d-LIMONENE

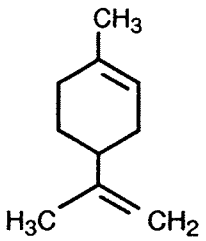
1. Exposure Data

1.1 Chemical and physical data

Limonene is, with the possible exception of α-pinene, the most frequently occurring natural monoterpene. It is a major constituent of the oils of citrus fruit peel and is found at lower levels in many fruits and vegetables. It occurs naturally in the *d* (or R)- and *l* (or S) optically active forms and as *dl* mixtures including the optically inactive racemate (dipentene). For example, the *d* form comprises 98–100% of the limonene in most citrus oils (family Rustaceae), whereas that in oil of citronella and oil of lemongrass (family Gramineae) is 96–100% *l*-limonene (Furia & Bellanca, 1975; Clayton & Clayton, 1981; Sax & Lewis, 1987; Mosandl *et al.*, 1990).

1.1.1 *Synonyms, structural and molecular formulae*

Chem. Abstr. Serv. Reg. No.: 5989-27-5
Deleted CAS Reg. Nos.: 7705-13-7; 95327-98-3
Chem. Abstr. Name: (R)-1-Methyl-4-(1-methylethenyl)cyclohexene
Synonyms: Cajaputene; carvene; cinene; (+)-dipentene; *d*-(+)-limonene; D-(+)-limonene; (+)-limonene; (R)-limonene; (R)-(+)-limonene; (+)-*para*-mentha-1,8-diene; (R)-(+)-*para*-mentha-1,8-diene; 1-methyl-4-isopropenyl cyclohexene-1; Refchole



$C_{10}H_{16}$ Mol. wt: 136.24

1.1.2 *Chemical and physical properties*

(*a*) *Description*: Colourless liquid (Sax & Lewis, 1987; Budavari, 1989) with a pleasant, lemon-like odour (US National Toxicology Program, 1990)
(*b*) *Melting-point*: -74.3 °C (Lide, 1991)
(*c*) *Boiling-point*: 175.5–176 °C (Budavari, 1989)

(*d*) *Density*: 0.8411 g/cm^3 at 20 °C/4 °C (Sax & Lewis, 1987)
(*e*) *Solubility*: Insoluble in water; soluble in benzene, carbon tetrachloride, diethyl ether, ethanol and petroleum ether (Lide, 1991; STN International, 1992); slightly soluble in glycerine (Flavor & Extract Manufacturers' Association, 1991)
(*f*) *Refractive index*: n_D^{20}, 1.4730 (Lide, 1991)
(*g*) *Optical rotation*: $[\alpha]_D^{20}$ + 125.6° (Lide, 1991)
(*h*) *Spectroscopy data*: Infrared, nuclear magnetic resonance and mass spectral data have been reported (Aldrich Chemical Co., 1992; STN International, 1992).
(*i*) *Stability*: Oxidizes to film in air (Sax & Lewis, 1987); must be stored away from light and air at –18 °C (Ranganna *et al.*, 1983)

1.1.3 *Trade names, technical products and impurities*

d-Limonene is available commercially in an untreated 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 (Florida Chemical Co., 1991a,b,c).

1.1.4 *Analysis*

Bertsch *et al.* (1974) described an analytical method in which trace quantities of organic materials, including limonene, in air are adsorbed on a porous polymer and separated by capillary gas chromatography (GC).

d-Limonene has been measured in a range of natural products, such as orange juice, by GC and head-space analysis (Massaldi & King, 1974; Marsili, 1986) and in packaging materials by thermal desorption (Lloyd, 1984). Searle (1989) described a procedure for monitoring airborne limonene vapour by GC (detection limit, 5 μg). GC with flame ionization detection has been used to analyse carrot volatiles collected on porous polymer traps. Samples were ground (blending), sliced or grated, and volatiles were collected on the polymer traps and eluted for analysis (Simon *et al.*, 1980).

Oil recoverable by distillation from orange, tangerine and grapefruit juices is at least 98% *d*-limonene. The *d*-limonene content of such oils has been determined by co-distillation with isopropanol, acidification and titration with potassium bromide–potassium bromate solution (Boland, 1984). The distribution of optical isomers of limonene has been determined in various essential oils using multidimensional GC, by coupling chiral and nonchiral columns (Mosandl *et al.*, 1990).

1.2 Production and use

1.2.1 *Production*

d-Limonene was first recovered as a commercial product during the 1941–42 Florida (USA) citrus season, from the steam evaporater condensate in the production of citrus molasses. By 1946, commercial production in Florida was common (Schulz, 1972).

The principal sources of *d*-limonene are the oils of orange, grapefruit and lemon (Verghese, 1968). It is the main volatile constituent of citrus peel oil, and the collected volatile portion of oil is usually referred to as *d*-limonene in the trade (Gerow, 1974). *d*-Limonene may be obtained by steam distillation of citrus peels and pulp resulting from the production of juice and cold-pressed oils or from deterpenation of citrus oils. It is sometimes redistilled (Furia & Bellanca, 1975).

Citrus peel oil can contain up to 95% *d*-limonene and stripper oil over that amount. Stripper oil is the oil recovered during concentration of the liquor which separates from the peel during pressing. The press liquor is concentrated to give citrus molasses, and the vapour which separates during concentration is condensed to yield stripper oil. In commercial practice, the essential peel oil is extracted by mechanical rupturing of oil sacs in the sub-epidermal layer (flavedo) of the peel and expression of the oil as an aqueous emulsion, from which it is separated by centrifuging (Ranganna *et al.*, 1983).

d-Limonene also occurs in other oils and essences obtained during the processing of citrus juice, including: juice oil, deoiler oil (oil separated from juice by centrifuging or decantation), essence oil (oil obtained from the recovery unit during concentration of fruit juices) and aroma oil (oil obtained by distillation of the aqueous discharge from the centrifuge used to separate pressed oil) (Ranganna *et al.*, 1983).

Annual worldwide production of *d*-limonene and orange oil/essence oil (95% *d*-limonene) has recently been approximately 45 000 tonnes. Citrus plantings under way in southern Florida, Brazil, Venezuela, Mexico, the Caribbean basin and elsewhere are expected to increase that figure to 73 000 tonnes annually within a decade (Florida Chemical Co., 1991a). The production of *d*-limonene in Florida in 1989 and 1990 was estimated to be 8600 and 6800–7700 tonnes, respectively (Anon., 1989). Limonene production in Florida in 1971 was estimated at approximately 4500 tonnes; an additional 450 tonnes of terpenes were recovered from 'folding' cold pressed oils (Schulz, 1972). In 1990, 450 tonnes of *d*-limonene were produced in California (USA), and Brazilian production was estimated to have been between 4100 and 8200 tonnes. Brazilian *d*-limonene is used almost exclusively by resin producers (Topfer, 1990).

In 1990, Brazil was the largest producer of orange oil, while Florida led in production of tangerine and grapefruit oil (Anon., 1990); Mexico supplies $\geq$ 80% of the world's lime oil (Anon., 1988a). In 1979, Italian production of essential oil from citrus fruit was as follows: oranges, 380 tonnes; lemon, 550 tonnes; bergamot, 100 tonnes; and mandarine, 30 tonnes (Anon., 1981).

Table 1 shows US imports of four citrus oils in 1981–90 and the major sources of the oils. In 1985–87, average US exports of orange oil were 1500 tonnes per year (Anon., 1988b). In 1983, Japan imported 200 tonnes of lemon oil (Anon., 1984).

1.2.2 *Use*

For nearly 50 years, *d*-limonene and orange oil/essence oil (95% *d*-limonene) have been used widely as flavour and fragrance additives in perfume, soap, food and beverages. *d*-Limonene has been used in non-alcoholic beverages, ice cream and ices, sweets, baked goods, gelatins and puddings, and chewing gum. It is also used as a chemical intermediate in the production of *l*-carvone, in terpene resin manufacture as a wetting and dispersing agent

Table 1. US imports of citrus oils

Year	Type of oil	Amount (thousand tonnes)	Major sources (decreasing order)
1981	Orange	2.1	Brazil
	Lemon	0.3	Argentina, Italy
	Lime	0.5	Mexico, Brazil, Haiti, Peru
	Grapefruit	0.04	Israel, Belize
1985	Orange	4.5	Brazil, Israel, Belize
	Lemon	1.0	Argentina, Italy
	Lime	0.6	Mexico, Peru, Brazil, Haiti
	Grapefruit	0.09	Israel, Brazil, Belize
1990	Orange	6.5	Brazil, Mexico
	Lemon	1.4	Argentina, Italy, Spain
	Lime	0.9	Mexico, Peru
	Grapefruit	0.3	Israel

From Yokoyama *et al.* (1988); US Department of Commerce (1991)

and in the preparation of sulfurized terpene lubricating oil additives. *d*-Limonene is also an important organic monomer in the synthesis of tackifying resins for adhesives. It has been used as a solvent, cleaner and odour in, e.g., the petroleum industry (Schulz, 1972; Furia & Bellanca, 1975; Sax & Lewis, 1987; Florida Chemical Co., 1991a,b).

Because *d*-limonene is a natural product with low toxicity for mammals and high acute toxicity for bark beetles, fruit flies and cat fleas, it has been proposed as an alternative to synthetic insecticides. Karr and Coats (1988) found that *d*-limonene had limited insecticidal properties against German cockroaches, house flies, rice weevils and corn rootworms. It has been used in shampoos and sprays for the control of fleas on dogs and cats; one such product reportedly contained 78.2% *d*-limonene (Hooser *et al.*, 1986; Hooser, 1990).

d-Limonene has been used to dissolve retained cholesterol gallstones postoperatively (Igimi *et al.*, 1991).

1.3 Occurrence

1.3.1 *Foods and botanical species*

Limonene is widely distributed among citrus and other plant species. It has been reported in more than 300 essential oils, at concentrations up to 90–95%, and at lesser, although still appreciable, concentrations in foods (e.g., 800 mg/l in non-alcoholic beverages, 3000 mg/kg in chewing gum) (Flavor and Extract Manufacturers' Association, 1991) (Table 2). Botanical species in which limonene occurs and which are used in pharmaceutical and para-pharmaceutical products, cosmetics, foods and beverages are presented in Table 3.

Table 2. Some food products containing *d*-limonene

Source	Concentration (ppm [mg/kg or mg/l])
Orange juice	0.4–219
Orange peel oil	740 000–970 000
Lemon oil	484 000
Lemon peel oil	520 000–810 000
Grapefruit juice	15.7–86
Grapefruit peel oil	837 000–973 000
Mandarine peel oil	660 000
Lime peel oil (cold press)	437 000–681 000
Lime peel oil (distilled)	428 000–523 000
Pomelo peel oil	861 900
Bilberry	0.08
Bilberry juice	Trace–0.007
Cranberry juice	0.02–0.12
Black currant	0.30
Currant leaves	5.6
Currant buds	77
Guava pulp	0.0002–30
Muscat grape	0.07–0.11
Papaya pulp	0.1–0.5
Peach	0.26–2600
Raspberry	Trace–0.1
Carrot	0.06–15.2
Celery leaves (fresh)	214
Celery root	0.8–26.8
Celery oil	128 000–150 000
Capsicum annuum (bell pepper)	0.2
Aniseed oil	3100 (67 in seeds)
Cinnamomum zeylanicum bark oil	Trace–5000
Nutmeg oil	20 000–130 000
Cumin oil	5000
Yellow ginger	7000
Pepper	2550–9950
Pepper oil	222 000
Black pepper oil	263 000
Chicken (heated)	0.0006–0.007
Coffee	1.7
Green tea	1.0
Mango	0.004–40
Dill herb	3.3–51
Dill root	48
Dill seed	2522
Kiwifruit	Trace
Illicium anisatum oil	15 000
Mentha pulegium oil	410

Table 2 (contd)

Source	Concentration (ppm [mg/kg or mg/l])
Origanum oil	4000
Bergamot oil	250 000–320 000

From Flavor and Extract Manufacturers' Association (1975); Shaw (1979); Saleh *et al.* (1985); Maarse & Visscher (1988); Flavor and Extract Manufacturers' Association (1991)

Table 3. Occurrence of limonene in various botanical species

Common name Scientific name Synonyms Family	Plant part used	Volatile oil (%) (limonene)[a]	Country or region of cultivation or growth
Angelica *Angelica archangelica* L. Garden angelica, European angelica Umbelliferae or Apiaceae	Rhizome, root, fruit, stem	0.3–1% (major)	Belgium, Hungary, Germany
Anise *Pimpinella anisum* L. Aniseed, sweet cumin, illicium, Chinese anise Umbelliferae or Apiaceae	Dried fruit	1–4% (constituent)	Widely cultivated
Sweet bay *Laurus nobilis* L. Laurel, Grecian laurel Lauraceae	Dried leaf	0.3–3.1% (minor)	Widely cultivated
West Indian bay *Pimenta racemosa* L. Mill. Myrcia, bay rum tree Myrtaceae	Leaf	3.9% (minor)	Venezuela, Puerto Rico, Caribbean Islands
Bergamot *Citrus bergamia* Rutaceae	Fruit peel	– (minor)	Italy
Bois de Rose oil *Aniba rosaeodora* Ducke Rosewood oil, Cayenne rosewood oil Lauraceae	Wood	– (minor)	Amazon region, wild
Buchu *Agathosma betulia* or *A. crenulata* Bookoo, buku, diosma Rutaceae	Dried leaf	1.0–3.5% (major, *d*-)	Cape Province, South Africa
Cananga oil *Cananga odorata* Annonaceae	Flower	– (major)	Java, Malaysia, Philippines, Moluccas

Table 3 (contd)

Common name Scientific name Synonyms Family	Plant part used	Volatile oil (%) (limonene)[a]	Country or region of cultivation or growth
Caraway *Carum carvi* L. Caraway fruit, carum Umbelliferae or Apiaceae	Dried fruit	2–8% (40%)	Widely cultivated
Cardamom *Elettaria cardamomum* L. Cardamom seed Zingiberaceae	Dried fruit, seed	3–8% (minor)	India, Sri Lanka, Lao People's Democratic Republic, Guatemala, El Salvador
Carrot *Daucus carota* L. Queen Anne's lace, carrot, wild carrot Umbelliferae or Apiaceae	Dried fruit, root	– (constituent)	Widely cultivated
Cascarilla *Croton eluteria* L. Sweetwood bark, sweetbark Euphorbiaceae	Dried bark	1.5–3% (major, *d*-)	West Indies, Mexico, Colombia, Ecuador
Celery *Apium graveolens* L. Celery seed, celery fruit Umbelliferae or Apiaceae	Seed (dried fruit)	2% (60%, *d*-)	France, India
Citronella *Cymbopogon nardus* L., *C. winterianus* Ceylon or Lenabatu citronella oil, Java or Maha Pengiri citronella oil Gramineae	Dried grass	– (constituent)	Sri Lanka, Java, Taiwan, Hainan Island, Malaysia, Africa, Central America, South America
Clary sage *Salvia sclarea* L. Clary wort, muscatel sage, clear eye, see bright Labiatae or Lamiaceae	Flowering top, leaf	0.1–0.15% (minor)	Widely cultivated
Coriander *Coriandrum sativum* L. Umbelliferae or Apiaceae	Dried fruit, leaf	0.2–2.6% (constituent, *d*-)	Widely cultivated
Cubebs *Piper cubeba* L. Cubeba, tailed pepper Piperaceae	Dried fruit	10–20% (constituent)	Southeast Asia
Cumin *Cuminum cyminum* L. Cummin, cumin seed Umbelliferae or Apiaceae	Dried fruit	2–5% (constituent)	Egypt, Iran, India, Morocco, Turkey, former USSR

Table 3 (contd)

Common name Scientific name Synonyms Family	Plant part used	Volatile oil (%) (limonene)[a]	Country or region of cultivation or growth
Dill, Indian dill *Anethum graveolens* L., *A. sowa* European Indian dill, East Indian dill Umbelliferae or Apiaceae	Dried fruit/ whole herb	1.2–7.7%/ 2.5–4% (major, *d*-)	Widely cultivated, India, Japan
Eucalyptus *Eucalyptus globulus* Blue gum, fever tree, gum tree Myrtaceae	Leaf	0.5–3.5% (major, *d*-)	Widely cultivated
Fennel *Foeniculum vulgare* Florence fennel, finocchio Umbelliferae or Apiaceae	Dried fruit	1.5–8.6% (2–6%) (constituent)	Widely cultivated
Galbanum *Ferula gummosa* Galbanum resin, galbanum gum Umbelliferae or Apiaceae	Exudate from stem	5–26% (major, *d*-)	Middle East, western Asia
Grapefruit *Citrus paradisi* Rutaceae	Fruit peel	– (90%)	USA, West Indies, Brazil, Israel, Portugal, Nigeria
Hops *Humulus lupulus* L. European hops, common hops Moraceae or Cannabaceae	Strobile	0.3–1% (minor)	Widely cultivated
Horehound *Marrubium vulgare* L. Marrubium, hoarhound Labiatae or Lamiaceae	Flowering top, dried leaf	Trace (constituent)	Europe, Asia, North America
Juniper *Juniperus communis* L. Juniper berries Cupressaceae	Dried cone	0.2–3.42% (1–2%) (minor)	Italy, Hungary, France, former Yugoslavia, Austria, former Czechoslovakia, former USSR, Germany, Poland, Spain
Labdanum *Cistus ladaniferus* L. Ambreine, rockrose Cistaceae	Leaf, twig	– (constituent)	Mediterranean region
Lavender, spike lavender *Lavandula angustifolia*, *L. latifolia* Garden lavender, aspic Labiatae or Lamiaceae	Flowering top/ dried flower	0.5–1.5%/ 0.5–1% (constituent)	Mediterranean region

Table 3 (contd)

Common name Scientific name Synonyms Family	Plant part used	Volatile oil (%) (limonene)[a]	Country or region of cultivation or growth
Lemon *Citrus limon* L. Cedro oil Rutaceae	Fruit, peel, leaf, twig	– (70%)	Widely cultivated, especially in USA, Italy, Cyprus, Guinea
Lime *Citrus aurantifolia* Rutaceae	Fruit, peel	– (major, *d*-)	Florida, West Indies, Central America
Mint *Mentha piperita* L., *M. spicata* L. Peppermint, spearmint Labiatae or Lamiaceae	Dried leaf/ whole herb	0.1–1%/ 0.7% (minor/minor)	Widely cultivated, especially in USA, Japan, Taiwan, Brazil
Myrrh *Commiphora molmol* Myrrha, gum myrrh Burseraceae	Exudate from bark	1.5–17% (constituent)	Northeast Africa, southwest Asia, especially in Yemen, Somalia, Ethiopia
Olibanum *Boswellia carteri* Frankincense, olibanum gum Burseraceae	Exudate from bark	3–10% (major)	Red Sea region, northeast Africa
Orange (bitter) *Citrus aurantium* L., *C. vulgaris*, *C. bigaradia* Rutaceae	Fruit, peel, flower, leaf, twig	1–2.5% (major, *d*-)	China, southern Europe, USA
Orange (sweet) *Citrus sinensis* L. Rutaceae	Fruit, peel	1.5–2% ($\geq$ 90%, *d*-)	Widely cultivated, especially in the USA, Mediterranean countries, Brazil
Pepper *Piper nigrum* L. Black pepper, white pepper Piperaceae	Dried fruit	2–4% (constituent)	India, Indonesia, Malaysia, China
Pine needle *Pinus mugo*, *P. sylvestris* L. Dwarf pine, Scotch pine Pinaceae	Leaf, twig	– (major, *d*-)	Europe, USA, western Asia
Rosemary *Rosmarinus officinalis* L. Labiatae or Lamiaceae	Flowering top, dried leaf	0.5% (major)	Widely cultivated, especially in California, United Kingdom, France, Spain, Portugal, Yugoslavia, Morocco, China
Rue *Ruta graveolens* L. Common rue, garden rue Rutaceae	Whole herb	0.1% (minor)	Widely cultivated

Table 3 (contd)

Common name Scientific name Synonyms Family	Plant part used	Volatile oil (%) (limonene)[a]	Country or region of cultivation or growth
Sage *Salvia lavandulaefolia* Spanish sage, Dalmatian sage Labiatae or Lamiaceae	Leaf	- (1–41%)	Spain, France
Savory *Satureja hortensis* L., *S. montana* L. Summer savory, winter savory Labiatae or Lamiaceae	Dried leaf/ stem	1%/1.6% (major)	Europe, USA, Mediterranean region
Tagetes *Tagetes erecta, T. patula, T. minuta* African marigold, Aztec marigold, French marigold, Mexican marigold Compositae or Asteraceae	Whole herb	- (constituent)	Widely cultivated
Tamarind *Tamarindus indica* L. Tamarindo Leguminosae or Fabaceae	Dried fruit	- (constituent)	Widely cultivated

From Leung (1980); -, not available

[a]Limonene content in the volatile oil, expressed as percentage (when available), otherwise noted as a constituent, major (constituent) or minor (constituent); stereoisomer not given if unspecified

Frozen reconstituted orange juice samples contained 219 ppm (mg/l) limonene [isomer unspecified]. Oxidation did not occur to a significant extent over four weeks of storage in glass (Marsili, 1986). When citrus juices are packed aseptically into laminated cartons, the *d*-limonene content of the juice is reduced by about 25% within 14 days' storage owing to absorption by the polyethylene (Mannheim *et al.*, 1987). Limonene has been found in packaging material at 25 ppm (μg/g) (Lloyd, 1984).

Limonene [isomers unspecified] was found to represent 32.4% of total terpenes in *Pinus greggii* and 0.5% in *P. pringlei*, two pine species indigenous to Mexico (Lockhart, 1990).

Daily US per-caput consumption of *d*-limonene, as a result both of its natural occurrence in food and of its presence as a flavour, was estimated to be 0.27 mg/kg bw per day for a 60-kg individual (Flavor and Extract Manufacturers' Association, 1991). Intake of *d*-limonene can vary considerably, however, depending on the types of food consumed. Citrus juice products are among the richest sources of *d*-limonene: intake owing to consumption of these products may approach 1 mg/kg bw per day for adults and 2 mg/kg bw per day for young children (US Department of Agriculture, 1982).

Annual US consumption of *d*-limonene from a variety of foods was calculated as follows: carrots, 5879 kg; celery leaves, 165 379 kg; heated chicken, 3.5 kg; roasted coffee, 1896 kg; cranberries, 3.0 kg; muscat grapes, 1.2 kg; grapefruit juice, 254 320 kg; lemon oil, 465 750 kg; mango, 736 kg; nutmeg, 17 250 kg; orange juice, 154 560 kg; oregano, 508 kg; peaches,

209 kg); pepper, 234 312 kg; raspberries, 2.5 kg; and green tea, 184 kg. Total annual US consumption of *d*-limonene as a result of its natural occurrence in these foods was 1300 tonnes. A survey in 1982 indicated that annual US consumption of *d*-limonene as a flavouring additive was 68 tonnes (Stofberg & Grundschober, 1987).

1.3.2 *Air*

d-Limonene was detected in the air of 81% of the mobile homes surveyed in Texas (USA) during a survey of air quality. Levels of *d*-limonene analysed by GC–mass spectrometry were 0.01–29 ppb [0.06–162 μg/m^3], with a mean of 2.2 ppb [13 μg/m^3] (Connor *et al.*, 1985). Limonene [isomer unspecified] was detected at 0–5.7 ppb [32 μg/m^3] in air samples taken at various locations around Houston, Texas. It was present in all of the more than 150 samples analysed by GC–mass spectrometry over a 15-month period (Bertsch *et al.*, 1974).

1.3.3 *Biological fluids*

Limonene [isomer unspecified] has been detected in human urine (Zlatkis & Liebich, 1971), as have its metabolites (Kodama *et al.*, 1974).

1.4 Regulations and guidelines

d-Limonene is generally recognized as safe for human consumption as a synthetic flavouring substance by the US Food and Drug Administration (1991).

2. Studies of Cancer in Humans

No data were available to the Working Group.

3. Studies of Cancer in Experimental Animals

3.1 Oral administration

3.1.1 *Mouse*

Groups of 50 male and 50 female B6C3F$_1$ mice, eight to nine weeks of age, received 0, 250 or 500 (males) and 0, 500 or 1000 (females) mg/kg bw *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) (US National Toxicology Program, 1990).

3.1.2 *Rat*

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 bw *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 (US National Toxicology Program, 1990).

3.2 Intraperitoneal administration

Mouse

In a screening assay based on the accelerated induction of lung tumours in a strain highly susceptible to development of this neoplasm, groups of 15 male and 15 female A/He mice, six to eight weeks old, received intraperitoneal injections of 0.2 g/kg bw or 1 g/kg bw (maximal tolerated dose) *d*-limonene in tricaprylin [purity 85-99%] three times per week for eight weeks (total doses, 4.8 and 24 g/kg bw). Vehicle control groups of 80 males and 80 females received intraperitoneal injections of 0.1 ml tricaprylin on the same schedule. The experiment was terminated 24 weeks after the first injection, and the lungs were removed and surface nodules counted. Survival was comparable between the groups. Lung tumour incidence was not increased; males—control, 22/77 (28%), low-dose, 1/15 (7%); and high-dose, 3/15 (20%); females—control, 15/77 (20%); low-dose, 2/15 (13%); and high-dose, 2/13 (15%) (Stoner *et al.*, 1973).

3.3 Administration with known carcinogens

3.3.1 *Oral administration*

(*a*) *Mouse*

Groups of 20–30 [exact number unspecified] male and female (both sexes being represented almost equally) stock albino mice [strain unspecified], > 6–8 weeks of age, received a single dose of 50 μg benzo[*a*]pyrene in 0.2 ml polyethylene glycol or polyethylene glycol alone by stomach tube and no further treatment, or they subsequently received 40 weekly intubations of 0.05 ml *d*-limonene [concentration unspecified] contaminated with < 0.1% *para*-cymene. A further group of 20–30 male and female mice served as untreated controls. In the group that received benzo[*a*]pyrene plus *d*-limonene, 5/23 mice that survived > 60 days and were autopsied within 24 h after death had a total of eight forestomach papillomas; in the group that received benzo[*a*]pyrene alone, 2/17 mice had a total of two forestomach papillomas; and in the group that received polyethylene glycol and *d*-limonene, 2/15 mice had a total of three forestomach papillomas. None of the 18 mice that survived more than 60 days in the untreated control group had a forestomach tumour (Field & Roe, 1965). [The Working Group noted the limited reporting.]

Two groups of 15 female A/J mice, nine weeks of age, were administered 0.2 mmol [27.3 mg] *d*-limonene (99% pure) in 0.2 ml cottonseed oil or cottonseed oil alone by oral gavage once a week for eight weeks; 1 h later they received a gavage of 20 mg/kg bw

N-nitrosodiethylamine (NDEA) in 0.2 ml water. Mice were autopsied 26 weeks after the first dose of NDEA, but only forestomachs and lungs were examined for tumours. All of the animals that received cottonseed oil plus NDEA had forestomach papillomas; 11/15 (73%) had more than 30 papillomas/stomach, and 4/15 (27%) also had carcinomas of the forestomach. In addition, the average number of pulmonary adenomas/mouse in this group was 10.4. Of the animals that received *d*-limonene prior to NDEA, only 10/15 (67%) had stomach papillomas ($p < 0.05$, two-sided U-test of Wilcoxon, Mann and Whitney); no mouse had more than 30 papillomas/stomach ($p < 0.001$), and none was shown to have a carcinoma of the forestomach ($p < 0.05$). The average number of pulmonary adenomas/mouse in this group was 6.5 ($p < 0.05$) (all p values are *versus* vehicle controls) (Wattenberg *et al.*, 1989). [The Working Group noted the limited number of tissues examined.]

Two groups of female A/J mice, nine weeks of age, received oral intubations of 0.5 mg/mouse 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), in 0.1 ml tricaprylin twice a week for eight weeks. One hour prior to each administration of NNK, one group of 15 mice each received 25 mg *d*-limonene (99% pure) in 0.2 ml cottonseed oil by oral gavage and one group of 20 mice received cottonseed oil alone. Mice were autopsied 28 weeks after the initial administration of NNK, but only stomachs and lungs were examined for tumours. Of the animals that received cottonseed oil plus NNK, 90% (18/20) had forestomach papillomas, with an average of 2.8 papillomas/mouse; one mouse also had a stomach carcinoma. In the group that received *d*-limonene plus NNK, no forestomach papilloma or carcinoma was observed ($p < 0.001$). Mice that received cottonseed vehicle plus NNK had 50.8 ± 2.6 (standard error) pulmonary adenomas/mouse, while those that received *d*-limonene plus NNK had only 15.3 ± 2.1 pulmonary adenomas/mouse ($p < 0.001$) (Wattenberg & Coccia, 1991). [The Working Group noted the limited number of tissues examined.]

Two groups of female A/J mice, nine weeks of age, each received an intraperitoneal injection of 2 mg NNK in 0.1 ml saline. One hour prior to the injection of NNK, one group of 14 mice each received 25 mg *d*-limonene (99% pure) in 0.2 ml cottonseed oil by oral gavage and one group of 15 mice received cottonseed oil alone. Mice were autopsied 28 weeks after the initial injection of NNK, but only stomachs and lungs were examined. Neither group of mice developed stomach papillomas, but mice that received cottonseed oil plus NNK had an average of 11.2 ± 1.1 pulmonary adenomas/mouse, while those that received *d*-limonene plus NNK had an average of 2.5 ± 0.7 pulmonary adenomas/mouse ($p < 0.001$) (Wattenberg & Coccia, 1991). [The Working Group noted the limited number of tissues examined.]

(*b*) *Rat*

Groups of 25 female Sprague-Dawley rats, 47 days old, were fed diets containing 0, 1000 or 10 000 mg/kg *d*-limonene (> 99% pure) one week prior to a single oral administration by gavage of 65 mg/kg bw 7,12-dimethylbenz[*a*]anthracene (DMBA) in sesame oil [volume unspecified]. The diets were continued for a further 27 weeks, during which time the animals were weighed weekly and palpated for tumours. The experiment was terminated 27 weeks after DMBA administration. In animals fed *d*-limonene, the time to development of the first mammary tumour was reported to have been longer than that in controls (Elegbede *et al.*, 1984). [The Working Group noted that the number of tumour-bearing animals was not given.]

Groups of 30 six-week-old female Sprague-Dawley rats were fed diets containing 5% *d*-limonene [purity unspecified] for one week before administration by gastric intubation of a single dose of 65 mg/kg bw DMBA. The *d*-limonene diet was continued for a further week, after which time animals were returned to basal diet. A second group of 30 females received DMBA by gastric intubation, followed one week later by administration of 5% *d*-limonene in the diet for 25 weeks. A third group of 30 females received DMBA and was maintained on basal diet, thus serving as controls. The experiment was terminated 25 weeks after the DMBA treatment. Tumours larger than 350 mm^3 were surgically resected, and all resected tumours and those found at necropsy were examined histologically. More than 95% of the mammary tumours were carcinomas. In animals that received *d*-limonene one week prior to and one week after DMBA, tumour latency was significantly increased ($p < 0.005$); no effect on latency was seen in animals fed *d*-limonene one week after DMBA for 25 weeks. The number of tumours per rat was reduced in both groups fed *d*-limonene ($p < 0.05$) (Elson *et al.*, 1988). [The Working Group noted the lack of detailed reporting.]

In a study reported as a short communication, groups of female Wistar Furth rats [number per group unspecified], 64–69 days old, were fed diets containing 5% *d*-limonene [purity unspecified], 5% orange oil [percentage of *d*-limonene unspecified] or basal diet throughout the study. After two weeks, the animals received a single intravenous injection of 50 mg/kg bw *N*-methyl-*N*-nitrosourea (MNU). All animals survived to the end of the experiment at 23 weeks. Feeding of both orange oil and *d*-limonene decreased tumour incidence ($p < 0.001$): At the end of the experiment, 80% of controls had mammary carcinomas, while the incidence in orange oil-treated animals was 47% and that in *d*-limonene-treated animals was 45% (Maltzman *et al.*, 1989). [The Working Group noted the lack of detailed reporting.]

In a study reported in a brief communication, groups of female Wistar Furth rats [numbers unspecified], 64–69 days old, were fed diets containing 5% *d*-limonene [purity unspecified] or basal diet. After two weeks, the animals received a single intravenous injection of 50 mg/kg bw MNU, and the *d*-limonene diet was continued for a further week, after which time the rats were returned to basal diet. A further group of females [number unspecified] received a single intravenous injection of MNU and one week later were fed diets containing 5% *d*-limonene until the end of the experiment. All animals survived to the end of the experiment at 23 weeks. Administration of *d*-limonene two weeks before and one week after MNU treatment did not affect the incidence or number of mammary tumours. Rats given *d*-limonene from one week after MNU treatment until the end of the experiment had about half the average number of tumours per rat as MNU-treated controls (Maltzman *et al.*, 1989). [The Working Group noted the lack of detailed reporting.]

Two groups, of 31 and 32 female Sprague-Dawley rats, six weeks old, were fed diets containing 0 and 1% (w/w) *d*-limonene (99.9% pure), respectively, for two weeks and then received 65 mg/kg bw DMBA (> 95% pure) once by gastric intubation; the two groups were continued on their respective diets for 20 weeks. *d*-Limonene caused a significant reduction in the incidence of mammary tumours: 58 in rats fed *d*-limonene plus DMBA (1.8 tumours/rat; median latency, 84 days) and 81 in rats fed DMBA alone (2.6 tumours/rat; median latency, 70 days). Two further groups of 52 female Sprague-Dawley rats, six weeks old, were fed diets containing 0 or 0.5% (w/w) *d*-limonene (99.9% pure) for two weeks and

then received 65 mg/kg bw DMBA once by gastric intubation; the diets were continued for a further week, after which time both groups were continued on basal diet until 20 weeks. Administration of *d*-limonene for two weeks prior to and one week after DMBA did not significantly reduce the incidence of mammary tumours: 156 in the *d*-limonene plus DMBA-treated rats (3.0 tumours/rat; median latency, 61 days) compared to 129 in rats fed DMBA alone (2.5 tumours/rat; median latency, 58 days) (Russin *et al.*, 1989).

Groups of 31–38 male Fischer 344 and 31–37 male NBR rats, eight weeks of age, were given 0 or 0.05% (500 mg/l) *N*-nitrosoethylhydroxyethylamine (NEHEA) in the drinking-water for two weeks; they were then given tap-water and treated by oral gavage with 150 mg/kg bw *d*-limonene (> 99% pure) in 3 ml/kg corn oil daily on five days a week for 30 weeks. The livers and kidneys were examined, and other tumours were noted grossly. In Fischer 344 rats, 9/31 treated with NEHEA and *d*-limonene had renal adenomas, compared with 1/30 treated with NEHEA alone and none of 31 rats given *d*-limonene alone and none of 31 untreated controls ($p < 0.05$). The numbers of atypical hyperplasias in the kidney were 15.5 ± 1.5/rat treated with NEHEA and *d*-limonene, 1.2 ± 0.2/rat treated with NEHEA alone, 0.4 ± 0.1/rat treated with *d*-limonene alone and none in untreated controls. NBR rats did not develop renal adenomas, and no difference in the number of atypical hyperplasias was seen after subsequent administration of *d*-limonene (0.2 ± 0.1/rat in both groups), after feeding of *d*-limonene alone (0.1 ± 0.0/rat) or after no treatment (0.1 ± 0.1/rat). In Fischer rats, but not in NBR rats, the number of liver tumorus was reduced by administration of *d*-limonene (Dietrich & Swenberg, 1991).

3.3.2 *Skin application*

The Working Group was aware of a series of studies on orange oil (which contains *d*-limonene) in which a promoting effect on mouse skin carcinogenesis initiated by DMBA was reported (Roe, 1959; Roe & Peirce, 1960). Because of limitations in the conduct and reporting of these studies, they were not reviewed.

Mouse

Groups of 50 female ICR/Ha Swiss mice, six to eight weeks of age, each received topical applications on shaved back skin three times a week for 440 days of 10 mg limonene [stereochemistry and purity unspecified] in 0.1 ml acetone, 10 mg limonene simultaneously with 5 μg benzo[*a*]pyrene in 0.1 ml acetone, 5 μg benzo[*a*]pyrene alone or 0.1 ml acetone alone. A group of 100 females served as untreated controls. Only tumours that persisted 30 days or more were counted in the cumulative totals. Animals that developed skin carcinomas were killed approximately two months after the tumours had been classified as malignant or when they were moribund. No skin tumour was seen at 440 days in untreated controls, vehicle controls or mice that received limonene alone. In the benzo[*a*]pyrene-treated group, 16/50 mice [number of survivors not specified] had a total of 26 papillomas and 12 carcinomas (first papilloma seen at 210 days). Of mice that received limonene plus benzo[*a*]pyrene, 13/50 [number of survivors unspecified] had a total of 13 papillomas and four carcinomas (first papilloma seen at 295 days). The authors concluded that limonene had slightly inhibited benzo[*a*]pyrene carcinogenesis (Van Duuren & Goldschmidt, 1976). [The Working Group noted that no statistical analysis was performed and that this conclusion appears to be based

on the lowered multiplicity of tumours and decreased number of malignant tumours in those given limonene.]

3.3.3 *Topical application and feeding*

Mouse

Groups of 24 female CD-1 mice, eight weeks of age, each received a single topical application of 0.2 μmol (51.2 μg) DMBA in 0.2 ml acetone or acetone alone on the shaved back. On day 7 after DMBA treatment, one DMBA-treated and one acetone-treated group were fed a diet containing 1% *d*-limonene (> 99% pure). On day 14 after DMBA treatment, three other groups received topical applications of 0.2 ml *d*-limonene (630 mmol, 1:1) in acetone, 10 nmol 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in acetone or acetone alone twice a week. Beginning seven weeks after DMBA treatment, the number of mice bearing papillomas and the number of papillomas were recorded weekly. The experiment was terminated 40 weeks after DMBA treatment. All mice that received DMBA plus TPA rapidly developed skin tumours, whereas mice that received TPA alone or DMBA alone did not. DMBA-treated mice fed 1% *d*-limonene in the diet did not develop skin tumours, nor did untreated mice that received an application of *d*-limonene alone on the skin. In DMBA-treated mice that received skin applications of *d*-limonene, the incidence of skin papillomas/mouse was increased slightly (Elegbede *et al.*, 1986). [The Working Group noted the lack of detailed reporting.]

3.3.4 *Subcutaneous injection*

Mouse

Three groups of 50 male C57Bl/6 Jax mice [age unspecified] each received a subcutaneous injection of 25 μg benzo[*rst*]pentaphene (dibenzo[*a*,*i*]pyrene; DBP) in 0.1 ml tricaprylin and, 24 h later, a subcutaneous injection (at about the same site) of 0.2 ml 75% (v/v) orange oil (containing about 45–50% *d*-limonene) or 75% (v/v) orange oil (containing about 85–90% *d*-limonene) or no additional treatment. A vehicle-control group of 50 males received an initial subcutaneous injection of 0.1 tricaprylin only. Two further groups of 50 male C57Bl/6 Jax mice [age unspecified] received a single subcutaneous injection of 0.2 ml 75% (v/v) orange oil (containing about 45–50% limonene) or 75% (v/v) orange oil (containing about 85–90%) with no DBP pretreatment. The animals were observed for up to two years. Neither tricaprylin alone nor either of the two orange oils alone caused subcutaneous tumours (survival at two years: tricaprylin, 33/50; 45% limonene, 26/50; 84% limonene, 30/50). Mice that received DBP alone had a higher incidence of subcutaneous tumours than did the group that also received orange oil containing 45–50% limonene, and the group that also received orange oil containing 85–90% limonene had an even lower incidence (Homburger *et al.*, 1971).

Groups of 50 male C57Bl/6 Jax mice [age unspecified] each received a subcutaneous injection of 25 μg DBP in 0.1 ml tricaprylin and, 24, 48, 72 and 96 h later, subcutaneous injections (at about the same site) of either 0.05 ml 75% (v/v) *d*-limonene in tricaprylin, 0.05 ml 75% (v/v) autoxidized *d*-limonene (containing 6% hydroperoxides) in tricaprylin, 0.05 ml hydroperoxides alone [75% solution in tricaprylin unspecified], 0.05 ml orange oil

(containing 85–90% limonene; control 1), 0.05 ml tricaprylin alone (control 2) or no further treatment (positive control). Tumour growth was reported to be reduced by *d*-limonene and by the hydroperoxides of *d*-limonene (Homburger *et al.*, 1971). [The Working Group noted the inadequate reporting.]

3.3.5 *Intravenous and intraperitoneal injection*

Mouse

Groups of 50 female A/Jax mice, two to three months old, each received a single subcutaneous injection of 500 μg DBP in 0.1 ml peanut oil or 0.1 ml peanut oil alone, followed after 24 h by weekly intravenous injections into the tail vein (later, intraperitoneal injections) of either 1% (v/v) orange oil (containing 45–50% limonene [stereoisomer, volume and diluent unspecified]), 1% (v/v) orange oil (containing 85–90% limonene) [volume and diluent unspecified] or 0.1 ml diluent. At the end of 13 weeks, the incidence of lung tumours was 21% in mice receiving peanut oil and diluent, 74% in mice receiving DBP plus diluent and 43 and 44% in mice receiving DBP plus either of the orange oils. In a second experiment, groups of 50 female A/Jax mice, seven weeks old, received a single subcutaneous injection of 500 μg/mouse DBP in 0.1 ml peanut oil or 0.1 ml peanut oil alone, followed after 24 h by weekly intravenous injections into the tail vein of either 1% (v/v) *d*-limonene, 1% (v/v) of its hydroperoxide or 0.1 ml diluent. At the end of 16 weeks, the incidences of lung adenomas were 27% in mice receiving peanut oil and diluent, 75% in mice receiving DBP and diluent, 40% in mice receiving DBP plus *d*-limonene and 50% in mice receiving DBP plus *d*-limonene hydroperoxide. The two orange oils and *d*-limonene hydroperoxide given alone without DBP pretreatment had no significant effect on the incidence of lung adenomas, whereas *d*-limonene alone reduced the incidence of lung adenomas from 27% (diluent controls) to 7% ($p < 0.05$) (Homburger *et al.*, 1971).

4. Other Relevant Data

4.1 Absorption, distribution, metabolism and excretion

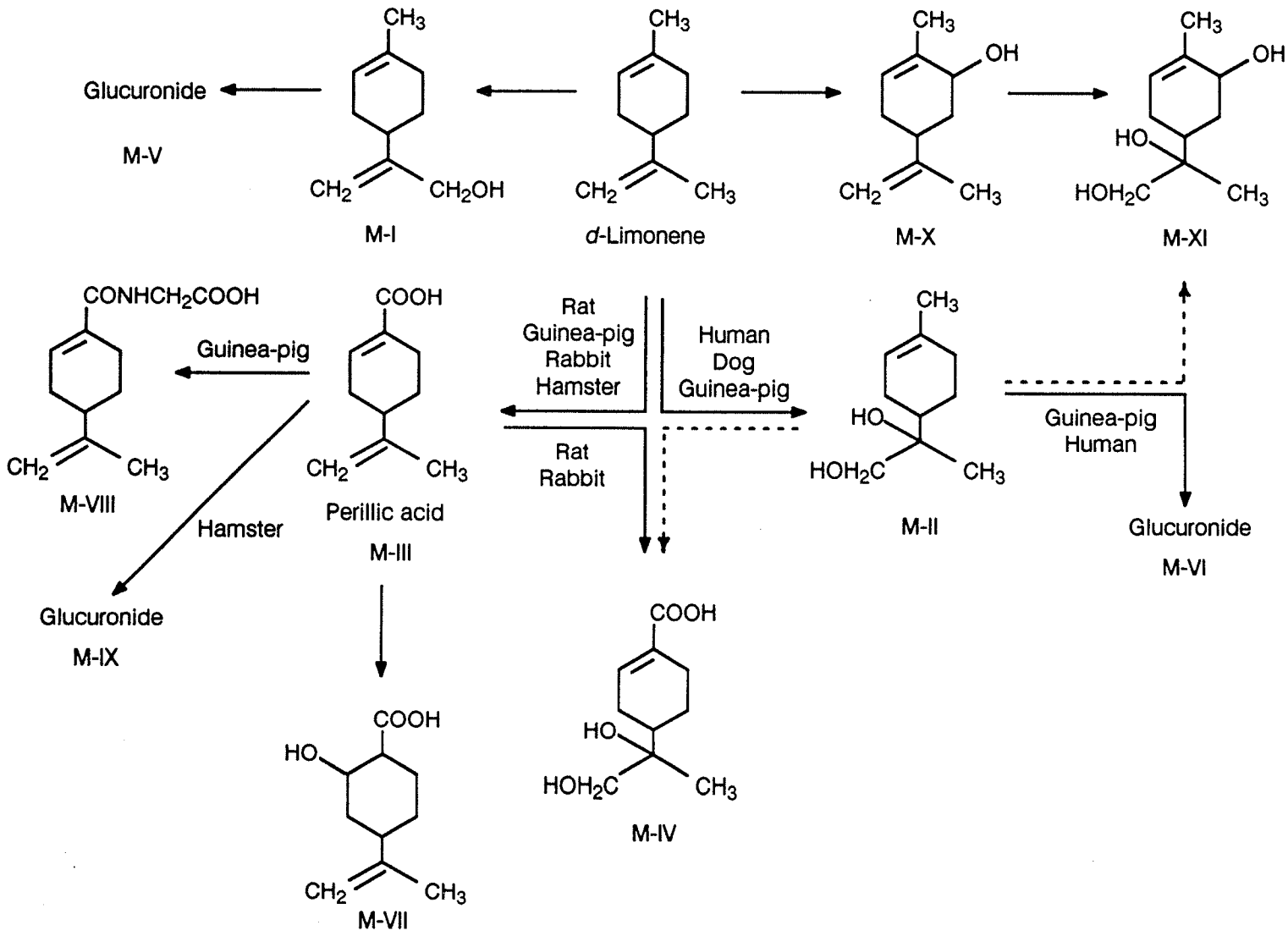
4.1.1 *Humans*

d-Limonene is absorbed in the gastrointestinal tract. Two male volunteers administered ^{14}C-*d*-limonene at 1.6 g orally excreted 55–83% of the dose in their urine within 48 h. The major urinary metabolite isolated was 8-hydroxy-*para*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid (M-VI, Fig. 1) (Kodama *et al.*, 1976).

4.1.2 *Experimental systems*

^{14}C-*d*-Limonene was absorbed rapidly following administration (800 mg/kg; 4.15 μCi/animal) by stomach tube to male Wistar rats. Radiolabel levels were maximal in the blood after 2 h; large amounts of radiolabel were also observed in the liver (maximal after 1 h) and the kidneys (maximal after 2 h). Negligible concentrations were found in blood and organs after 48 h (Igimi *et al.*, 1974).

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



From Kodama *et al.* (1976)

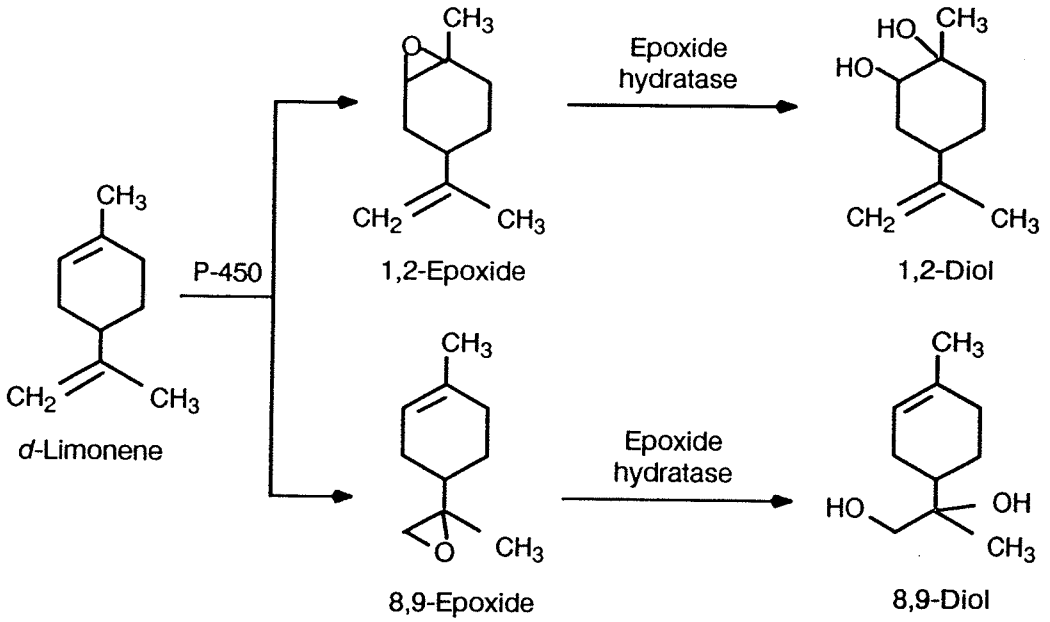
M-I, *p*-Mentha-1,8-dien-10-ol; M-II, *p*-menth-1-ene-8,9-diol; M-IV, perillic acid-8,9-diol; M-V, *p*-mentha-1,8-dien-10-yl-β-D-glucopyranosiduronic acid; M-VI, 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid; M-VII, 2-hydroxy-*p*-menth-8-en-7-oic acid; M-VIII, perillylglycine; M-IX, perillyl-β-D-glucopyranosiduronic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

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). Bile-duct cannulated rats administered *d*-limonene orally excreted 25% of the dose in the bile within 24 h (Igimi *et al.*, 1974).

Following oral administration of *d*-limonene to rabbits, the urinary metabolites isolated were *para*-mentha-1,8-dien-10-ol (M-I), *para*-menth-1-ene-8,9-diol (M-II), perillic acid (M-III), perillic acid-8,9-diol (M-IV), *para*-mentha-1,8-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) (see Fig. 1). Following oral administration of *d*-limonene to dogs and rats, a further five 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-1,8-dien-6-ol (M-X) and probably *para*-menth-1-ene-6,8,9-triol (M-XI). The major urinary metabolite was M-IV in rats and rabbits, M-IX in hamsters, M-II in dogs and M-VI in guinea-pigs (Kodama *et al.*, 1976).

d-Limonene was metabolized 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 (Watabe *et al.*, 1980, 1981) (See Fig. 2).

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



From Watabe *et al.* (1980)

4.2 Toxic effects

4.2.1 *Humans*

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

4.2.2 *Experimental systems*

LD_{50} values for *d*-limonene were reported in male and female mice to be 5.6 and 6.6 (oral), 1.3 and 1.3 (intraperitoneal) and > 41.5 and > 41.5 (subcutaneous) g/kg bw, respectively; those in male and female rats were reported to be 4.4 and 5.1 (oral), 3.6 and 4.5 (intraperitoneal), > 20.2 and > 20.2 (subcutaneous) and 0.125 and 0.11 (intravenous) g/kg bw, respectively (Tsuji *et al.*, 1975a). The acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits were reported to exceed 5 g/kg bw (Opdyke, 1975).

After daily oral administration of *d*-limonene at 277–2770 mg/kg bw to male and female Sprague-Dawley rats for one month, the highest dose was found to have caused a slight decrease in body weight and food consumption. On histological examination, granular casts were observed in the kidney of males, but no significant change was found in the other organs (Tsuji *et al.*, 1975a).

d-Limonene did not cause renal disease in NCI Black Reiter (NBR) male rats. These rats do not synthesize $\alpha_{2\mu}$-globulin, which is normally present in the hyaline droplets that are found in male Fischer 344 rats with *d*-limonene-induced nephrotoxicity (Dietrich & Swenberg, 1991).

A dose-related increase in relative liver and kidney weights was observed in young adult male Fischer 344 rats administered 75, 150 or 300 mg/kg bw *d*-limonene daily by gavage on five days per week and killed on study days 6 or 27. Dose-related formation of hyaline droplets was also observed in the kidneys. α_{2u}-Globulin was detected in larger amounts in the renal cortical tissue of animals treated with *d*-limonene than in controls. Alterations considered to be 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 (Kanerva *et al.*, 1987).

Chronic 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 mineralization and epithelial hyperplasia and increased severity of spontaneous nephropathy (US National Toxicology Program, 1990).

After single administration of 14, 41, 136 or 409 mg/kg bw *d*-limonene by gavage to male and female Sprague-Dawley rats, a dose-related accumulation of hyaline droplets was observed in proximal renal tubules only in male rats. After administration of ^{14}C-labelled *d*-limonene at 409 mg/kg bw, 2.5 times more radiolabel was accumulated in renal tissue of males than in females; the label was found to be reversibly bound to protein (Lehman-McKeeman *et al.*, 1989). In contrast, adult male and female beagle dogs administered *d*-limonene at 100 or 1000 mg/kg bw (maximal tolerated dose for emesis) per day by gavage twice daily for six months had increased kidney weights but showed no histopathological change, hyaline droplet accumulation or nephropathy (Webb *et al.*, 1990).

In a chronic toxicity study in beagle dogs, oral doses of more than 340 mg/kg bw (females) and 1000 mg/kg bw (males) per day for six months resulted in protein casts in the renal tubules. Daily doses of more than 1000 mg/kg bw (females) and 3024 mg/kg bw (males) resulted in slight weight loss in some animals due to frequent vomiting (Tsuji *et al.*, 1975b).

d-Limonene is a mildly toxic skin irritant when applied at full strength to intact or scratched rabbit skin for 24 h under occlusion (Opdyke, 1975). Continuous perfusion of

0.5% *d*-limonene (0.5–0.6 ml [420–504 mg]/min) into the gall-bladder of rabbits for 6 h irritated particularly the mucous membranes and the common bile duct (Tsuji *et al.*, 1975c).

4.2.3 *Mechanisms of toxicity*

Treatment of male rats with *d*-limonene leads to a characteristic nephrotoxicity, a key feature of which is the accumulation in proximal tubule cells of hyaline droplets containing $\alpha_{2\mu}$-globulin. $\alpha_{2\mu}$-Globulin is the major low-molecular-weight protein excreted in male rat urine; it is present at much lower levels in females.

A number of studies have shown that *d*-limonene and *d*-limonene-1,2-oxide bind specifically, but reversibly, to $\alpha_{2\mu}$-globulin; the binding of *d*-limonene-1,2-oxide resulted in reduced degradation of the protein by lysosomal proteinases *in vitro* (Lehman-McKeeman *et al.*, 1990). Cell death and proliferation were enhanced in renal tubules of Fischer 344 rats treated with *d*-limonene; no enhancement of cell proliferation occurred in NBR rats, which do not synthesize $\alpha_{2\mu}$-globulin (Dietrich & Swenberg, 1991).

In mature male rats, approximately 50 mg of $\alpha_{2\mu}$-globulin are filtered per day, about 40% being excreted in urine and 60% being reabsorbed and catabolized (Neuhaus *et al.*, 1981; Baetcke *et al.*, 1991). Female rats excrete 100–300 times less $\alpha_{2\mu}$-globulin in urine (Borghoff *et al.*, 1990; Baetcke *et al.*, 1991; Dietrich & Swenberg, 1991). Mice excrete large amounts of a structurally similar protein in a sex-dependent manner; however, this protein does not bind to *d*-limonene, nor is it reabsorbed in the kidney (Lehman-McKeeman & Caudill, 1992a).

$\alpha_{2\mu}$-Globulin belongs to a superfamily of proteins called lipocalins, which are widely distributed among mammalian species and bind, stabilize and transport hydrophobic ligands such as retinol and steroid hormones (Lehman-McKeeman & Caudill, 1992b). Normal human urine contains very little of this class of proteins, although a sex-dependent protein (urine protein 1) has been identified, which occurs in male urine at concentrations five times higher than in female urine. The concentration in male human urine is four to five times lower than that of $\alpha_{2\mu}$-globulin in male rat urine (Bernard *et al.*, 1989; Baetcke *et al.*, 1991). It is structurally related to rabbit uteroglobulin and not to the rat protein (Jackson *et al.*, 1988). It does not bind to *d*-limonene. Of the lipocalins studied, only that in male rat kidney binds to limonene, whereas those of mice, hamsters, guinea-pigs, dogs and humans do not (Lehmann-McKeeman & Caudill, 1992b).

4.3 Reproductive and developmental toxicity

4.3.1 *Humans*

No data were available to the Working Group.

4.3.2 *Experimental systems*

Mice, rats and rabbits were treated orally during the period of organogenesis with daily doses of *d*-limonene up to 2363 mg/kg bw (mice), 2869 mg/kg bw (rats) and 1000 mg/kg bw (rabbits). The highest dose was lethal to < 40% of pregnant rabbits, and several rat dams died. The studies consistently showed impaired weight gain in the dams and delayed prenatal

development, which was restored to normal during the postnatal period. In mice and rabbits, anomalies of the ribs were observed in the offspring (Tsuiji *et al.*, 1975d; Kodama *et al.*, 1977a, b).

4.4 Genetic and related effects

4.4.1 *Humans*

No data were available to the Working Group.

4.4.2 *Experimental systems* (see also Table 4 and Appendices 1 and 2)

d-Limonene was not mutagenic to *Salmonella typhimurium*. In single studies, it did not induce differential toxicity in *Bacillus subtilis* strains, sister chromatid exchange or chromosomal aberrations in Chinese hamster ovary cells, trifluorothymidine resistance in mouse lymphoma L5178Y cells or transformation in rat tracheal cells *in vitro*. *d*-Limonene-1,2-oxide did not induce unscheduled DNA synthesis in primary cultures of rat hepatocytes.

5. Summary of Data Reported and Evaluation

5.1 Exposure data

d-Limonene is found widely in citrus and many other plant species and is a major constituent of many essential oils. It is used extensively as a component of flavourings and fragrances, as a chemical intermediate and as an insect repellant. Widespread exposures occur through consumption of fruits, vegetables and products containing essential oils. Consumption of *d*-limonene has been estimated to be 0.2–2 mg/kg bw per day.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

d-Limonene has been tested for carcinogenicity by oral gavage in one study in mice and one study in rats. In mice, no treatment-related tumour was observed. It significantly increased the combined incidence of renal-cell adenomas and carcinomas and induced renal tubular hyperplasia in male rats.

In a two-stage experiment, oral treatment with *d*-limonene after administration of *N*-nitrosoethylhydroxyethylamine enhanced the development of renal adenomas and renal tubular hyperplasia in male Fischer 344 rats, which synthesize $\alpha_{2\mu}$-globulin, but not in male NBR rats, in which there is no evidence that $\alpha_{2\mu}$-globulin is synthesized in measurable quantities.

5.4 Other relevant data

In men, oral intake of *d*-limonene induced transient proteinuria. *d*-Limonene induced nephrotoxicity in male Fischer 344 but not NBR rats.

Table 4. Genetic and related effects of *d*-limonene and its metabolites

Test system	Result		Dose (LED/HID)[a]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
***d*-Limonene**				
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	1360.0000	Watabe *et al.* (1980)
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	1667.0000	Haworth *et al.* (1983)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	1360.0000	Watabe *et al.* (1980)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	1667.0000	Haworth *et al.* (1983)
SA7, *Salmonella typhimurium* TA1535, reverse mutation	–	–	1360.0000	Watabe *et al.* (1980)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	1667.0000	Haworth *et al.* (1983)
SA8, *Salmonella typhimurium* TA1538, reverse mutation	–	–	1360.0000	Watabe *et al.* (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	1360.0000	Watabe *et al.* (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	1667.0000	Haworth *et al.* (1983)
G51, Gene mutation, mouse lymphoma L5178Y cells, TFT resistance *in vitro*	–	–	60.0000	US National Toxicology Program (1990)
SIC, Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	162.0000	US National Toxicology Program (1990)
CIC, Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	–	500.0000	US National Toxicology Program (1990)
Transformation, rat tracheal cells *in vitro*[b]	–	0	3.0000	Steele *et al.* (1990)
***d*-Limonene-1,2-oxide**				
URP, Unscheduled DNA synthesis, primary rat hepatocytes *in vitro*	–	0	13.6000	von der Hude *et al.* (1990)
Essential oils containing *d*-limonene				
BSD, *Bacillus subtilis, rec* strains, differential toxicity	–	0	4 mg/plate	Zani *et al.* (1991)
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	200 µg/plate	Zani *et al.* (1991)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	200 µg/plate	Zani *et al.* (1991)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	200 µg/plate	Zani *et al.* (1991)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	200 µg/plate	Zani *et al.* (1991)

–, negative; 0, not tested

[a]In-vitro tests, µg/ml

[b]Not on profile

No data were available on the genetic and related effects of *d*-limonene in humans. In a small number of studies with a variety of end-points, *d*-limonene showed no evidence of genotoxic activity.

5.5 Evaluation[1]

No data were available on the carcinogenicity of *d*-limonene to humans.
There is *limited evidence* in experimental animals for the carcinogenicity of *d*-limonene.

Overall evaluation

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

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[1]For definition of the italicized terms, see Preamble, pp. 26–29.

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