell 1993).

1.4 Environmental occurrence

1.4.1 Air

Limonene (enantiomer form not given) concentrations in indoor air were measured in Berlin between 1988 and 1999. The median level was 8 µg/m^3 but levels up to 2490 µg/m^3 had been measured. It was noted that concentrations of limonene were increasing significantly over the period of investigation. (Schleibinger et al. 2001).

Indoor concentrations of limonene (no specification of enantiomer) in northern Italy ranged from 10 to 480 µg/m^3 (mean 140 µg/m^3) (De Bortoli et al. 1986 – quoted from CICAD 1998) whereas concentrations ranged from 1.6 to 78 µg/m^3 (mean 18 µg/m^3) in 17 residences in Ruston, Washington (Montgomery & Kalman 1989 – quoted from CICAD 1998). In 754 randomly selected residences in Canada, indoor concentrations of limonene ranged from 9 to 30 µg/m^3 (Fellein & Otson 1993 – quoted from CICAD 1998).

d-Limonene has been detected in indoor and outdoor air in various locations in Texas, USA in concentrations from 0.01 to 29 ppb (up to 162 µg/m^3) (IARC 1993).

Limonene (enantiomer form not given) concentrations in air were measured in Los Angeles, USA. Indoor concentration was around 0.04 mg/m^3 while outdoor concentration was found to be 0.002 mg/m^3. ((Wallace et al. 1991 - quoted from Karlberg & Lindell 1993 and from IARC 1999).

1.4.2 Water

No data have been found.

1.4.3 Soil

No data have been found.

1.4.4 Foodstuffs

d-Limonene is one of the most commonly occurring terpenes in nature, occurring in citrus and a wide variety of other plant species. It is a major constituent of oil of citrus rind, dill oil, oil of cumin, neroli, bergamot and caraway (NTP 1991 - quoted from IARC 1999).

1.5 Environmental fate

1.5.1 Air

If released to the atmosphere, *d*-limonene is expected to rapidly undergo gas-phase oxidation reactions with photochemically produced hydroxyl radicals, ozone, and at night with nitrate radicals. Calculated half-lives for these processes are 2.3-2.6 hours, 25-26 minutes and 3.1 minutes, respectively. (HSDB 2001).

1.5.2 Water

If released to water, *d*-limonene may bioconcentrate in fish and aquatic organisms and it may significantly adsorb to sediment and suspended organic matter. It is expected to rapidly volatilise from water to the atmosphere. The estimated half-life for volatilisation of *d*-limonene from a model river is 3.4 hours, although adsorption to sediment and suspended organic matter may attenuate the rate of this process. (HSDB 2001).

1.5.3 Soil

If released to soil *d*-limonene is expected to exhibit low to slight mobility. It is expected to rapidly volatilise from both dry and moist soil to the atmosphere although strong adsorption to soil may attenuate the rate of this process. (HSDB 2001).

1.5.4 Bioaccumulation

No data have been found.

1.6 Human exposure

Exposure of the general population may occur by inhalation or dermal contact due to presence of *d*-limonene in the atmosphere as a result of releases from natural sources or by ingestion of food in which it is contained or use of consumer products scented with pure *d*-limonene or essential oils containing it. (HSDB 2001).

The average daily intake via foods has been estimated to about 0.3 mg/kg b.w. in the USA (Flavor and Extract Manufacturers Association 1991 – quoted from CICAD 1998).

2 Toxicokinetics

2.1 Absorption and distribution

2.1.1 Inhalation

The toxicokinetics of *d*-limonene were studied in human volunteers exposed by inhalation (2 hours, work load 50 W) in an exposure chamber on three different occasions. The exposure concentrations were approximately 10 (control concentration), 225, and 450 mg/m^3 *d*-limonene. The relative pulmonary uptake was high, between 68% and 63% of the amount supplied was found in plasma. The lung clearance rate in 4 hours was 1.1 litre/kg per hour for 225 mg/m^3 and 1.4 litre/kg per hour for 450 mg/m^3, and the lung clearance rate in 21 hours was 1.1 litre/kg per hour for 450 mg/m^3. (Falk-Filipsson et al. 1993).

2.1.2 Oral intake

d-Limonene is absorbed from the gastro-intestinal tract. Two male volunteers given 1.6 g [^{14}C]*d*-limonene orally excreted 52-83 % of the dose in the urine within 48 hours. (Kodama et al. 1976 - quoted from IARC 1999).

17 women and 15 men with advanced metastatic solid tumours received an average of three treatment cycles of 21 days (one dose on day 1, then three daily doses on days 4-21) at doses ranging from 0.5 to 12 g/m^2 body surface area orally administered *d*-limonene. The maximal plasma concentration was attained at 1-6 h. The mean peak plasma concentrations of *d*-limonene were 11-20 µmo1/l. (Vigushin et al. 1998 - quoted from IARC 1999).

When [^{14}C]*d*-limonene was administered orally to male and female Sprague-Dawley rats at a dose of 409 mg/kg b.w., the renal concentration of *d*-limonene equivalents was about 2.5 times higher in males than females, and approximately 40% of the radiolabel in male rat kidneys was bound reversibly to renal proteins. (Lehman-McKeeman et al. 1989 - quoted from IARC 1999).

[^{14}C]*d*-Limonene was absorbed after administration by gavage of 800 mg/kg b.w. (4.15 µCi/animal) to male Wistar rats. The radiolabel concentration in blood was maximal after 2 hours, and large amounts of radiolabel were also observed in the liver (maximal after 1 hour) and kidneys (maximal after 2 hours). Negligible concentrations were found in blood and organs after 48 hours. (Igimi et al. 1974 - quoted from IARC 1999 and from JECFA 1992).

2.1.3 Dermal contact

In an experimental human study, it was found that the blood level following 2 hours exposure of one hand to 98% pure *d*-limonene was low compared to exposure via inhalation (minimum 63 % of the amount supplied). The maximum level (approximately 1.4 x 10^{-6} mol/l) of *d*-limonene was reached in the arterial capillary blood in the non-exposed hand 140 minutes after initiation of exposure. (Falk et al. 1991 – quoted from Karlberg & Lindell 1993).

d-Limonene was rapidly absorbed (43 minutes) through the intact, shaved abdominal skin of mice. (Meyer & Meyer 1959 - quoted from JECFA 1992).

Twelve Long-Evans male rats were administered single topical doses of 5 mg/kg b.w. ^{14}C-limonene; the treated area was then occluded for 3 hours (2 males) or 6 hours (10 males). Following occlusion, the residual dose was removed and the treated area was re-occluded. Authors reported that peak concentrations of radioactivity in tissue samples were measured 3-6 hours after dosing in the gastrointestinal tract (0.1-0.4% dose/g), livers and kidneys (0.08-0.2% dose/g), and thyroid and fat (0.02-0.06% dose/g); except for the gastrointestinal tract, concentrations of radioactivity in all tissues were appreciably lower at 24 hours. After 6 hours of exposure, 48% of the radioactivity was recovered in the skin. Total mean recovery of radioactivity was reported to be approximately 76%. (Research Institute for Fragrance Materials Inc. 1990 - quoted from JECFA 1992).

2.2 Elimination

2.2.1 Inhalation

The toxicokinetics of *d*-limonene were studied in human volunteers exposed by inhalation. The exposure concentrations were approximately 10 (control concentration), 225, and 450 mg/m^3 *d*-limonene. Three linear phases of elimination could be distinguished in the time studied: an initial phase of slope α (0-15 minutes after exposure), an intermediate phase of slope β for rapid elimination (16-319 minutes after exposure) and a terminal phase of slope γ for slow elimination (320-1300 minutes after exposure). The plasma half-life of *d*-limonene was approximately 2.6 minutes for the α phase, 32 minutes for the β phase and 75 minutes for the γ phase. (Falk-Filipsson et al. 1993).

2.2.2 Oral intake

Two male volunteers given 1.6 g [^{14}C]*d*-limonene orally excreted 52-83 % of the dose in their urine within 48 hours. The major urinary metabolite was 8-hydroxy-*p*-menth-1-en-9-yl-β-*D*-glucopyranosiduronic acid (Kodama et al. 1976 - quoted from IARC 1999).

A pilot study was conducted in healthy volunteers (five women, two men) to investigate the metabolism of orally administered *d*-limonene. After the subjects had ingested 100 mg/kg *d*-limonene in a custard, their blood was drawn at 0 and 24 hours for blood chemistry and at 0, 4 and 24 hours for analysis of metabolites. Gas chromatography-mass spectrometry indicated the presence of five *d*-limonene metabolites in plasma: two major peaks were identified as dihydroperillic acid and perillic acid and a third major peak was limonene-l,2-diol. Two minor peaks were found to be the respective methyl esters of the acids. Limonene itself was only a minor component. In all subjects, the metabolite concentrations were higher at 4 hours than at 24 hours, but a half-life value was not determined. (Crowell et al. 1994 - quoted from IARC 1999).

The toxicokinetics of *d*-limonene were studied in two women and one man who received 0.5-12 g/m^2 body surface area per day orally for 21 days, and plasma and urine samples were collected on days 1 and 21. The metabolites were characterized and their structures elucidated by liquid chromatography-mass spectrometry and nuclear magnetic resonance spectrometry. Five major metabolites were detected in

plasma: limonene-l,2-diol, limonene-8,9-diol, perillic acid, an isomer of perillic acid and dihydroperillic acid. The urinary metabolites comprised the glucuronides of the two isomers of perillic acid, limonene-8,9-diol and a monohydroxylated limonene. The results are consistent with those of previously published studies in humans and in animals, but this study was the first in which limonene-8,9-diol and an additional isomer of perillic acid were identified. (Poon et al. 1996 - quoted from IARC 1999).

d-Limonene administered orally to 17 women and 15 men with advanced metastatic solid tumours at an average of three treatment cycles of each 21 days (one dose on day 1, then three daily doses on days 4-21) at daily doses ranging from 0.5 to 12 g/m^2 body surface area. The predominant metabolites were perillic acid (21-71 µmol/l), dihydroperillic acid (17-28 µmol/l), limonene-l,2-diol (10-21 µmo1/l), uroterpinol (14-45 µmol/l) and an isomer of perillic acid. After reaching these peaks, the plasma concentrations decreased according to first-order kinetics. The values for the integrated area under the curve for time-concentration showed little variation with administered dose. There was no accumulation of the parent or metabolites after a treatment cycle. (Vigushin et al. 1998 - quoted from IARC 1999).

Urinary recovery of [^{14}C]*d*-limonene was 77-96% within three days in rats, guinea-pigs, hamsters and dogs; faecal recovery was 2-9% within three days (Kodama et al. 1976 - quoted from JECFA 1992 and from IARC 1999).

Bile-duct-cannulated rats given *d*-limonene orally excreted 25% of the dose in the bile within 24 hours. Approximately 60% of *d*-limonene was excreted in the urine, 5% in the faeces, and 2% was expired. (Igimi et al. 1974 - quoted from JECFA 1992 and from IARC 1999).

Kodama et al. (1974 - quoted from JECFA 1992) reported that 72% of *d*-limonene metabolites were excreted in male rabbit urine 72 hours after oral administration, while 7% was found in the faeces.

After oral administration of *d*-limonene to rabbits, the urinary metabolites isolated were *para*-mentha-l,8-dien-10-o1 (M-I on Figure 1), *para*-menth-1-en-8,9-diol (M-II), perillic acid (M-III), peril1ic acid-8,9-diol (M-IV), *para*-mentha-l,8-dien-10-yl-β-D-glucopyranosiduronic acid (M-V) and 8-hydroxy-*para*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid (M-VI) (Kodama et al. 1974 – quoted from IARC 1999).

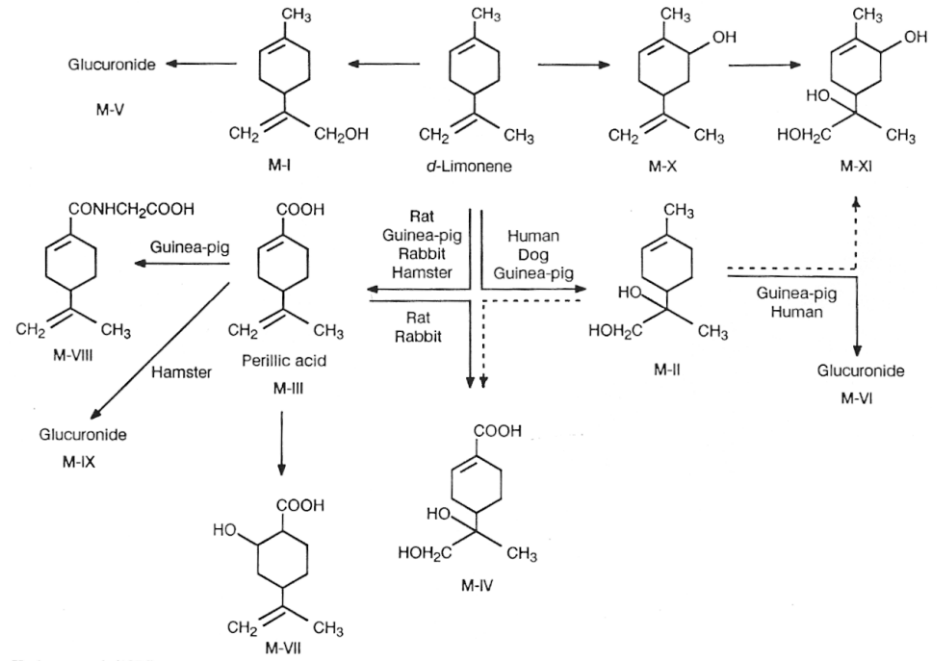
After oral administration of *d*-limonene to dogs and rats, five other urinary metabolites were isolated: 2-hydroxy-*para*-menth-8-en-7-oic acid (M-VII), perillylglycine (M-VIII), perillyl-β-*D*-glucopyranosiduronic acid (M-IX), *para*-mentha-l,8-dien-6-ol (M-X) and probably *para*-menth-l-ene-6,8,9-triol (M-XI) (Kodama et al. 1976 – quoted from IARC 1999).

The major urinary metabolite was M-IV in rats and rabbits, M-IX in Syrian hamsters, M-II in dogs and M-VI in guinea-pigs. (Kodama et al. 1976 – quoted from IARC 1999).

In humans, about 25-30 % of an oral dose of *d*-limonene was found in urine as *d*-limonene-8,9-diol (M-II) and its glucuronide (M-VI); about 7-11 % was eliminated as perillic acid (M-III) and its metabolites (Smith et al. 1969 – quoted from CICAD 1998). *d*-Limonene-8,9-diol is most probably formed via *d*-limonene-8,9-epoxide (Kodama et al. 1976, Watabe et al. 1981 – both quoted from CICAD 1998). In another study, perillic acid (M-III) was reported to be the principal metabolite in both rats and humans (Crowell et al. 1992 – quoted from CICAD 1998).

The possible metabolic pathways of *d*-limonene are shown in Figure 1. (Kodama et al. 1976 – quoted from IARC 1993*).
* IARC 1999 shows a 1,2-unsaturated M-VII compound.

Figure 1. Possible metabolic pathways of *d*-limonene



From Kodama *et al.* (1976)
M-I, *p*-Mentha-1,8-dien-10-ol; M-II, *p*-menth-1-ene-8,9-diol; M-IV, perillic acid-8,9-diol; M-V, *p*-mentha-1,8-dien-10-yl-β-D-glucopyranosiduronic acid; M-VI, 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid; M-VII, 2-hydroxy-*p*-menth-8-en-7-oic acid; M-VIII, perillylglycine; M-IX, perillyl-β-D-glucopyranosiduronic acid; M-X, *p*-mentha-1,8-dien-6-ol; M-XI, *p*-menth-1-ene-6,8,9-triol

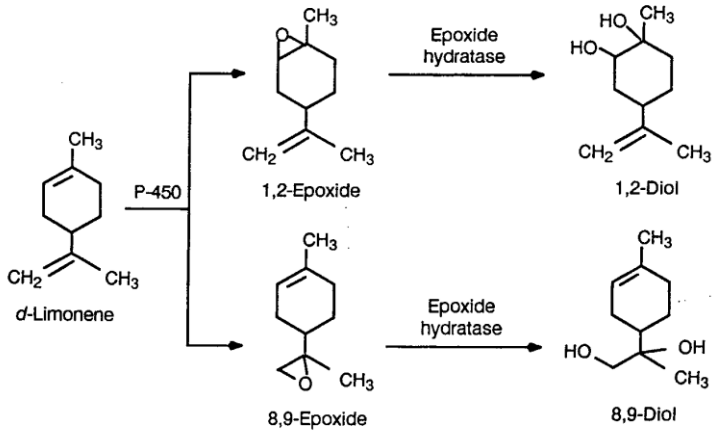
2.2.3 Dermal contact

Twelve Long-Evans male rats were administered single topical doses of 5 mg/kg. At the 24-72-hour sampling times, 8-12% was excreted in urine, 1-3% was excreted in faeces, and 14-18% was expired in air. Total mean recovery of radioactivity was reported to be approximately 76%. (Research Institute for Fragrance Materials Inc. 1990 - quoted from JECFA 1992).

2.2.4 *In vitro* metabolites

Under alkaline extraction conditions, *d*-limonene was metabolised by rat liver microsomes *in vitro* to the glycols *d*-limonene 8,9-diol and *d*-limonene 1,2-diol *via* the 8,9- and 1,2-epoxides (see Figure 2) (Watabe et al. 1980, 1981 - quoted from IARC 1999). Under neutral extraction conditions, no hydrolysis of *d*-limonene-l,2-epoxide to its corresponding diol was observed (Lehman-McKeeman et al. 1989 - quoted from IARC 1999). In rat liver microsomes, epoxidation of *d*-limonene at the 1,2 double bond occurs only in the *cis* orientation, whereas in mouse liver microsomes both *cis* and *trans* isomers of this epoxide are formed (Lehman-McKeeman & Caudill 1992a - quoted from IARC 1999).

Figure 2. Oxidation of *d*-limonene double bonds by rat liver microsomes



From Watabe *et al.* (1980)

Watabe 1980 – quoted from IARC 1993.

The major metabolites bound to the renal protein fraction in male Sprague-Dawley rats were identified as *d*-limonene-l,2-epoxide (> 80%), parent *d*-limonene and the 1,2-diol representing minor components of the protein-bound moieties. The renal protein to which these metabolites bound was identified as alpha$_{2u}$-globulin by high-performance liquid chromatography. (Lehman-McKeeman et al. 1989 - quoted from IARC 1999).

2.3 Mode of action

2.3.1 Mechanism for tumour formation and nephrotoxicity associated with α_{2u}-globulin

A discussion for the criteria for establishing that an agent causes renal tumours in male rats through a response associated with α_{2u}-globulin took place in October 1998 (based on the work of Capen et al. 1999) and the following list was concluded by IARC (October 1998-panel or Working Group).

All the following criteria (IARC 1999) are met by *d*-limonene, which produces hyaline droplet nephropathy and causes renal tubular tumours in male rats, which are therefore concluded to happen through an α_{2u}-globulin-associated response:

- lack of genotoxic activity (the agent and/or a metabolite) on the basis of an overall evaluation of results obtained *in vitro* and *in vivo;*
- nephropathy and renal tumourigenicity seen only in male rats;
- induction in shorter studies of the characteristic sequence of histopathological changes, of which protein droplet accumulation is obligatory;
- identification of the protein that accumulates in tubular cells as α_{2u}-globulin; reversible binding of the chemical or metabolite to α_{2u}-globulin;
- induction of sustained increased cell proliferation in the renal cortex;

- and similarities in dose-response relationship of the tumour outcome with those for histopathological end-points (protein droplets, α_{2u}-globulin accumulation, cell proliferation).

The unique specificity of the syndrome of renal toxicity in male rats due to α_{2u}-globulin is demonstrated by the lack of toxicity and of renal tumours in mice. Mice synthesise mouse urinary protein, which shares nearly 90% sequence identity to α_{2u}-globulin; however, *d*-limonene-1,2-epoxide does not bind to the mouse protein and it does not produce a similar syndrome in mice. Additionally, there is a lack of response in female rats, which synthesise many other proteins of the α_{2u}-globulin superfamily. *d*-Limonene has no carcinogenic activity at any other site in male rats. The most abundant α_{2u}-globulin superfamily protein in human kidney and plasma is α_1-acid glycoprotein, and this protein does not bind to agents that induce α_{2u}-globulin nephropathy in rats.
Taken together, there is no evidence that any human protein can contribute to a renal syndrome similar to α_{2u}-globulin nephropathy, and thus no evidence that *d*-limonene is carcinogenic in humans by a mechanism similar to α_{2u}-globulin nephropathy.
Consequently, all of the mechanistic data support the conclusion that the renal tumours in male rats produced by *d*-limonene are not relevant to humans.
(IARC 1999).

3 Human toxicity

3.1 Single dose toxicity

3.1.1 Inhalation

Human volunteers (8 healthy men) exposed by inhalation (2 hours, work load 50 W) in an exposure chamber to approximately 10, 225, and 450 mg/ m^3 *d*-limonene did not experience any irritative symptoms or symptoms related to the CNS. A 2% decrease in vital capacity was observed after exposure to *d*-limonene at the high exposure level compared to 10 mg/m^3 (statistically ($p < 0.01$) significant but not considered clinically or functionally significant by the authors). (Falk-Filipsson et al. 1993).

3.1.2 Oral intake

Five healthy adult male volunteers who received a single oral dose of 20 g *d*-limonene all developed transient proteinuria, non-bloody diarrhoea and tenesmus. The results of functional tests of the liver, kidney and pancreas were normal. (Igimi et al. 1976 - quoted from IARC 1999).

3.1.3 Dermal contact

In an experimental human study, it was found that 2 hours exposure of one hand to 98% pure *d*-limonene caused a burning sensation combined with painful itching starting within few minutes after beginning of the study and ever increasing during the exposure. The itching decreased toward the end of the exposure, while the burning sensation continuously increased for 10 minutes after cessation of the exposure. The skin on the upper side of the hand was swollen and moderately reddened. The swelling disappeared 1.5 hours after cessation of the exposure. Purpura (small bleedings in the skin) appeared 4.5 hours later and reached maximum after 1-2 days. The symptoms persisted for several weeks. (Falk et al. 1991 - quoted from Karlberg & Lindell 1993).

3.2 Repeated dose toxicity

3.2.1 Inhalation

No information has been found.

3.2.2 Oral intake

In the phase-I clinical trial of orally administered *d*-limonene described in section 2.2.2, toxicity was limited to gastrointestinal symptoms (irritation, nausea, diarrhoea) and was dose-related over the range 6.5-12 g/m^2 body surface area per day. (Vigushin et al. 1998 - quoted from IARC 1999).

3.2.3 Dermal contact

d-Limonene was earlier considered to be one of the substances responsible for perfume allergies. However, no reports have been found on type I allergy to *d*-limonene. (Karlberg & Lindell 1993).

Among 179 patients with contact allergy to perfume only 2 exhibited positive reaction in a patch-test against *d*-limonene (Santucci et al. 1987 - quoted from Karlberg & Lindell 1993).

Contact allergy to both *d*- and *l*-limonene was reported in a patient originally sensitised with turpentine (Dooms-Goossens et al. 1977 - quoted from Karlberg & Lindell 1993).

No experimental sensitisation was experienced with *d*-limonene by a Human Maximization Test in 25 volunteers (Greif 1967 - quoted from Karlberg & Lindell 1993).

Karlberg & Lindell (1993) did not find any reporting of *d*-limonene as the single cause for allergic contact eczema.

3.2.4 *In vitro* studies

A culture of human lung fibroblasts exposed to a mixture of *d*-limonene and 50 % oxygen under conditions most similar to the *in vivo* situation did not increase the toxicity of *d*-limonene beyond an additive effect for concentrations of 200-300 µM. The results did not suggest limonene 1,2-epoxide as the active compound in limonene toxicity. The detoxification of limonene in human lung cells did not involve the glutathione system. (Rolseth et al. 2002).

3.3 Toxicity to reproduction

No information has been found.

3.4 Mutagenic and genotoxic effects

No information has been found.

3.5 Carcinogenic effects

No information has been found.

4 Animal toxicity

4.1 Single dose toxicity

4.1.1 Inhalation

Both enantiomer forms of airborne limonene induced a mild bronchoconstrictive effect in concentrations above 1000 ppm for 30 minutes in conscious BALB/c mice. The exposure concentration for decreasing the respiratory rate (breaths per minute) by 50 % was 1076 ppm (6090 mg/m^3) for *d*-limonene but 1467 ppm for *l*-limonene for the first 10 minutes of the exposure period. The effects were reported to be caused by decreased trigeminal reflex due to sensory irritation. No pulmonary or general anaesthetic effects were recorded for the highest concentrations tested (1600 ppm for *d*-limonene and >2400 ppm for *l*-limonene). (Larsen et al. 2000).

Significant sensory irritation resulting in 33 % reduction of the mean respiratory rate in mice was obtained by exposure during 30 minutes to a 16 second old mixture of ozone (initially 4 ppm after reaction <0.03 ppm) and 48 ppm *d*-limonene (272 mg/m^3). Addition of the sensory irritation effects of a large number of unexpected chemically identified reaction products could not explain all of the observed sensory irritation effect, which suggested that one or more strong airway irritant(s) had been formed (Clausen et al. 2001).

Inhalational exposure for 60 minutes to oxidation products of ozone and *d*-limonene resulted in development of airflow limitations that persisted for at least 45 minutes post-exposure. The airflow limitation was exacerbated in mice that were exposed to *d*-limonene alone immediately following exposure to the oxidation products. All effects from limonene/ozone exposures were reversible within 6 hours. (Rohr et al. 2002).

4.1.2 Oral intake

Oral LD_{50}-values have been summarised in Figure 4.1.

Groups of control (6 animals) and experimental (4 animals/dose group) male Wistar rats were administered single gavage doses of 0, 200, 400, 600, 800, or 1200 mg/kg b.w. *d*-limonene in 2% tragacanth solution in a total volume of 4 ml/kg. Authors reported that no effects were observed on liver triglycerides, microsomal proteins, cytochrome b5 and drug metabolising enzymes. (Ariyoshi et al. 1975 – quoted from JECFA 1992).

4.1.3 Dermal contact

A dermal LD_{50}-value of above 5000 mg/kg has been reported for rabbits (IARC 1999).

Figure 4.1. LD_{50}-values in experimental animals (from IARC 1999).

Species	Sex	Route	LD_{50}	Reference
Mouse	Male & female	Oral	5600 mg/kg (M) 6600 mg/kg (F)	Tsuji et al. (1975)
Rat	Male & female	Oral	4400 mg/kg (M) 5100 mg/kg (F)	Tsuji et al. (1975)
Rat	Information not given	Oral	> 5000 mg/kg	Opdyke (1975)
Rabbit	Information not given	Dermal	> 5000 mg/kg	Opdyke (1975)

4.2 Irritation

4.2.1 Skin irritation

The skin irritating effect of a commercial insect repellent containing 78.2% *d*-limonene and a non-ionic tenside (polysorbate 80), has been studied on the cat. Cats in various groups were dipped once into the insect repellent in various concentrations of *d*-limonene in water (6.5×10^{-2} mol/l – 1 mol/l). No skin irritation was seen at the recommended concentration, but a more concentrated solution (1 mol/l) resulted in reddening and scratch-marks in the peri-anal area and scrotum. Histopathology revealed mild to moderate acute inflammation in epidermis and multiple ulcerations. (Hooser et al. 1986 – quoted from Karlberg & Lindell 1993).

d-Limonene applied undiluted to intact or abraded rabbit skin for 24 hours under occlusion was moderately irritating (no strain, number of animals, or score were given) (Opdyke 1975 – quoted from Nord 1993).

Applications of *d*-limonene solutions – 25 or 40% v/v in 95% ethanol – caused no long term irritation in the ears of 25 rabbits. However, application of undiluted *d*-limonene caused skin redness. (Lacy et al. 1987 – quoted from Nord 1993).

4.2.2 Eye irritation

In a study in rabbits, *d*-limonene caused irritation to the eye (Tsuji et al. 1974 – quoted from CICAD 1998).

4.3 Sensitisation

Although *d*-limonene was once considered the main allergen in citrus fruits, data from more recent studies in animals have revealed air-oxidized *d*-limonene rather than unoxidised *d*-limonene to be the sensitising agent.

When limonene (unspecified form and unknown purity of the test material) was tested in four different sensitisation tests with guinea-pigs (Open Epicutaneous Test, Draize Test and a test with Freund's Complete Adjuvant) it was sensitising in all but Draize Test (Klecak et al. 1977 – quoted from CICAD 1998).

In another study in mice, *d*-limonene did not induce sensitisation (Maisey & Miller 1986 – quoted from CICAD 1998).

Hydroperoxides and other oxidation products of *d*-limonene formed on exposure to the air have proved to be potent contact allergens when tested with Freund's Com-

plete Adjuvant in guinea-pigs, whereas unoxidised *d*-limonene did not cause any sensitisation (Karlberg et al. 1991, 1992 – quoted from CICAD 1998).

4.4 Repeated dose toxicity

4.4.1 Inhalation

No information has been found.

4.4.2 Oral intake

4.4.2.1 Rats

A dose-related increase in relative liver and kidney weights was observed in groups of 5 young adult male Fischer 344 rats given 75, 150 or 300 mg/kg b.w. *d*-limonene daily by gavage on five days per week and killed on study days 6 or 27. With Mallory-Heidenhain staining, a dose-related formation of hyaline droplets was observed histologically in the kidneys. Hyaline droplet nephropathy was associated with increased concentrations of $alpha_{2u}$-globulin in renal cortical homogenates separated by two-dimensional gel electrophoresis. The concentrations of other renal proteins were not increased by *d*-limonene treatment. Alterations considered being sequelae of the hyaline droplet response, including granular casts in the outer zone of the medulla and multiple cortical changes collectively classified as chronic nephrosis, were observed in the kidneys of all rats killed on day 27. Only male rats were studied. (Kanerva et al. 1987 - quoted from IARC 1999).

In a 30-day study, groups of 5 male and female rats were exposed daily to 0, 277, 554, 1385 or 2770 mg *d*-limonene/kg b.w. orally. A general decrease in food intake and a dose-related decrease in body weight were seen in groups of exposed males, but little or no effect on neither organ weights nor relative organ weights was observed. No significant changes were seen in urinalysis, haematology or biochemical values. The following tissues were examined histopathologically: adrenals, duodenum, heart, kidneys, liver, lungs, lymph nodes, pancreas, pituitary, spleen, stomach, testes/ovaries, thymus and thyroids. Authors reported that no significant changes were noted except that granular casts were seen in the kidneys of most exposed male rats (0/5, 3/5, 5/5, 5/5 or 4/5 animals exposed to 0, 277, 554, 1385 or 2770 mg/kg b.w./day respectively). (Tsuji et al. 1975a – quoted from JECFA 1992).

Groups of 5 F344/N rats of each sex were given daily gavage doses of 0, 413, 825, 1650, 3300 or 6600 mg *d*-limonene/kg b.w. in 10 ml/kg b.w. corn oil 5 days/week over a 16 day period (12 total doses). All rats receiving 6600 mg *d*-limonene/kg b.w./day as well as 5/5 male and 3/5 female rats that were exposed to 3300 mg/b.w./day died. The authors reported that no clinical signs were seen in rats receiving 1650 mg *d*-limonene/kg b.w./day or lower, and that no compound-related histopathologically effects were seen in any rats. (NTP 1990 – quoted from JECFA 1992).
d-Limonene given orally (by gavage) for four days at 1650 mg/kg b.w. per day caused no renal toxicity in male NCI Black Reiter rats, which do not synthesize the $alpha_{2u}$-globulin that is normally present in hyaline droplets found in male Fischer 344 rats with *d*-limonene-induced nephrotoxicity. (Dietrich & Swenberg 1991a).

Male Wistar rats were treated with gavage doses of 0 or 400 mg/kg b.w. *d*-limonene in 2% tragacanth solution in a total volume of 4 ml/kg for 2, 3, 15 or 30

days; animals were killed 24 hours following the last dose. Authors reported that, following repeated treatment for 30 days, relative liver weight and hepatic phospholipid content were slightly increased, and liver and serum cholesterol were decreased 49% and 8%, respectively. In addition, palmitic, linoleic and arachidonic acids were increased, and stearic acid was decreased in the liver; aminopyrine demethylase and aniline hydroxylase were increased 26% and 22%, respectively, and cytochrome P-450 and b5 were increased by 31% and 30%, respectively. (Ariyoshi et al. 1975 – quoted from JECFA 1992).

d-Limonene given orally in corn oil by gavage at 150 mg/kg b.w. increased renal-cell proliferation in male Fischer 344 rats, particularly in the P_2 segment of the renal proximal tubular epithelium, after 4 or 31 weeks of exposure. Cell proliferation, determined by bromodeoxyuridine labelling, was increased approximately fivefold over that in control rats. No increase in renal-cell proliferation was observed in male NBR rats treated similarly in the same experiment. (Dietrich & Swenberg 1991b).

d-Limonene increased renal-cell proliferation in response to hyaline droplet exacerbation in Fischer 344 rats dosed orally for 91 days at 0, 5, 30, 75 or 150 mg/kg b.w. No formation of hyaline droplets was noted at the lowest dose, and there was no increase in proliferating cell nuclear antigen-labelled renal proximal tubular cells. At doses of 30 mg/kg b.w. *d*-limonene and higher, both hyaline droplet formation and the percentage of labelled cells were increased. At the highest dose, the percentage of antigen-labelled cells was increased by about six times over that in controls, and the cells were localized to the P_2 segment of the proximal tubule (Lehman-McKeeman 1997 – quoted from IARC 1999).

Oral administration of 75 or 150 mg *d*-limonene to male Fischer 344/N rats on five days per week for two years was associated with dose-related alterations to the kidney, such as increased incidences of mineralisation and epithelial hyperplasia and increased severity of chronic progressive nephropathy. In the same study, no signs of toxicity, including renal hyaline droplet formation, were observed in female Fischer 344/N rats dosed with 300 or 600 mg/kg b.w. (NTP 1990 - quoted from IARC 1999).

4.4.2.2 Mice

Male and female B6C3F 1 mice were treated orally on five days a week for two years with doses of 250 or 500 and 500 or 1000 mg/kg b.w., respectively. The mean body weights of females at the high dose were 5-15% lower than those of controls after week 28, but no compound-related toxicity was observed in animals of either sex. (NTP 1990 - quoted from IARC 1999).

The ability of *d*-limonene to cause hyaline droplet nephropathy was evaluated in C57BL/6-derived transgenic mice engineered to express $alpha_{2u}$-globulin. These mice excreted approximately 30% less $alpha_{2u}$-globulin than male rats. $alpha_{2u}$-Globulin was detected in the kidney by immunoblotting; after *d*-limonene treatment at 150 mg/kg b.w. for three days, the concentration of $alpha_{2u}$-globulin was increased threefold relative to untreated controls. Spontaneous hyaline droplet formation was not seen in control transgenic mice, but small droplets were observed after *d*-limonene treatment (Lehman-McKeeman & Caudill 1994 - quoted from IARC 1999).

4.4.2.3 Dogs

In Beagle dogs, oral doses of more than 340 mg/kg b.w. (females) and 1000 mg/kg b.w. (male) per day for six months resulted in protein casts in the renal tubules. Daily doses of more than 1000 mg/kg b.w. (females) and 3024 mg/kg b.w. (males) resulted in slight weight loss due to frequent vomiting in some animals. (Tsuji et al. 1975b – quoted from IARC 1999).

In another study in adult beagle dogs, *d*-limonene at l00 or 1000 mg/kg b.w. (maximal tolerated dose for emesis) per day (by gavage twice daily) for six months increased kidney weights but induced no histopathological changes, hyaline droplet accumulation or nephropathy (Webb et al. 1990 - quoted from IARC 1999).

4.4.3 Dermal contact

No data have been found.

4.5 Toxicity to reproduction

No data have been found considering the effects of *d*-limonene on fertility.

Pregnant Wistar rats (15 per group) were given *d*-limonene orally from day 9 to 15 of gestation at the doses of 0, 591 or 2869 mg/kg b.w. In the high dose group, the maternal body weight gain was decreased and several dams died. An increased number of dead foetuses was also seen in this group. Significantly delayed ossifications of metacarpal bone and proximal phalanx were seen in foetuses of the high dose group. Further a tendency for decreased total body weight as well as decreased weights of thymus, spleen and ovaries were seen among the foetuses in the high dose group. NOAEL for both maternal and embryonal toxicity in this study was 591 mg/kg b.w. (Tsuji et al. 1975 – quoted from Nord 1993).

Pregnant mice (ICR) were given oral doses of 0, 591, or 2363 mg/kg b.w. *d*-limonene from days 7 through 12 of gestation. Authors reported significant decreases in body weight gain of dams in the high-dose group; foetuses of dams exposed to the high-dose showed increased incidences of lumbar rib, fused rib, delayed ossification, and decreased body weight gain relative to foetuses of control dams. NOAEL for both maternal and embryonal toxicity in this study was 591 mg/kg b.w. (Kodama et al. 1977a – quoted from JECFA 1992).

Pregnant Japanese white rabbits were given oral doses of 0, 250, 500, or 1000 mg/kg b.w. *d*-limonene from day 6 to day 18 of gestation. Significant decreases in body weight gain in dams given 250 or 500 mg/kg b.w./day *d*-limonene were observed, and survival in dams given 1000 mg/kg b.w./day was significantly reduced (33% mortality). From examination of the foetuses, authors concluded that *d*-limonene was not teratogenic in rabbits (incomplete lobulation of the lungs and delayed ossification were not reported to statistically differ from those found in the control group). (Kodama et al.1977b – quoted from JECFA 1992).
Nord (1993), when quoting the same reference (published in Japanese only), came to the result that the NOAEL for maternal toxicity as well as embryotoxicity was 250 mg/kg b.w./day.

4.6 Mutagenic and genotoxic effects

The results of the various tests on mutagenicity and genotoxicity are summarised in Table 4.4.

Table 4.4. Mutagenicity and genotoxicity test performed with limonene and metabolites. From IARC (1999).

Test System	Results[a]		Dose[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
d-Limonene				
Salmonella typhimurium TA100, TA98, TA1535, TA1537, TA1538, reverse mutation	-	-	2720 µg/ plate	Watabe et al. (1981)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, reverse mutation	-	-	3333 µg/ plate	Haworth et al. (1983)
Gene mutation, mouse lymphoma L5178Y cells, *tk* locus *in vitro*	-	-	60	NTP (1990)
Sister chromatic exchange, Chinese hamster ovary cells *in vitro*	-	-	162	NTP (1990)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	-	-	500	NTP (1990)
Inhibition of intercellular communication mouse 3PC cells *in vitro*	+	NT	136	Jansen & Jongen (1996)
Cell transformation, Syrian hamster embryo cells	-	NT	50	Oshiro et al. (1998)
Mammalian spot test, mouse *in vivo*	-		215 ip x 3	Fahrig (1982)
d-Limonene-1,2-oxide				
Escherichia coli PQ37, induction of SOS repair	-	-	500	von der Hude et al. (1990)
Salmonella typhimurium TA100, TA1535, reverse mutation	-	-	500 µg/ plate	von der Hude et al. (1990)
Unscheduled DNA synthesis, primary rat hepatocytes *in vitro*	-	NT	15	von der Hude et al. (1990)
Essential oils containing *d*-limonene				
Bacillus subtilis rec strains, differential toxicity	-	NT	30 µL/plate	Zani et al. (1991)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, reverse mutation	-	-	2.5 µL/plate	Zani et al. (1991)

[a] +, positive; -, negative; NT, not tested

[b] Expressed as either lowest effective dose or highest ineffective dose (in positive and negative tests, respectively). Unless otherwise stated, *in-vitro* test doses, µg/ml; *in-vivo* test doses, mg/kg b.w. per day; ip, intraperitoneal.

4.6.1 *In vitro* studies

d-Limonene was not mutagenic to *Salmonella typhimurium*. In single studies, it did not induce sister chromatid exchange, chromosomal aberrations, trifluorothymidine resistance, or transformation of rodent cells *in vitro*.
d-Limonene has been found to inhibit gap-junctional intercellular communication in mouse primary keratinocytes and derived cell lines.

Essential oils containing *d*-limonene did not induce differential toxicity in *Bacillus subtilis,* nor did they induce reverse mutation in S. *typhimurium*.

The metabolite *d*-limonene-1,2-oxide gave negative results in the SOS chromotest. It was not mutagenic to *Salmonella typhimurium* and did not induce unscheduled DNA synthesis in rat hepatocytes *in vitro*.

4.6.2 *In vivo* studies

d-Limonene gave negative results in the mammalian spot (mice) test even at toxic doses and did not inhibit the transformation of rat tracheal epithelial cells by benzo[*a*]pyrene (Steele et al. 1990 - quoted from IARC 1999).

4.7 Carcinogenic effects

Groups of 50 male and 50 female Fischer 344/N rats, seven to eight weeks of age, received 0, 75 or 150 (males) and 0, 300 or 600 (females) mg/kg b.w. *d*-limonene (> 99% pure) in corn oil by gavage on five days a week for 103 weeks. The experiment was terminated after 105 weeks. In males, treatment-related increases were observed in the incidences of renal tubular hyperplasia (vehicle control, 0/50; low-dose, 4/50; high-dose 7/50), renal tubular-cell adenoma (vehicle control, 0/50; low-dose, 4/50; high-dose 8/50; $p < 0.01$, trend test), and renal tubular-cell adenocarcinoma (vehicle control, 0/50; low-dose, 4/50; high-dose 3/50). The incidence of lesions of the kidney was not increased in female rats. (NTP 1990 - quoted from IARC 1993).

Groups of 50 male and 50 femaleB6C3F_1 mice, eight to nine weeks of age, received 0, 250 or 500 (males) and 0, 500 or 1000 (females) mg/kg b.w. *d*-limonene (> 99% pure) in corn oil by gavage on five days a week for 103 weeks. The experiment was terminated after 105 weeks. No significant increase in the incidence of neoplasms was observed. The incidence of neoplasms (adenomas and carcinomas combined) of the anterior pituitary was lower in high-dose females than in controls (2/48 versus 12/49). (NTP 1990 - quoted from IARC 1993).

A number of studies on oral administration of *d*-limonene synchronous with administration of known carcinogens in rats and mice were reviewed by the IARC (1993) and IARC (1999). These studies are found to be of no relevance in this evaluation.

5 Regulations

5.1 Ambient air

-

5.2 Drinking water

-

5.3 Soil

-

5.4 Occupational Exposure Limits

Denmark:	-
ACGIH:	-
Germany:	-
Sweden:	The 8-hour time weighted exposure limit for *d*-limonene is 150 mg/m^3 (25 ppm) and the short-term (15 minutes) exposure limit is 300 mg/m^3 (50 ppm).

5.5 EU-Classification

d-Limonene, *l*-limonene and limonene are classified for flammability (R10 - flammable), for irritative effects (Xi;R38 – irritating to skin), for sensitisation (R43 – may cause sensitisation by skin contact), and for environmental toxicity (N;R50/53 – very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment) (MM 2002).

5.6 IARC

d-Limonene is not classifiable as to its carcinogenicity to humans (Group 3) (IARC 1999).

5.7 US-EPA

The data on *d*-limonene were reviewed by the US-EPA and considered to be inadequate for the derivation of an inhalation RfC (IRIS 2001).

6 Summary and evaluation

6.1 Description

d-Limonene is a colourless liquid with a characteristic citrus odour. It is practically insoluble in water but is soluble in most organic solvents. It has a vapour pressure of 1.43 mmHg (at 20°C).

Various odour thresholds have been reported in the literature:
2.5 mg/m^3
Based on recognition: 2.2-2.3 mg/m^3, 0.0059 mg/m^3
Based on detection: 0.056-5.6 mg/m^3, 4.7 mg/m^3, 0.7 mg/m^3.

6.2 Environment

d-Limonene occurs naturally in citrus and in a variety of other plants and is used as an additive for flavouring foods and as a fragrance for cosmetics and household chemicals. Limonene (no information about enantiomer form given) is one of the minor constituents of turpentine. *d*-Limonene may be found in the environment in relation to either of these occurrences.

The median level of indoor concentrations of limonene of unknown enantiomer form measured in Berlin between 1988 and 1999 was 8 µg/m^3 and maximum concentration was 2490 µg/m^3. A concentration of limonene (enantiomer form not given) was found in indoor air (northern Italy) between 10 and 480 µg/m^3 (mean 140 µg/m^3). Outdoor concentrations measured in Los Angeles were 2 µg/m^3. *d*-Limonene concentrations were detected in indoor and outdoor air in various locations in Texas, from 0.01 to 29 ppb (0.06 to 0.162 mg/m^3).

6.3 Human exposure

Exposure to *d*-limonene occurs from its presence in foods and consumer products via inhalation, dermal contact, or per oral. The average daily intake has been estimated to about 0.3 mg/kg b.w. from the foods.

6.4 Toxicokinetics

d-Limonene is well absorbed via the lungs in humans (up to about 70%) and the gastro-intestinal tract (up to more than 80%) in humans and most experimental animals. Peak concentrations in plasma can be measured around two hours after exposure in most species. There are conflicting results with respect to dermal absorption. Peak plasma concentrations in man were measured after two hours continuous exposure, and were low compared to concentrations after inhalation. Forty-eight percent of a dermally applied dose was retained in the skin of rats after 6 hours.

The main metabolites of *d*-limonene are formed via oxidation, to perillic acid derivatives (in rat, guinea-pig, rabbit and hamster) or to *p*-menth-1-ene-8,9-diol derivatives in humans, dogs, and guinea-pigs. Both types of oxidation products are

excreted as glucuronides mainly via the urine. Very small amounts of *d*-limonene-1,2-epoxide have been discovered in intact animals but is formed by rat liver microsomes *in vitro*. This compound is nephrotoxic to animals, which produce the α_{2u}-globulin (male rats of several strains).

Most of the metabolites are excreted during the first 24 hours after administration of *d*-limonene and very little parent *d*-limonene or metabolites are persisting after 72 hours. A half-life of 1.25 hours has been calculated meaning that around 3 days will be needed to clear a body completely after inhalational exposure to 450 mg/m^3 for two hours.

6.5 Mode of action

The unique specificity of the syndrome of renal toxicity in male rats due to α_{2u}-globulin is demonstrated by the lack of toxicity and of renal tumours in mice. Mice synthesise mouse urinary protein, which shares nearly 90% sequence identity to α_{2u}-globulin; however, *d*-limonene-1,2-epoxide does not bind to the mouse protein and it does not produce a similar syndrome in mice. Additionally, there is a lack of response in female rats, which synthesise many other proteins of the α_{2u}-globulin superfamily. *d*-Limonene has no carcinogenic activity at any other site in male rats. The most abundant α_{2u}-globulin superfamily protein in human kidney and plasma is α_1-acid glycoprotein and this protein does not bind to agents that induce α_{2u}-globulin nephropathy in rats.

6.6 Human toxicity

A 2% decrease in vital capacity after 2 hours of inhalational exposure to 450 mg/m^3 *d*-limonene when compared to that measured after exposure to 10 mg/m^3 *d*-limonene was the only toxic effect observed (statistically but not clinically significant). No irritative symptoms or toxic effects on the central nervous system were recorded.

Transient proteinuria, non-bloody diarrhoea, and tenesmus were seen after a single oral dose of 20 g *d*-limonene in all of five adult males.
Gastro-intestinal symptoms: irritation, nausea, and diarrhoea were dose-related to 21 days testing with daily oral doses from 6.5 to 12 g/m^2 body surface.

Prolonged skin contact with undiluted *d*-limonene was moderately irritating.

Only two out of 179 patients with contact allergy against perfume gave positive reactions in a patch test with *d*-limonene and no experimental sensitisation was experienced in 25 volunteers with *d*-limonene in a Human Maximization Test.

Tests with human lung cells exposed *in vitro* to *d*-limonene and 50 % oxygen did not suggest limonene 1,2-epoxide as responsible for limonene toxicity. Limonene detoxification was not primarily by mechanisms involving glutathione.

No data regarding toxicity to reproduction, mutagenic and genotoxic effects, and carcinogenic effects in humans have been found.

6.7 Animal toxicity

6.7.1 Single dose toxicity

Oral LD_{50}-values in rats and mice from 4400 to 6600 mg/kg b.w. have been reported for *d*-limonene. For rabbits, the dermal LD_{50}-value reported was above 5000 mg/kg b.w.

d-Limonene at a concentration of 1076 ppm (6090 mg/m^3) caused a 50% decrease in the respiratory rate (RD_{50}) in BALB/c mice exposed by inhalation due to sensory irritation.

d-Limonene (48 ppm) and ozone (4 ppm initially <0.03 ppm after reaction) reaction mixture resulted in 33 % decrease in the respiratory rate in BALB/c mice exposed by inhalation. The compounds identified in the reaction mixture could not account for the sensory irritation. An extended exposure to the reaction mixture exacerbated the airflow limitation of immediately subsequent exposure to *d*-limonene alone.

Undiluted *d*-limonene has been reported to be moderately irritating to the skin of rabbits.

When limonene (unspecified form and unknown purity of the test material) was tested in four different sensitisation tests with guinea-pigs (Open Epicutaneous Test, Draize Test and a test with Freund's Complete Adjuvant) it was sensitising in all but Draize Test. When tested with Freund's Complete Adjuvant in guinea-pigs, unoxidised *d*-limonene did not cause any sensitisation. In mice, *d*-limonene did not induce sensitisation.

6.7.2 Repeated dose toxicity

No data have been found regarding effects following repeated inhalation or dermal application of *d*-limonene in experimental animals.

In male rats producing α_{2u}-globulin (Fischer strain, Sprague-Dawley strain, and others), nephrotoxicity is observed following repeated dosing with fairly low oral doses of *d*-limonene. In most studies, hyaline droplet nephropathy was observed at the lowest dose level tested so that no NOAEL could be established (LOAELs of 30, 75, 277, 400, or 150 mg/kg b.w. have been observed). In one study (91-day), a NOAEL of 5 mg/kg b.w./day was reported for hyaline droplet formation in male F344 rats. The highest NOAEL reported was 1650 mg/kg b.w./day (12 doses to F344 rats or 4 doses to NCI Black Reiter rats (not a α_{2u}-globulin producing strain).

In all other test animals, but male rats of α_{2u}-globulin producing strains, the only compound related effects identified following repeated oral dosing with *d*-limonene were slight decreases in body weight gain and food consumption. No compound related histological changes were identified in these animals. Daily oral doses tested were up to 2770 mg/kg b.w./day for one month or 600 mg/kg b.w./day (5/7 days) for two years in female Fischer 344/N rats, and up to 500 and 1000 mg/kg b.w./day (5/7 days) for two years in male and female mice, respectively. NOAELs were found to be between 500/600 mg/kg b.w./day (mice/female rats) in 2-year studies and 1650 mg/kg b.w./day for 16 days in female rats.

Beagle dogs given daily oral doses of up to 1000 mg/kg b.w./day for 6 months showed increased kidney weights but no histological changes; vomiting was frequent and thus slight weight loss occurred.

6.7.3 Toxicity to reproduction

No data have been found regarding fertility and regarding developmental toxicity following inhalation or dermal application of *d*-limonene in experimental animals

Daily oral dosing during organogenesis that consistently resulted in decreased weight gains in the dams (from 250 or 500 mg/kg b.w./day in rabbits and above 2000 mg/kg b.w./day in rats and mice) resulted in delayed prenatal development. A NOAEL of 591 mg/kg b.w./day was observed for both maternal and developmental toxicity in rats and mice; a NOAEL for maternal and developmental toxicity in rabbits cannot be established.

6.7.4 Mutagenic and genotoxic effects

d-Limonene, essential oils containing *d*-limonene, or the metabolite *d*-limonene-1,2-oxide were neither genotoxic nor mutagenic in any of the assays performed *in vitro* or *in vivo*.

6.7.5 Carcinogenic effects

d-Limonene has been tested for carcinogenicity by oral gavage in one study in rats and in one study in mice. *d*-Limonene significantly increased the combined incidence of renal-cell adenomas and carcinomas and induced renal tubular hyperplasia in male rats; in female rats and in mice, no treatment related tumours were seen.

6.8 Evaluation

The oxidation products of ozone and the unsaturated hydrocarbon limonene have been shown to cause enhance upper airway irritation in mice compared with ozone and limonene alone. Furthermore, it has been shown that the oxidation products may have moderate-lasting adverse effects on both the upper airways and pulmonary regions. (Rohr et al. 2002).

d-Limonene is well absorbed from the lungs in humans (up to about 70%) following inhalation and from the gastro-intestinal tract (up to more than 80%) in humans and most experimental animals; conflicting results regarding dermal absorption have been reported. The main metabolites of *d*-limonene are formed via oxidation in both humans and experimental animals; however, species differences have been observed regarding which metabolites are formed (perillic acid derivatives in rat, guinea-pig, rabbit and hamster; *p*-menth-1-ene-8,9-diol derivatives in humans, dogs, and guinea-pigs). Minor amounts of *d*-limonene-1,2-epoxide have been detected in intact animals, but is formed by rat and mouse liver microsomes *in vitro*. The major metabolite bound to the renal protein fraction in male rats was identified as *d*-limonene-1,2-epoxide (more than 80%) and this compound is considered to be nephrotoxic to animals, which produce α_{2u}-globulin (male rats of several strains).

d-Limonene has a very low acute toxic potential in experimental animals following oral administration or dermal application as evidence by the LD_{50}-values; no data are available for inhalation.
In humans, a 2% decrease in vital capacity following inhalation of 450 mg/m^3 *d*-limonene was reported when compared to that measured after exposure to 10 mg/m^3 (statistically but not clinically significant); no irritative symptoms or effects on the central nervous system were recorded. Mice exposed by inhalation to about 1076 ppm (6090 mg/m^3) *d*-limonene had a decrease in respiratory rate of 50% (RD_{50}) whereas exposure to a reaction mixture of 48 ppm and 4 ppm ozone cause a decrease in respiratory rate of 33% and further exacerbated the airflow limitation of an immediately following exposure to *d*-limonene alone.

Undiluted *d*-limonene is considered as being moderately irritating to the skin of both humans and rabbits; this is consistent with the EU-classification (irritating to skin).

d-Limonene has been regarded as the major allergen in citrus fruit and considered to be one of the agents responsible for perfume allergies. No reports have been found on type I allergy to *d*-limonene and *d*-limonene has never been reported as being the single cause for allergic contact eczema. Of 179 patients with contact allergy against perfume, only 2 patients gave positive reactions in a patch test with *d*-limonene. No sensitisation was experienced in 25 volunteers with *d*-limonene in a Human Maximization Test.
Limonene, of unspecified form and unknown purity, has shown a positive result for skin sensitising properties in 3 of 4 different sensitisation tests with guinea-pigs. However, when *d*-limonene was tested in guinea pigs and in mice, no sensitisation occurred. Data from more recent studies in animals have revealed that air-oxidised *d*-limonene rather than unoxidised *d*-limonene is the sensitising agent and hydroperoxides and other oxidation products of *d*-limonene formed by exposure to the air have proved to be potent contact allergens when tested with Freund's Complete Adjuvant in guinea-pigs.
Overall, *d*-limonene itself in a pure unoxidised form does not have a skin sensitising potential whereas upon contact with air, a skin sensitising potential may be expressed. *d*-Limonene is classified as a skin sensitiser under the EU Dangerous Substances and Preparations Directive due to its ability to form allergenic oxidation products.

Only oral repeated dose toxicity studies are available in experimental animals with no studies being available in humans. The predominant effect observed is hyaline droplet nephrotoxicity in male rats (only in male rats producing α_{2u}-globulin, e.g., Fischer 344 and Sprague-Dawley, but not NCI Black Reiter rats) at fairly low oral doses; a NOAEL of 5 mg/kg b.w./day (90-day study) has reported for hyaline droplet formation in male F344 rats. In Beagle dogs, increased kidney weights but no histological changes were observed following oral doses of up to 1000 mg/kg b.w./day (6 months). In all other test animals, but male rats of α_{2u}-globulin producing strains, the only compound related effects identified following repeated oral dosing with *d*-limonene were slight decreases in body weight gain and food consumption; no compound related histological changes were identified in these animals.

No data regarding toxicity to reproduction in humans have been found, and no data regarding fertility and regarding developmental toxicity following inhalation or dermal application of *d*-limonene in experimental animals.
Developmental toxicity studies in rats, mice, and rabbits indicate delayed prenatal development following oral dosing during organogenesis, but only at dose levels that consistently resulted in decreased weight gains in the dams. A NOAEL of 591

mg/kg b.w./day is considered for both maternal and developmental toxicity in rats and mice; a NOAEL for maternal and developmental toxicity in rabbits cannot be established based on the available data.

d-Limonene is not considered to possess a genotoxic or a mutagenic potential as the substance itself, essential oils containing *d*-limonene, or the metabolite *d*-limonene-1,2-oxide were negative in any of the assays performed *in vitro* or *in vivo*. No data in humans have been found.

No data regarding carcinogenic effects in humans have been found. When tested by oral gavage in a 2-year study in rats and in mice, *d*-limonene significantly increased the incidence of renal-cell adenomas and carcinomas and induced renal tubular hyperplasia in male rats; no carcinogenic activity was observed at any other site in male rats, and no treatment related tumours were seen in female rats or in mice of both sexes.
The unique specificity of the syndrome of renal toxicity in male rats producing α_{2u}-globulin is demonstrated by the lack of renal toxicity and of renal tumours in mice, in female rats, and in male rats (NCI Black Reiter rats), which do not synthesise α_{2u}-globulin. The major metabolite bound to the renal protein fraction in male rats has identified as *d*-limonene-1,2-epoxide (more than 80%) and this compound is considered to be the nephrotoxic metabolite in animals, which produce α_{2u}-globulin (male rats of several strains). Mice synthesise a protein, which shares nearly 90% sequence identity to the rat α_{2u}-globulin; however, *d*-limonene-1,2-epoxide does not bind to the mouse protein and it does not produce a similar syndrome in mice as in male rats. Additionally, there is a lack of response in female rats, which synthesise many other proteins of the α_{2u}-globulin superfamily, but not the male rat specific protein. The most abundant α_{2u}-globulin superfamily protein in human kidney and plasma is α_1-acid glycoprotein and this protein does not bind to substances that induce α_{2u}-globulin nephropathy in rats.
Overall, it is concluded that *d*-limonene produces renal tumours in male rats by a non-DNA-reactive mechanism, through an α_{2u}-globulin-associated response, which is not relevant to humans.

6.8.1 Critical effect and NOAEL

The critical effect following exposure to *d*-limonene is considered to be the skin sensitising potential of the oxidation products of *d*-limonene if oxidised by contact with air (but not to the pure, unoxidised substance itself); no data are available regarding a potential for respiratory sensitisation. However, based on the available data, a NOAEL or LOAEL for the critical effect cannot be established.

In experimental animals, the critical effect in male rats of α_{2u}-globulin producing strains is hyaline droplet nephrotoxicity, including renal tumours; however, due to the male rat specific α_{2u}-globulin syndrome, these renal effects are not relevant to humans. None of the other effects observed in experimental animals (slight decreases in body weight gain and food consumption) are considered as being critical effects.

In human volunteers, no irritative symptoms were recorded following inhalation of 450 mg/m^3 *d*-limonene for 2 hours. Mice exposed by inhalation to about 1076 ppm (6090 mg/m^3) *d*-limonene had a decrease in respiratory rate of 50% (RD_{50}). No data are available regarding effects of *d*-limonene following inhalation repeatedly. For the purpose of estimating a quality criterion in air, 450 mg/m^3 is considered as being a NOAEC for irritative symptoms in humans.

7 Quality criterion in air

The quality criterion in air is calculated based on a NOAEL of 450 mg/m^3 for irritative symptoms in humans.

$$QC_{air} = \frac{NOAEC}{UF_I * UF_{II} * UF_{III}} = \frac{450\ mg/m^3}{1 * 10 * 10}$$

$$= 4.5\ mg/m^3$$

The uncertainty factor UF_I is set to 1 as human data are used. The UF_{II} is set to 10 in order to reflecting the range in biological sensitivity within the human population. The UF_{III} is set to 10 because of the limitations in the available data on *d*-limonene, i.e. no data on effects following inhalation repeatedly, a NOAEC but not a LOAEC has been reported for irritative effects in humans, a NOAEL or LOAEL cannot be established for the critical effect (skin sensitisation), and no data available regarding respiratory sensitisation.

A quality criterion of 4.5 mg/m^3 has been calculated. A health based C-value of 4.5 mg/m^3 and placing in Main Group 2 is proposed.

Varying, but low odour thresholds in air have been reported for *d*-limonene ranging from about 0.006 to 5.6 mg/m^3. Both odour thresholds based on detection as well as on recognition have been reported; however, the reported thresholds overlap and do not allow to conclude on a 50% odour threshold.
The C-value of 4.5 mg/m^3 established based on health effects is not considered to take into account the discomfort from the odour.

7.1.1 C-value

A health based C-value of 4.5 mg/m^3, Main Group 2.

It should be noted that the health based C-value is not considered to take into account the discomfort from the odour.

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Evaluation of health hazards by exposure to d-Limonene and proposal of a health-based quality criterion for ambient air

The Danish Environmental Protection Agency has requested an evaluation of health hazards by exposure to d-Limonene. This resulted in 2006 in the present report which includes a health-based quality criterion for the substance in ambient air.

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