Food and Chemical Toxicology 49 (2011) 2471–2494

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

The FEMA GRAS assessment of aliphatic and aromatic terpene hydrocarbons used as flavor ingredients

T.B. Adams [a,*], C. Lucas Gavin [a], M.M. McGowen [a], W.J. Waddell [b], S.M. Cohen [c], V.J. Feron [d], L.J. Marnett [e], I.C. Munro [f], P.S. Portoghese [g], I.M.C.M. Rietjens [h], R.L. Smith [i]

[a] Flavor and Extract Manufacturers Association, 1620 I Street, NW, Suite 925, Washington, DC 20006, USA
[b] Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, USA
[c] Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198, USA
[d] Business Unit Toxicology and Applied Pharmacology, TNO Quality of Life, Zeist, The Netherlands
[e] Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[f] CanTox Health Sciences International, Mississauga, Ontario, Canada
[g] Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, USA
[h] Department of Toxicology, Wageningen University, Wageningen, The Netherlands
[i] Division of Biomedical Sciences, Section of Molecular Toxicology, Imperial College School of Medicine, South Kensington, London SW7 2AZ, UK

ARTICLE INFO

Article history:
Received 17 December 2010
Accepted 1 June 2011
Available online 2 July 2011

Keywords:
Terpene hydrocarbons
Aliphatic hydrocarbons
Flavoring ingredients
FEMA GRAS

ABSTRACT

This publication is the thirteenth in a series of safety evaluations performed by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA). In 1993, the Panel initiated a comprehensive program to re-evaluate the safety of more than 1700 GRAS flavoring substances under conditions of intended use. Since then, the number of flavoring substances has grown to more than 2600 substances. Elements that are fundamental to the safety evaluation of flavor ingredients include exposure, structural analogy, metabolism, pharmacokinetics and toxicology. Flavor ingredients are evaluated individually and in the context of the available scientific information on the group of structurally related substances. Scientific data relevant to the safety evaluation of the use of aliphatic and aromatic terpene hydrocarbons as flavoring ingredients are evaluated. The group of aliphatic and aromatic terpene hydrocarbons was reaffirmed as GRAS (GRASr) based, in part, on their self-limiting properties as flavoring substances in food; their rapid absorption, metabolic detoxication, and excretion in humans and other animals; their low level of flavor use; the wide margins of safety between the conservative estimates of intake and the no-observed-adverse effect levels determined from subchronic and chronic studies and the lack of significant genotoxic potential.

Contents

* Corresponding author.
E-mail address: tadams@therobertsgroup.net (T.B. Adams).

0278-6915/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2011.06.011

1. Introduction

Aliphatic and terpene hydrocarbons are ubiquitous through the food chain, therefore it is not surprising that they serve as effective flavoring ingredients. They are often used to convey citrus, pine, balsamic, woody and fruity notes. Consumers are exposed to aliphatic and terpene hydrocarbons from a variety of ingested and environmental sources (Helmig et al., 1999a,b; Guenther et al., 2000). The volume of use as flavor ingredients for aliphatic and aromatic terpene hydrocarbons prompted the FEMA Expert Panel to conduct this review of the relevant literature in support of the safe use of this group in food.

2. Chemical identity

This summary presents the key scientific data relevant to the safety evaluation of 17 aliphatic and aromatic terpene hydrocarbons used as flavoring ingredients. All substances in the group are unsaturated and include (1) three branched-chain aliphatic hydrocarbons and one structurally related unsaturated linear hydrocarbon, (2) six alkyl-substituted alicyclic and six bicyclic hydrocarbons, and (3) one alkyl-substituted aromatic hydrocarbon (Table 1).

3. Flavor use, natural occurrence in food, and exposure

The total annual volume of the 17 flavoring ingredients in this group is approximately 249,000 kg in the USA (Gavin and Williams, 2008). Greater than 99.9% of the total volume in the USA is accounted for by naturally occurring acyclic (e.g., myrcene), monocyclic (e.g., limonene), bicyclic (e.g., α-pinene), and monoaromatic (e.g., *p*-cymene) terpene hydrocarbons. The *per capita* intake[1] of each agent is reported in Table 1. The use of D-limonene (No. 5) itself accounts for approximately 92% of the total annual volume reported in the USA. The *per capita* intake of D-limonene in the USA is 27,905 μg/person per day. β-Caryophyllene (No. 16), α- and β-pinene (Nos. 12 and 13), α-phellandrene (No. 9), terpinolene (No. 6), myrcene (No. 2), and *p*-mentha-1,4-diene (No. 8) account for the majority of the reported annual volumes of use for other terpene hydrocarbons. The daily *per capita* intakes of these other flavoring agents are in the range of 114–760 μg/person per day.

All 17 substances (Nos. 1–17) are ubiquitous in the food supply and have been reported to occur naturally in coffee, alcoholic beverages, baked and fried potato, heated beans, tea, bread and cheese (Nijssen et al., 2010).

Substances in this group are products of plant biosynthesis formed via the isoprene pathway. Quantitative natural occurrence data for 17 aliphatic terpene hydrocarbons in the group demonstrate that their consumption occurs predominantly as natural components of traditional food (i.e., consumption ratio > 1) (Stofberg and Kirschman, 1985; Stofberg and Grundschober, 1987). Their ubiquitous presence in plants is reflected by the fact that essential oils derived from fruits, spices, vegetables, tree barks, roots, leaves, etc. have been shown to contain many of these terpene hydrocarbons (see Table 1).

4. Absorption, distribution, excretion, and metabolism

4.1. Absorption, distribution and excretion

4.1.1. Acyclic branched-chain hydrocarbons

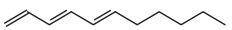
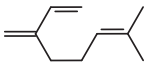
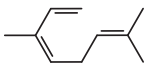
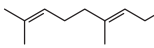
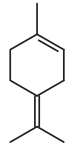
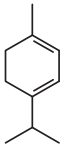
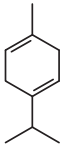
Greater than 70% of an oral dose of 400–700 mg/kg bw of myrcene given daily for 2 days to male albino rabbits was excreted in the urine as diol metabolites collected over 3 days (Ishida et al., 1981). The same metabolites were observed in the urine of adult male IISC rats given 800 mg/kg bw of myrcene daily by oral intubation for 20 days (Madyastha and Srivatsan, 1987).

4.1.2. Monocyclic hydrocarbons

Monocyclic hydrocarbons, such as D-limonene, administered orally are rapidly absorbed and distributed throughout the body. Following oral administration to humans, D-limonene was distributed preferentially to fatty tissues; this is probably due to high oil–blood partition coefficient and a long half-life during the slow elimination phase (Falk et al., 1990a; Falk-Fillipsson et al., 1993).

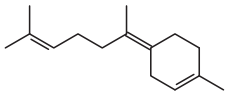
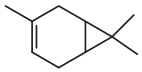
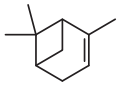
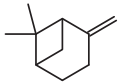
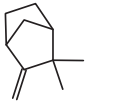
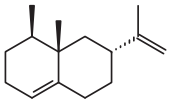
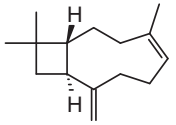
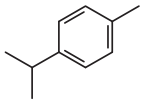
[1] Intake (μg/person/day) calculated as follows: [((annual volume, kg) × (1 × 10^9 μg/kg)/(population x survey correction factor × 365 days)], where population (10%, "eaters only") = 28 × 10^6 for the USA; where correction factor = 0.8 for the Gavin et al., USA survey representing the assumption that only 80% of the annual flavor volume was reported in the poundage surveys (Gavin et al., 2008). Intake (μg/kg bw/d) calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

Table 1
Identity and exposure data for aliphatic and aromatic terpene hydrocarbons used as flavor ingredients.

Flavoring ingredient	FEMA No.	CAS No. and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				μg/d	μg/kg bw/d		
1. 1,3,5-Undecatriene	3795	16356-11-9 	2	0.3	0.005	+	NA
2. Myrcene	2762	123-35-3 	1338	164	3	66,842	50
3. 3,7-Dimethyl-1,3,6-octatriene	3539	13877-91-3 	295	36	0.6	102	0.3
4. Farnesene	3839	502-61-4 	1	0.2	0.003	+	NA
5. D-Limonene	2633	5989-27-5	228,157	27906	465	1,300,995	6
6. Terpinolene	3046	586-62-9 	1678	205	3	25,220	15
7. *p*-Mentha-1,3-diene	3558	99-86-5 	485	59	1	24,720	51
8. *p*-Mentha-1,4-diene	3559	99-85-4 	930	114	2	98,317	106
9. α-Phellandrene	2856	99-83-2 	2232	273	5	133,334	60

(*continued on next page*)

Table 1 (*continued*)

Flavoring ingredient	FEMA No.	CAS No. and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				μg/d	μg/kg bw/d		
10. Bisabolene	3331	495-62-5 	54	7	0.1	33,865	627
11. 3-Carene	3821	13466-78-9 	113	14	0.2	+	NA
12. α-Pinene	2902	80-56-8 	2590	317	5	189,253	73
13. β-Pinene	2903	127-91-3 	1823	223	4	275,128	151
14. Camphene	2229	79-92-5 	8	1	0.02	27,350	3419
15. Valencene	3443	4630-07-3	322	39	0.7	444	1
16. β-Caryophyllene	2252	87-44-5 	6214	760	13	279,587	45
17. *p*-Cymene	2356	1195-32-0  	2644	323	5	29,302	11

[a] Intake (μg/person/day) calculated as follows: [((annual volume, kg) × (1 × 109 μg/kg)/(population × survey correction factor × 365 days)], where population (10%, "eaters only") = 26 × 10^6 for the USA; where correction factor 0.8 for the Gavin et al., USA survey representing the assumption that only 80% of the annual flavor volume, respectively was reported in the poundage surveys (Gavin et al., 2008). Intake (μg/kg bw/d) caculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[b] Quantitative data for the United States reported by Stofberg and Grundschober (1987).

[c] The consumption ratio is calculated as follows: (annual consumption via food, kg)/(most recent reported volume as a flavoring substance, kg); NA = data not available.

Blood clearance was 1.1 L/kg bw/h in males exposed via inhalation to D-limonene for 2 h at 450 mg/m^3 (Falk-Fillipsson et al., 1993).

In male volunteers, 50–80% of a 1600 mg oral dose of [^{14}C]-D-limonene was excreted in the urine with less than 10% appearing in the faeces within 2 days (Kodama et al., 1976). Volunteers exposed via inhalation to 450 mg/m^3 D-limonene showed a triphasic pattern of appearance and elimination from the blood, with half-lives of about 3 (appearance), 33 (fast elimination), and 750 min (slow elimination), respectively (Falk-Fillipsson et al., 1993).

Following the oral administration of 800 mg/kg of 9-[^{14}C]-D-limonene to male rats, higher amounts of radioactivity were initially reported in the liver, kidney and blood (C_{max} at 2 h) than in other tissues, but these levels were negligible at 48 h (Igimi et al., 1974). Approximately 60% of the administered radioactivity was recovered from the urine, with 5% from faeces and 2% from expired CO_2 after 48 h. In a more recent dietary administration study, BALB/c mice were fed a diet containing 10% D-limonene (ca. 15,000 mg/kg bw/day) and plasma levels of the principle metabolite, perillic alcohol, ranged from 42.8 to 67.3 μM (6.60–1.38 μg/mL) after 3 days exposure (Del Toro-Arreola et al., 2005).

4.1.3. Bicyclic hydrocarbons

Being the most common C_{10} hydrocarbon in nature, the pharmacokinetics of α-pinene has been investigated via several routes of exposure. Human volunteers were exposed to an atmosphere containing 0, 10, 225, or 450 mg/m^3 of (+)-α-pinene (Falk et al., 1990b) or 3-carene (Falk et al., 1991) for 2 h in an exposure chamber on four occasions. Volunteers exercised on a bicycle ergometer during exposure. Total pulmonary uptake of (+)-α-pinene increased linearly with dose, with 40% and 58% uptake occurring at 10 and 450 mg/m^3, respectively. Uptake of 3-carene was 61% and 70% at 10 and 450 mg/m^3, respectively. There was no difference in pulmonary uptake between the (+)- and (−)-α-pinene enantiomers at 450 mg/m^3. Clearance of α-pinene and 3-carene from the blood was rapid (1.1 and 0.9 L/hr/kg, respectively), indicating that α-pinene and 3-carene are rapidly distributed and metabolized. Blood levels of either substance at the two lower doses (10 and 225 mg/m^3) were below detection limits 4 h after exposure. Elimination was considered triphasic with (+) and (−)-α-pinene exhibiting a rapid initial appearance phase (4.8 and 5.6 min, respectively), a rapid elimination phase (30 and 48 min, respectively) and a slow elimination phase (695 and 555 min, respectively). Less than 0.001% of the total uptake of α-pinene or 3-carene was eliminated unchanged in the urine during and immediately after exposure. Pulmonary function was determined by measuring the forced expiratory volume (FEV), the vital capacity (VC), peak expiratory flow (PEF), residual volume (RV), mean expiratory flow at 50% of the VC (MEF_{50}), airway resistance (R_{aw}), and conductance (sG_{aw}) in a body plethysmograph. There was no evidence of changes in acute lung function during or 20 min after exposure to α-pinene and 3-carene. The authors concluded that short-term exposure to high atmospheric concentrations (greater than 10,000 mg/m^3) of α-pinene did not result in acute changes to lung function under exercising conditions.

Five humans were exposed for 4 or 6 h to an atmosphere containing 6.4 or 3.2 ppm (24 and 12 mg/m^3, respectively) of a mixture of volatile organic substances including α-pinene (Ashley and Prah, 1997). At 6.4 ppm, the air concentration of α-pinene was 0.139 ppm (0.775 mg/m^3). The mean pre-exposure blood concentration of α-pinene of 0.035 ppb increased to an average concentration of 2.0 ppb during exposure (50–240 min). Thereafter (330–450 min), the mean blood concentration decreased to 0.15 ppb. At 3.2 ppm exposure, changes proportional to those observed at 6.4 ppm were recorded. Similar results were also recorded for a 6-h exposure (Ashley and Prah, 1997). In a similar study, workers exposed for 8 h to atmospheres containing 0.035, 070, or 0.105 ppm (131, 263 or 394 μg/m^3, respectively) of α-pinene showed effective blood concentrations (average difference between blood plateau levels and pre-exposure baseline levels) of 0.94, 1.9, or 3.5 ppb, respectively (3.5, 7 and 13 μg/m^3, respectively) (Kawai et al., 1992).

Respiratory elimination of unchanged (−)- and (+)-α-pinene was approximately 8% following inhalation exposure of volunteers to up to 450 mg/m^3 (Levin et al., 1992). Urinary elimination of *cis*- and *trans*-verbenol[2] accounted for 1–4% of the total uptake. Sawmill workers exposed to an atmosphere containing 40–300 mg/m^3 pinene for 3 days showed urinary levels of 10–50 μg/ml of *cis*- and *trans*-verbenol (Eriksson and Levin, 1990).

4.1.4. Aromatic hydrocarbons

The data for simple aromatic terpene hydrocarbons indicate that these substances are readily absorbed from the gastrointestinal tract, distributed in the body, and excreted in the urine.

In a study conducted with rats, 33 mg/kg bw of a structural homologue of cymene, [^{14}C]-cumene,[3] was administered as a single oral dose or repeated oral dose over 8 days (Research Triangle Institute, 1989). The elimination half-life for the homologue, cumene, was calculated to be 8.6 and 7.3 h in male and female rats, respectively. Additionally, 60–80% of an oral dose of 100 mg/kg bw of *p*-cymene given to rats was excreted unchanged in the urine within the following 48 h (Walde et al., 1983).

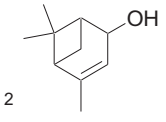
4.1.5. Summary of absorption, distribution and excretion

Being highly lipophilic, the aliphatic, alicyclic, and aromatic terpene hydrocarbons in this group cross biological membranes (gastrointestinal tract, skin, respiratory epithelia, etc.) by passive diffusion. For oral intake, the rate of diffusion depends mainly upon the concentration gradient between the gastrointestinal tract and portal blood. Compounds are rapidly absorbed and distributed to body tissues and then slowly eliminated primarily in the urine as polar oxidized metabolites.

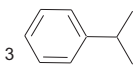
4.2. Enzyme Induction

In Phase I metabolism, the biotransformation of D-limonene, β-myrcene, and α-pinene, and β-pinene as well as the other group members are catalyzed by cytochromes P450 (CYP-450). In female rats, D-limonene (monocyclic hydrocarbon) and β-myrcene (acyclic hydrocarbon) are inducers (upon repeated administration) and competitive inhibitors of the same isoenzymes, specifically CYP2B1 (Miyazawa et al., 2002). In a study of the induction of liver monooxygenase by β-myrcene, liver microsomes from female rats treated via gavage with 1000 mg/kg bw/day β-myrcene for 1 or 3 days were isolated (De-Oliveira et al., 1997a). Activities of several markers of different CYP-450 enzymes were monitored, including pentoxyresorufin-*O*-dealkylatase (PROD) and benzyloxy-resorufin-*O*-dealkylase (BROD), which are selective markers of CYP2B1. β-Myrcene produced marked increases in the activities of both PROD and BROD, indicating induction of CYP2B1. Levels of apoproteins CYP2B1/2B2 were increased 8.2-fold after repeated treatment with β-myrcene.

[2]



[3]



Radioimmunoassay revealed that pinene (3- and 2.6-fold in males and females, respectively) and limonene (2-fold in females only) induced the CYP2B subfamily in liver microsomes isolated from rats following intraperitoneal injection of 40 mg/kg bw daily for 3 days (Austin et al., 1988). In this study, β-myrcene did not significantly induce CYP2B isoenzymes. In an anticarcinogenesis study, limonene induced CYP2B and 2C subfamilies, and epoxide hydrolase in female rat liver microsomes isolated from rats administered 1 or 5% limonene in the diet for 2 weeks (Maltzman et al., 1991). Cadinene and α-pinene both significantly increased by approximately 12- and 14-fold, respectively, the levels of CYP2B1 in hepatic microsomes obtained from male rats treated with 300 mg/kg bw of each compound via intraperitoneal injection (Hiroi et al., 1995). Additionally, cadinene significantly increased the levels of CYP3A2 (2-fold), while α-pinene (1.6-fold) significantly increased the levels of CYP4A2.

In studies, fractions designated CYP-450 and CYP-451 obtained from rat liver microsomes were incubated with α-pinene for intervals of 1–8 min (White and Agosin, 1980). Analysis of the homogenate revealed the presence of β-pinene and limonene together with smaller amounts of *trans*-verbenol, myrtenol,[4] verbenone,[5] and pinene oxide. The proportion of oxidized metabolites was greater in incubations with CYP-451 than in CYP-450. β-Caryophellene administered to rats and mice for four days at a dose level of 2500 mg/kg bw induced liver microsomal enzymes, as shown by decreased hexobarbital-induced sleep times for treated rats compared to controls (Ambrose, 1983).

D-Limonene (0.05–2 μM), β-myrcene (0.02–1 μM), and (+)-α-(0.00625–0.5 μM) and (−)-α-pinene (0.01–0.5 μM) produced a concentration-dependent reversible inhibition of PROD when incubated with liver microsomes from phenobarbital-treated rats (De-Oliveira et al., 1997b).

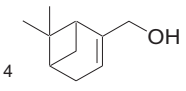
4.2.1. Summary of enzyme induction

Based on these data, acyclic, monocyclic, bicyclic, and aromatic terpene hydrocarbons induce an array of CYP isozymes that is responsible for the hydroxylation of the hydrocarbon eventually leading to more polar metabolites.

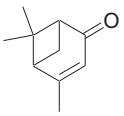
4.3. Metabolism

Available biochemical and pharmacokinetic data indicate that all of the aliphatic and aromatic terpene hydrocarbons in this group participate in similar pathways of metabolic detoxication. Being lipophilic, these hydrocarbons are rapidly absorbed by the gastrointestinal tract and oxidized to polar oxygenated metabolites via CYP-450 isoenzymes. The Phase I metabolites are then conjugated and excreted mainly in the urine. The substances in this group are oxidized either by side chain oxidation or epoxidation of the exocyclic or endocyclic double bond. Alkyl oxidation initially yields hydroxylated metabolites that may be excreted in conjugated form or undergo further oxidation, yielding more polar metabolites that are also excreted. If a double bond is present, epoxide metabolites may form that are detoxicated either by hydrolysis to yield diols, or by conjugation with glutathione to yield mercapturic acid derivatives.

4



5



4.3.1. Acyclic hydrocarbons

In mammals, including humans, the acyclic hydrocarbons such as β-myrcene are metabolized by CYP-450-mediated epoxidation to yield epoxides that are then converted to the corresponding diol via the action of epoxide hydrolase. The diols are then conjugated with glucuronic acid and excreted in the urine (Ishida et al., 1981; Madyastha and Srivatsan, 1987).

Both in rats and rabbits, the principal urinary metabolite following gavage administration of β-myrcene is myrcene-3,10-glycol, formed from the hydration of the epoxide intermediate. Epoxidation of the 3,10-double bond was reported to favor epoxidation of the 1,2-double bond (Ishida et al., 1981; Madyastha and Srivatsan, 1987). When rats were administered 800 mg/kg bw/day of myrcene orally via gavage, the principal metabolite isolated from the urine was 10-hydroxylinalool (myrcene-3,10-glycol) and, to a lesser extent, 7-methyl-3-methylene-oct-6-ene-1,2-diol (myrcene-1,2-glycol) (Madyastha and Srivatsan, 1987). Other minor metabolites included the hydroxy acids of both the 3,10- and 1,2-glycols (10-carboxylinalool and 2-hydroxy-7-methyl-3-methylene-oct-6-enoic acid, respectively) and a diol, 1-hydroxymethyl-4-isopropenylcyclohexanol, formed by intramolecular cyclization of an open chain metabolite.

Male rabbits were administered 400–700 mg/kg bw [^{14}C]β-myrcene via gavage and the urine was collected over 72 h (Ishida et al., 1981). Principal urinary metabolites identified were myrcene-3,10-glycol (40.7%), myrcene-1,2-glycol (20.8%), and uroterpenol (11.8%) (see Fig. 1). Additionally, the glycols underwent further oxidation to yield 2-hydroxymyrcene-carboxylic acid and 3-hydroxymyrcene-10-carboxylic acid. These results indicate that myrcene primarily undergoes epoxidation of the 3,10- and 1,2-double bonds followed by hydrolytic epoxide ring opening to yield the corresponding glycols. A minor metabolic pathway involves cyclization and subsequent formation of limonene as a transient intermediate which undergoes rapid oxidation to form uroterpenol.

4.3.2. Monocyclic hydrocarbons

Humans metabolize monocyclic hydrocarbons, such as limonene, by either allylic oxidation of the exocyclic methyl group to yield perillic acid derivatives (i.e., perillic acid and dihydroperillic acid), or by epoxidation and hydrolysis to yield diols (i.e., limonene-1,2-diol and limonene-8,9-diol (Crowell et al., 1994; Poon et al., 1996; Vigushin et al., 1998). Allylic oxidation is, by far, the major pathway for metabolism of limonene in humans. These metabolic products constitute the major plasma metabolites. Limonene (unchanged) and perillic acid derivatives (methyl ester) were also detected as minor plasma metabolites (Poon et al., 1996). Peak plasma levels for all metabolites were achieved 4–6 h after administration, with the exception of limonene-8,9-diol, which reached its peak level 1 hour after administration (Crowell et al., 1994). Phase II glucuronic acid conjugates have been identified in the urine of human volunteers for all major and minor metabolites. They include the glucuronic acid conjugates of perillic acid, dihydroperillic acid, limonene-8,9-diol, limonene-10-ol, limonene-6-ol, and limonene-7-ol (perillyl alcohol) (Kodama et al., 1974, 1976; Poon et al., 1996).

Similar to humans, the C_1 methyl substituent of limonene is oxidized in the rat to form perillic acid (see Fig. 2). Perillic acid can then be excreted unchanged, or as the glycine or glucuronic acid conjugate in the urine, or it can be further oxidized to perillic acid-8,9-diol or 2-hydroxy-*p*-menth-8-en-7-oic acid. Approximately 85% of the urinary limonene metabolites in the rat were identified as perillic acid or a metabolite of perillic acid (Kodama et al., 1976). Minor pathways reported for the rat include epoxidation of either the 1,2- or the 8,9- double bond, and subsequent hydrolysis to the diol.

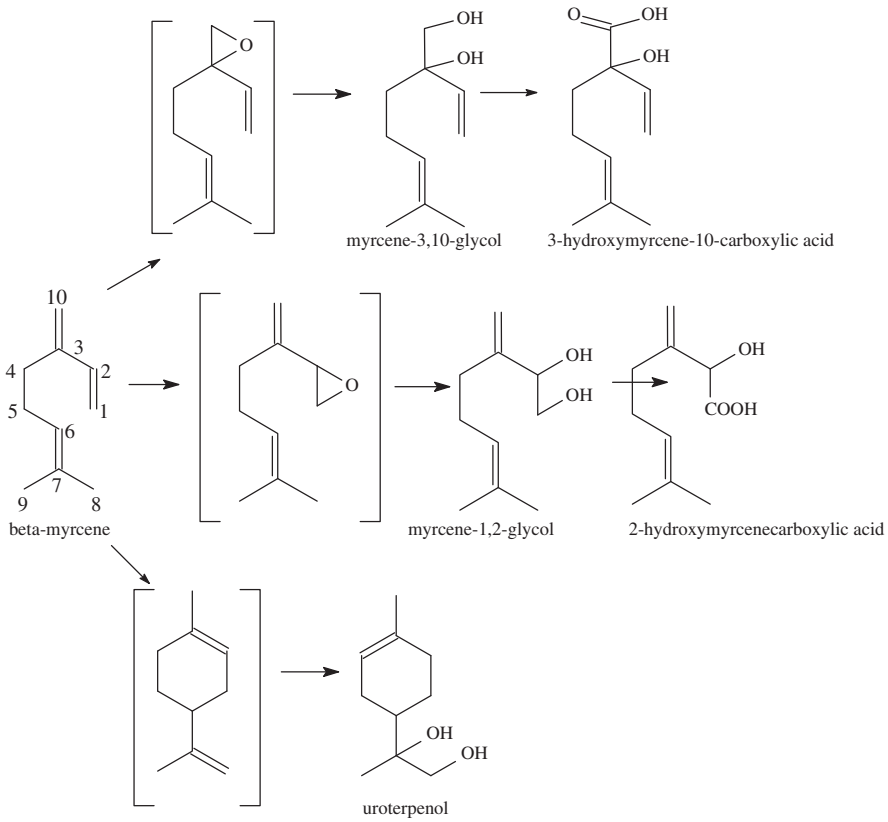


Fig. 1. Metabolism of β-myrcene in rabbits.

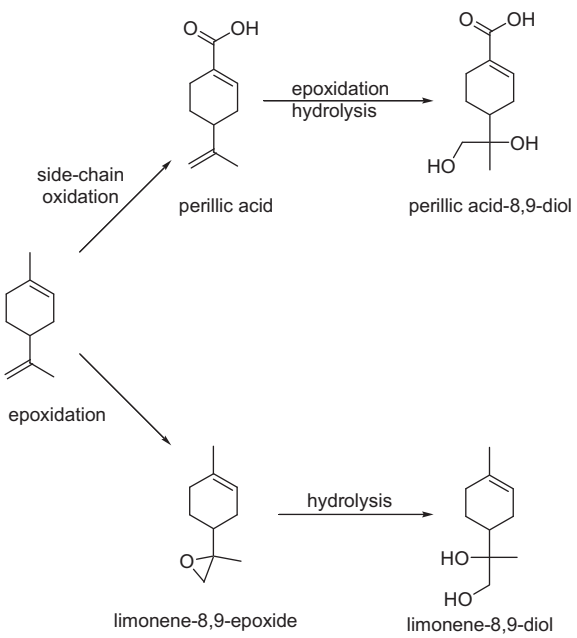


Fig. 2. Metabolism of D-limonene in rats.

Upon incubation with rat liver microsomes, the majority of D-limonene was converted to the 8,9-diol via its precursor the 8,9-epoxide and to a lesser extent, the 1,2-epoxide. The observation that 3,3,3-trichloropropene-1,2-oxide, an inhibitor of epoxide hydrolase, blocks hydrolysis of limonene epoxide, provides further evidence that the 8,9-diol forms from the 8,9-epoxide. Epoxidation of the C_8 double bond is favored over epoxidation of the C_1 double bond, due to the steric hindrance of the 1-methyl group. The 1,2-epoxide underwent a very low rate of microsomal hydrolysis (1% of the rate for the 8,9-epoxide). This, in part, explains the absence of the 1,2-diol as a microsomal metabolite (Watabe et al., 1981).

When male rat liver microsomes were incubated with either D- or L-limonene, perillyl alcohol formed by methyl group 7-hydroxylation and carveol formed by ring 6-hydroxylation. Incubation of limonene with female rat liver microsomes resulted in low activity for conversion to either alcohol. Cytochrome CYP-450 2C11 catalyzes the formation of the alcohol metabolites in rats. Use of phenobarbital-induced liver microsomes resulted in an increase in carveol metabolites, but not in perillyl alcohol metabolites. With fetal liver microsomes, rates of limonene hydroxylation were low or undetectable; however, after birth, limonene hydroxylation increased in males but not in females with formation of perillyl alcohol increasing the most rapidly. The relationship of the observed sex-related differences in metabolism (e.g., CYP450 2C11-induced hydroxylation in males) to sex-specific differences in toxicity is discussed in Section 5.3 of this paper (Miyazawa et al., 2002).

In male rats orally administered 3 mmol/kg bw (408 mg/kg bw) of [14C]-D-limonene, the 1,2-epoxide (82%), the 1,2-diol (5%), and D-limonene (13%) were detected in the renal proximal tubular cells where they were reversibly (40%) associated with α_u-globulin, a hepatic protein filtered by the glomeruli (Lehman-McKeeman et al., 1989). It has been determined that these protein-D-limonene metabolite associations in the P-2 section of the proximal tubule are a prerequisite for the observed nephrotoxicity in the male rat (Lehman-McKeeman et al., 1989). No proteins analogous to α_{2u}-globulin have been observed in man (Olson et al., 1990).

4.3.3. Bicyclic hydrocarbons

Analysis of urinary metabolites eliminated by human volunteers within 4 h following a 2-h exposure to 10–450 mg

(+)-α-pinene/m^3 in the above (see Section 4.1.3) pharmacokinetic study (Falk et al., 1990b) revealed that *cis*- and *trans*-verbenol in a ratio of 1:10, with 3.8% of the dose was eliminated at 10 mg/m^3, and 1.7% of the dose at 450 mg/m^3. Most of the verbenols were eliminated within 20 h. In a more extensive metabolic study, urine was collected from sawmill workers at the end of an 8–9 h work shift or from chamber-exposed individuals (Eriksson and Levin, 1996). Following hydrolysis of glucuronic acid conjugates, *cis*- and *trans*-verbenol were identified in the urine along with two diols, *cis*- and *trans*-4-hydroxymyrtenol, formed by methyl group hydroxylation of *cis*- and *trans*-verbenol. *trans*-4-Hydroxymyrtenal was also detected (see Fig. 3).

Analysis of the urinary metabolites of a patient attempting suicide with 400–500 mL of pine oil containing 57% α-pinene showed the presence of myrtenol, verbenol, and borneol (Koppel et al., 1981). Renal excretion reached a peak level 5 days after ingestion. The urine of normal humans has been shown to contain α-pinene, β-pinene, 3-carene, and camphene (Zlatkis et al., 1973).

The metabolic detoxication of bicyclic terpene hydrocarbons in humans can be predicted to approximate that in other mammals (see Fig. 3). Male albino rabbits (6/group) were given a single oral dose levels of 400–700 mg/kg bw of (+)-α-pinene, (−)-α-pinene, (±)-α-pinene, (−)-β-pinene, (−)-*cis*-pinane, or (+)-*δ*-3-carene (Ishida et al., 1981). The test substance was administered by stomach tube as a suspension in a water/polysorbate 80. Animals were housed individually and urine was collected daily for 3 days. Greater than 80% of each bicyclic terpene hydrocarbon was recovered in the urine as glucuronic acid conjugates of hydroxylated terpene hydrocarbons. The principal metabolite formed by allylic oxidation of the exocyclic methyl group of each of the three {(+), (−), or (±)} stereochemical forms of α-pinene was verbenol (see Fig. 3). Greater than 98% of (−)-α-pinene was converted to (−)-*trans*-verbenol, while 67% of (+)-α-pinene was converted to racemic *trans*-verbenol. In addition, the (+)-isomer was metabolized by allylic oxidation of the C_2 methyl group to yield myrtenol (15%) and small amounts of oxidized myrtenol, myrtenic acid.

The presence of an exocyclic alkene function in (−)-β-pinene provides for additional metabolic options. Allylic oxidation of the C_2 position yields (+)-*trans*-pinocarveol (11%), while epoxidation of the exocyclic alkene followed by reduction or hydration yields (−)-*trans*-10-pinanol (39%) and (−)-*l*-*p*-menthene-1,8-diol (30%), respectively (Ishida et al., 1981). Ring cleavage yields (−)-α-terpineol (5%) (see Fig. 3).

In a manner similar to α-pinene, 3-carene (No.11) undergoes oxidation of a ring allylic position followed by ring opening to yield (−)-*m*-mentha-4,6-dien-8-ol (72%) (Ishida et al., 1981). Smaller

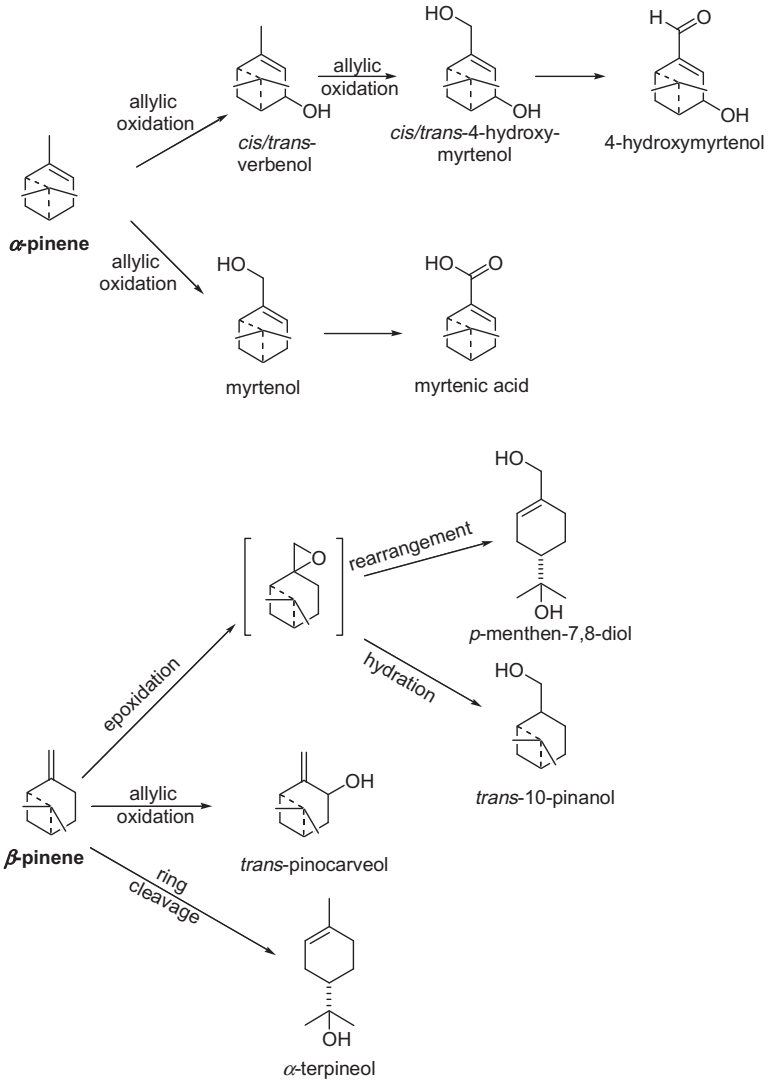


Fig. 3. Metabolism of α-pinene and β-pinene in animals.

amounts of metabolites obtained by hydroxylation at the gem-dimethyl group and allylic oxidation of the C_2 methyl group are obtained. Similarly, in rabbits, β-caryophyllene (No. 16) undergoes epoxidation of the endocylic 5,6-double bond and hydroxylation at the gem-dimethyl group. Epoxidation of the exocyclic 2,12-double bond was also reported (Asakawa et al., 1981, 1986; Ishida et al., 1979).

4.3.4. Aromatic hydrocarbons

The metabolism of *p*-cymene (No. 17) or its homologue, cumene (isopropyl benzene), has been studied extensively in several animals. In general, the studies indicate that *p*-cymene undergoes extensive oxidation of the methyl substituent and isopropyl side chain to yield polar oxygenated metabolites (Bakke and Scheline, 1970; Boyle et al., 1999; Matsumoto et al., 1992; Walde et al., 1983). These metabolites are either excreted unchanged in the urine, or undergo Phase II conjugation with glucuronic acid and/or glycine, followed by excretion in the urine.

In the rat, a larger percentage of metabolites were conjugated (i.e., 34.2% free versus 65.8% conjugated) when *p*-cymene was orally administered at 0.37 mmol/kg bw (50 mg/kg bw) than when it was administered at 1.49 mmol/kg bw (200 mg/kg bw) (i.e., 81.9% free versus 18.1% conjugated), suggesting saturation of the conjugation pathway (Boyle et al., 1999). Forty-eight hours after administration of the 50 mg/kg bw dose, 2-*p*-tolypropan-2-ol (39% of recovered dose) and 2-*p*-carboxyphenylpropan-2-ol (19% of recovered dose) were recovered from the urine. The former metabolite is the product of benzylic hydroxylation of the isopropyl substituent, whereas the latter metabolite is the product of benzylic hydroxylation of the isopropyl substituent and the methyl substituent.

Following an oral dose of 100 mg/kg bw of *p*-cymene given to rats, the principal urinary metabolites were *p*-isopropylbenzoic acid (19%) and 2-*p*-carboxyphenylpropionic acid (16%) (Walde et al., 1983). Other minor urinary metabolites excreted included 2-*p*-tolylpropan-1-ol (8%), 2-*p*-carboxyphenylpropan-2-ol (9%), 2-*p*-(hydroxymethyl)phenylpropionic acid (4%), 2-*p*-carboxyphenylpropan-1-ol (11%), and *p*-isopropylbenzoylglycine (2%). In the same study, when the same dose was given to guinea pigs, similar urinary metabolites were identified. The primary urinary metabolite, however, was *p*-isopropylbenzoylglycine (31%), indicating that conjugation with glycine was more prevalent in guinea pigs than in rats. In addition, where no ring hydroxylation of *p*-cymene was reported in rats, trace amounts of ring hydroxylation metabolites, carvacrol and hydroxycarvacrol, were detected in guinea pigs following oral exposure.

In a study designed to identify the stereochemistry of p-cymene metabolites, 10 g of test material was administered orally to two male and two female rabbits (Matsumoto et al., 1992). Urine was collected over the following 72 h and analysed for metabolites. Seven (7) different hydroxylated and carboxylated metabolites were recovered in the urine. Four were optically active and determined to be 2-(*p*-tolyl)-1-propanol, 2-(*p*-tolyl)-propanoic acid, *p*-(2-hydroxy-1-methylethyl)benzoic acid *p*-(1-carboxyethyl)benzoid acid. Three were optically inactive and determined to be 2-(*p*-tolyl)-2-propanol, *p*-isopropylbenzoic acid, and *p*-(1-hydroxy-1-methylethyl)benzoic acid. Oxidation of the methyl group of the isopropyl substituent yields 2-(*p*-tolyl)-1-propanol in an *R*/*S* ratio of 65:35. The (*R*)-alcohol is then further oxidized to (*R*)-2-(*p*-tolyl)propanoic acid, which undergoes complete stereochemical inversion to (*S*)-2-(*p*-tolyl)propanoic acid. This same type of stereoselective biotransformation has been reported for the metabolism of inflammatory agents in humans, most notably (*R*)-(−)ibuprofen (Lee et al., 1985; Rudy et al., 1995; Suri et al., 1997; Tan et al., 2002). Subsequently, the alcohol or acid metabolite may undergo oxidation of the tolyl methyl group to yield the corresponding hydroxy acid and diacid, respectively. If the tolyl methyl is oxidized before the isopropyl group, no stereochemical inversion is observed upon oxidation of the isopropyl side-chain. Based on the observed stereochemical changes, it is evident that *omega*-hydroxylation of *p*-cymene or *p*-isopropylbenzoic acid metabolite occurs preferentially at the *pro-S*-methyl group of the isopropyl substituent (see Fig. 4) (Matsumoto et al., 1992).

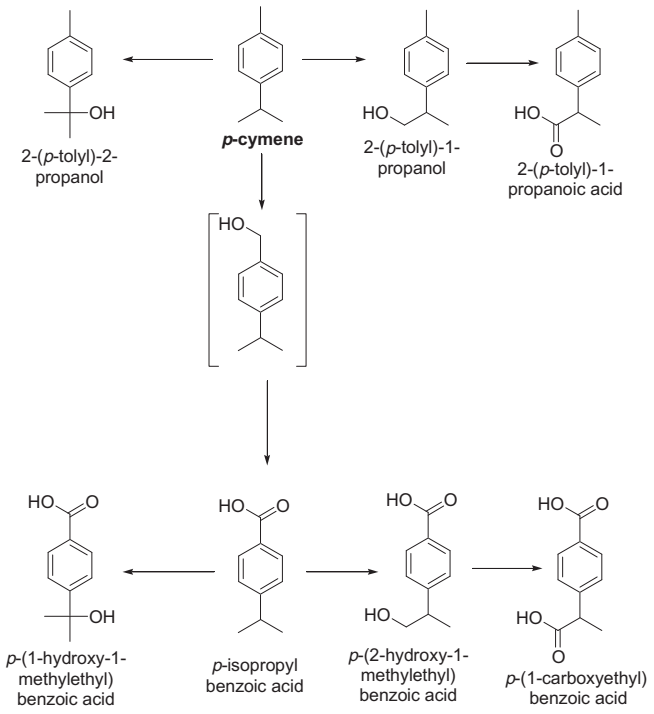


Fig. 4. Oxidative metabolism of p-cymene.

4.3.5. Summary of metabolism

The metabolic fate of acyclic, alicyclic, bicyclic and aromatic terpene hydrocarbons is remarkably similar. The presence of alkene functional groups and alkyl substituents allows for CYP450 catalyzed oxidation with allylic or benzylic hydroxylation being the most favorable pathway. These hydroxylation products are either conjugated with glucuronic acid and excreted in the urine or undergo further oxidation to yield the corresponding carboxylic acids. Epoxidation of the alkene function also occurs especially for sterically unhindered alkenes as are present in myrcene and limonene. The epoxide metabolite has not been observed in rodent or human studies for the sterically hindered bicyclic monoterpenes pinene and camphene. These epoxides are then hydrolyzed via the action of epoxide hydrolase to yield the corresponding diols (glycols) that are excreted either unchanged or as glucuronic acid conjugates or undergo subsequent oxidation to yield carboxylic acid derivatives or undergo conjugation with glutathione, which is the first step in mercapturic acid formation.

5. Toxicological studies

5.1. Acute toxicity

Oral LD_{50} values have been reported for 16 of the 17 substances in this group (see Table 2). LD_{50} values range from 1590 to greater than 8000 mg/kg bw in rats, and 2000 to greater than 13,360 mg/kg bw in mice. These values indicate that aliphatic and aromatic hydrocarbons exhibit low acute oral toxicity (Brownlee, 1940; Clark et al., 1979; Dogra et al., 1989; Hart and Wong,

Table 2
Acute studies and short- and long-term toxicity studies for aliphatic and aromatic terpene hydrocarbons used as flavor ingredients.

Flavoring ingredient		Acute oral studies		Short- and long-term studies			
		Oral LD_{50} mg/kg bw (species)	Reference	Species; sex[a]	Time (days)/route	NOAEL (mg/kg bw/day)	Reference
1	1,3,5-Undecatriene	2000–4000 (mouse) >8000 (rat)	Pellmont (1973) Pellmont (1973)	Rat; M, F	14/Diet	10[b,c]	Shapiro (1988)
2	Myrcene	>5000 (rat)	Moreno (1972b)	Mice; M, F Rat; M, F	90/Gavage 90/Gavage	<250 <250	NTP (2010) NTP (2010)
3	3,7-Dimethyl-1,3,6-octatriene	5000 (rat)	Moreno (1976)				
5	D-Limonene	5600 (M, mouse) 6600 (F, mouse)	Tsuji et al. (1975a)	Rat; M	6 or 27/Gavage	<75	Kanerva et al. (1987)
		>5000 (rat)	Moreno (1972a)	Rat; M	90/Gavage	5	Webb et al. (1989)
		4400 (M, rat) 5100 (F, rat)	Tsuji et al. (1975a)	Dog; M, F	180/Gavage	100	Webb et al. (1990)
				Dog; M, F	180/Oral	340 (M) <340 (F)	Tsuji et al. (1975b)
				Mouse; M, F	16/Gavage	1650	NTP (1990)
				Mouse; M, F	90/Gavage	500 (M) 1000 (F)	NTP (1990)
				Mouse; M, F	721/Gavage	250 (M) 500 (F)	NTP (1990)
				Rat; M, F	16/Gavage	1650	NTP (1990)
				Rat; M, F	90/Gavage	<150 (M) 1200 (F)	NTP (1990)
				Rat; M, F	721/Gavage	<75 (M) 300 (F)	NTP (1990)
6	Terpinolene	4.39 ml/kg (rat, 3784 mg/kg bw)[d]	Brownlee (1940)				
7	*p*-Mentha-1,3-diene	1680 (rat)	Moreno (1973a)				
8	*p*-Mentha-1,4-diene	3650 (rat)	Moreno (1973b)				
9	α-Phellandrene	>5700 (rat) 1.87 ml/kg (rat, 1590 mg/kg bw)[e]	Moreno (1972c) Brownlee (1940)				
10	Bisabolene	>5000 (mouse) >13,360 (mouse)	Moreno (1974a) Colalianni (1967)				
11	3-Carene	4800 (rat)	Moreno (1972d)				
12	α-Pinene	3700 (rat)	Moreno (1972e)	Rat; M, F Mouse; M, F	98/Inhalation 98/Inhalation	21[f] (M) 170[g] (F) 72[h] (M,F)	NTP (2006a,b)
13	β-Pinene	>5000 (rat)	Moreno (1975)	Rat; M, F	14/Diet	10[b,c]	Shapiro (1988)
14	Camphene	>5000 (rat)	Moreno (1974b)	Rat; M, F	28/Gavage	250	Hoechst (1991)
15	Valencene	>5000 (rat)	Moreno (1980)				
16	β-Caryophyllene	>5000 (rat)	Hart and Wong (1971)				
17	*p*-Cymene	4750 (rat)	Jenner et al. (1964)				

[a] M = Male; F = Female. If not listed, sex was not specified in the report.
[b] Study performed using a dose of 10 mg/kg bw/day of Galbelica, which is a solution composed of 80% β-pinene and 20% 1,3,5-undecatriene.
[c] Study performed with either a single dose or multiple doses that produced no adverse effect. The value is therefore not a true NOAEL, but is the highest dose level tested that produced no adverse effects. The actual NOAEL may be higher.
[d] Calculated using the specific gravity of terpinolene = 0.862 (Burdock, 2005).
[e] Calculated using the specific gravity of α-phellandrene = 0.835–0.865 (FCC, 1996).
[f] Dose converted from 25 ppm (see text).
[g] Dose converted from 200 ppm (see text).
[h] Dose converted from 200 ppm (see text).

1971; Hasegawa et al., 1989; Jenner et al., 1964; Keating, 1972; Levenstein, 1975; Moreno, 1972a–e, 1973a,b, 1974a,b, 1975, 1976, 1980; Pellmont, 1973; Schafer and Bowles, 1985; Tsuji et al., 1975a).

5.2. Short-term studies of toxicity

The results of short-term studies with representative aliphatic and aromatic terpene hydrocarbons are summarized in Table 2 and described below. The use of the term "significantly" indicates a statistically significant change from corresponding control values. Based on the results of studies on myrcene, limonene, pinene, and camphene there is consistent evidence of α_{2u}-globulin renal tubular effects in the male rat.

5.2.1. Myrcene (No. 2)

5.2.1.1. Mice. In a gavage study (NTP, 2010), groups (10/dose/sex) of male and female B6C3F$_1$ mice were administered 0, 250, 500, 1000, 2000, or 4000 mg/kg bw of β-myrcene daily, excluding weekends and holidays, by gavage for approximately 13 weeks and at least two consecutive days prior to necropsy. Body weights and clinical observations were made weekly and sperm morphology and vaginal cytology evaluations were conducted on animals in the control and three lowest dose groups. At termination of both study groups, blood was taken for clinical chemistry determinations, hematological examination and micronuclei evaluation. At necropsy, organ weights were measured and histopathological examination was performed on a wide variety of tissues.

All animals in the 4000 mg/kg bw/day group died within the first week while 9 of 10 males and 8 of 10 females of the 2000 mg/kg bw/day group died by Week 4. In animals that died prior to study termination, clinical signs included lethargy, abnormal breathing, or thin appearance. Group mean body weight gains were significantly depressed for males (−22.5%) but not females (−2.4%) in the 1000 mg/kg bw/day group. Females at the 500 mg/kg bw/day dose level showed significantly lower mean body weights (92% of controls) and body weight gains (77% of controls) than that of controls. No other significant changes were reported at the lower doses in either sex.

Animals at the two highest doses were not evaluated due to early mortality. For males, mean relative liver weight was significantly increased for the 1000 mg/kg bw/day group only. For females, mean absolute and relative (to body weight) liver weights were increased at 500 and 1000 mg/kg bw/day. In addition, the mean absolute right kidney weight of females administered 1000 mg/kg bw/day was significantly increased and the mean relative right kidney weight was significantly increased at 250, 500, and 1000 mg/kg bw/day in females. There was a significant decrease (up to ∼10%) in hematocrit (males), hemoglobin (males) and erythrocyte count (both sexes) values in animals from the 1000 mg/kg bw/day group. No other significant differences in organ weights or hematology parameters were reported. Histopathological evaluation revealed no abnormal findings in mice administered up to 1000 mg/kg bw/day of β-myrcene. Based on the changes in liver and kidney weights at all dose levels, a NOAEL for male and female mice could not be assigned.

5.2.1.2. Rats. In a 13-week gavage study (NTP, 2010), Core groups (10/dose/sex) of male and female F344N Fischer rats were administered 0, 250, 500, 1000, 2000, or 4000 mg/kg bw of β-myrcene daily, excluding weekends and holidays, by gavage. Body weights and clinical observations were made weekly and sperm morphology and vaginal cytology evaluations were conducted on animals in the control and three lowest dose groups. At termination of both study groups, blood was taken for clinical chemistry determinations, hematological examination and micronuclei evaluation. At necropsy, organ weights were measured and histopathological examination was performed on a wide variety of tissues. Right kidneys of male rats were frozen while left kidneys were processed for Mallory Heidenhain staining and hematoxylin and eosin staining for investigation of α_{2u}-globulin in male rats. Also, Special Study Groups (10/dose/sex) were given three doses of β-myrcene daily for 3 weeks and 2 days. Body weights were measured weekly and hematological examinations and blood chemical determination were performed at termination on Day 23. At termination, the left kidneys were frozen and the right kidneys were processed and microscopically examined for the presence of hyaline droplets using Mallory Heidenhain staining and hematoxylin and eosin staining.

All animals in the Core groups and Special Study groups at 4000 mg/kg bw/day group died within the first 12 days of the study. In the Core group, 2/10 males and 4/10 females died at 2000 mg/kg bw/day, 1/10 males and 1/10 females died at 1000 mg/kg bw/day, and 1/10 males died at 500 mg/kg bw/day. Similar lethality was reported in the Special group. Animals dying prior to study termination, showed lethargy, ruffled fur, abnormal breathing, or thin appearance. Mean body weights and body weight gains were significantly reduced in males (>10%) and females (6–10%) at 500, 1000, and 2000 mg/kg bw/day.

Except for recording lesions, any animals dying early in the study were not evaluated. Clinical pathology revealed significant decreases in leukocytes (−27% and −24%, respectively) and lymphocytes (−35% and −25%, respectively) in males and females of the 2000 mg/kg bw/day Special group at day 23. Creatinine levels were significantly increased (+16%) in 2000 mg/kg bw/day Special group males at day 23 but were significantly decreased (−13% to −29%) in 1000 and 2000 mg/kg bw/day Core group males and females and in 250 and 500 mg/kg bw/day Core group females at Week 14. A significant increase in mean absolute and relative liver and kidney weights was reported in male and female rats. Except for mean absolute liver weight values in males, this increase was dose dependent. Mean absolute thymus weight was significantly decreased in males beginning at 500 mg/kg bw/day and in females at 2000 mg/kg bw/day. Mean relative thymus weight was decreased only in males at 2000 mg/kg bw/day. No other clinical pathology parameters or organ weight changes were reported.

In Core group males, histopathological examination revealed consistent evidence of renal tubular hyaline droplet formation in dose groups surviving to Day 93 (250, 500, and 1000 mg/kg bw/day). In Special group males, hyaline droplet formation was seen in all groups including controls. Necrosis of the renal tubule was significantly increased in all treated animals of the Core group. Incidence of nephrosis was significantly higher in Core group males and females given 1000 or 2000 mg/kg bw/day. Control and test Core group animals all showed evidence of nephropathy at similar incidences and all males in the Core group showed evidence of porphyrin pigmentation of the Harderian gland (significant at doses of 500 mg/kg bw/day and higher). In addition to the reported kidney effects, other significant changes reported in Core group animals were increased degeneration of the olfactory epithelium (2000 mg/kg bw/day males and females), suppurative inflammation of the nose (2000 mg/kg bw/day females), chronic inflammation of the nose (1000 and 2000 mg/kg bw/day males and females), atrophy of the spleen (2000 mg/kg bw/day males and females), atrophy of the mesenteric lymph node (1000 mg/kg bw/day females, 2000 mg/kg bw/day males and females), and acute inflammation of the forestomach (2000 mg/kg bw/day females). Based on the α_{2u}-globulin renal tubular effects observed at all dose levels and in the control group, a NOAEL for male rats could not be assigned.

5.2.2. D-Limonene (No. 5)

5.2.2.1. Rats. Groups of five young adult male F344/N rats were administered D-limonene via gavage at dose levels of 0, 75, 150, or 300 mg/kg bw/day 5 days a week for up to 27 days (Kanerva et al., 1987). Observations included daily and final body weights, weekly food intake, and relative and absolute liver and kidney weights and light microscopy of the liver and kidneys. Examinations were conducted on animals that were killed on day 6 (after five doses) and day 27 (after 20 doses) of the study. The kidneys were examined for hyaline droplet formation, granular cast formation and nephrosis. Two-dimensional gel electrophoresis evaluation of protein profiles was conducted on samples of kidneys of the mid-dose group (150 mg/kg bw/day) killed on day 6. Relative (to body weight) liver and kidney weights were significantly increased in high-dose rats (300 mg/kg bw/day) day 6 and 27 of the study. Renal effects were noted, including the formation of hyaline droplets, which was dose-related (statistical significance not stated) and peaked by day 6 of the study. Mid-dose rats exhibited significantly greater levels of α_{2u}-globulin in renal cortical tissues compared to controls. In addition, dose-related granular cast formation (mid- and high-dose) and nephrosis (all doses) were present in the kidneys of treated animals killed on day 27. Based on the renal effects noted at all doses, no NOAEL for male rats could be assigned.

Groups of 5-week-old male Fischer 344 rats received 0, 2, 5, 10, 30, or 75 mg/kg bw/day of D-limonene via gavage 5 days/week for 13 weeks (Webb et al., 1989). Rats from selected dose groups were killed and necropsied throughout the study (days 8–29), with all remaining rats killed and necropsied at the end of the study. Interim necropsies were conducted on five rats of the 10 mg/kg bw/day dose group on days 8 or 15 of the study, as well as on five rats of the control and high-dose (75 mg/kg bw/day) groups on days 8, 15, 22, or 29 of the study. At the end of the 90-day study, all of the surviving rats were killed and their tissues were subjected to histopathological examination. Rats were observed daily for signs of toxicity and body weights were measured. Feed consumption was evaluated weekly. Linear regression analyses indicated significantly increased relative (to body weight) kidney and relative liver weights in high-dose rats. There were no histopathological changes noted in the livers of treated rats. However, histological examination of the kidneys, revealed changes characterized by hyaline droplet formation, granular casts and multiple cortical changes, all of which were classified as chronic nephrosis. Exacerbation of hyaline droplet formation was reported at the earliest necropsy, 8 days after administration of the 10 and 75 mg/kg bw/day doses. The authors reported a NOEL for nephrotoxicity of 5 mg D-limonene/kg bw/day.

Groups of 10 F344/N rats of each sex were administered 0, 150, 300, 600, 1200 or 2400 mg/kg bw/day of D-limonene in corn oil by gavage, 5 days/week for 13 weeks (NTP, 1990). The animals were housed five/cage and fed *ad libitum*. The animals were observed twice per day and weighed weekly. Necropsies were performed on all animals. Histological examinations were performed on all vehicle control and high-dose animals, and all female rats in the 1200 mg/kg bw/day dose group. Tissues from all major organs were examined. Kidneys were examined for all male rats. No statistical analysis were reported. Ninety percent of female rats (9/10) and 50% of male rats (5/10) receiving 2400 mg/kg bw/day D-limonene died within the first week of the study. The final mean body weights of male rats receiving the three highest doses (600, 1200, and 2400 mg/kg bw/day) were reported to be 6%, 12%, and 23% lower than that of the controls, respectively. In addition, the final body weight of the one surviving female rat at the highest dose was 11% lower compared to that of controls. All other treated animals had final mean body weights similar to those of controls. Rough hair coats, lethargy, and excessive lacrimation were observed for all animals at the two highest dose levels (1200 and 2400 mg/kg bw/day). A dose-related increase in severity of nephropathy was noted. The nephropathy was characterized by degeneration of epithelium in the convoluted tubules, granular casts with tubular lumens, primarily in the outer stripe of the outer medulla, and regeneration of the tubular epithelium. Hyaline droplets were observed in the epithelium of the proximal convoluted tubules in all groups of male rats, including vehicle controls. Upon further review to determine if there were differences in these findings between control and treated animals, two "blinded" pathologists determined no definite differences in the accumulation of hyaline droplets among the slides from different dose groups. Based on renal effects in all dose levels, no NOAEL could be assigned for male rats. A NOAEL of 1200 mg/kg bw/day was selected for female rats based on low survival at the highest dose.

5.2.2.2. Mice. Groups of ten B6C3F$_1$ mice of each sex were administered 0, 125, 250, 500, 1000 or 2000 mg/kg bw/day of D-limonene in corn oil by gavage, 5 days/week for 13 weeks (NTP, 1990). Animals were housed five per cage and fed *ad libitum*. The animals were observed twice per day and weighed weekly. Necropsies were performed on all animals. Histological examinations were performed on all vehicle control and high-dose animals. Tissues from all major organs were examined. One of 10 males and 2/10 females administered 2000 mg/kg bw/day D-limonene, and as well as 1/10 females administered 500 mg/kg bw/day D-limonene died before the end of the study. Several other animals also died as a result of gavage error. Final mean body weights were 10% lower than control for male mice and 2% lower than control for female mice at the two highest dose levels (1000 and 2000 mg/kg bw/day). An alveolar cell adenoma was reported in the lung of one female at the highest dose level (2000 mg/kg bw/day). Clinical signs of rough hair coats and decreased activity were reported for the animals at the 1000 and 2000 mg/kg bw/day levels.

5.2.2.3. Dogs. Groups of male and female beagle dogs (3/sex/group) were orally administered D-limonene at dose levels of 0, 0.4, 1.2, or 3.6 mL/kg bw/day (approximately, 0, 340, 1000, and 3000 mg/kg bw/day, respectively) for approximately 6 months (Tsuji et al., 1975b). Frequent vomiting was reported at dose levels exceeding 1000 and 3000 mg/kg bw/day for female and male dogs, respectively, as well as a decrease in body weight (statistical significance not reported) in some animals at these dose levels. Total cholesterol and blood sugar levels were decreased in both male and female dogs at a dose level of 3000 mg/kg bw/day; however, it was not reported if these effects were statistically significant. There were protein casts noted in the renal tubules of female and male dogs orally administered D-limonene at dose levels of 340 and 1000 g/kg bw/day, respectively; however, no histological changes were observed in the other organs examined.

D-Limonene was administered to groups of five male and five female adult beagle dogs in a 6-month study (Webb et al., 1990). Doses of 0, 100, or 1000 mg D-limonene/kg bw/day were administered by gavage in divided doses twice a day. Observations were made following each dose for 1 h to assess effects of treatment. Diarrhea and emesis occurred periodically with the same frequency in high- (1000 mg/kg bw/day) and low-dose (100 mg/kg bw/day) groups. At the termination of the experiment, blood and urine samples of D-limonene-treated dogs were comparable with control groups, with the exception of a 35% increase in serum cholesterol and a 2-fold increase in serum alkaline phosphatase levels in high-dose dogs of both sexes. No significant changes were reported in the body weights or consumption of feed in either treatment group. No histological abnormalities were noted in any major organ or tissue. No hyaline droplets or histopathological changes were reported in the kidneys of treated animals as was

reported in the earlier study conducted by Tsuji et al. (1975b). A small increase (not statistically significant) in relative kidney weight was reported in the low-dose group, but a significant increase in relative kidney weight was reported in the high-dose group. Since there were no nephrotoxic effects observed in the kidneys of male and female dogs administered either dose of D-limonene for 180 consecutive days, it was concluded by the authors that the effects of D-limonene on the male rat kidney reported in NTP (1990) were specific to that sex and species.

5.2.3. α-Pinene (No. 12)

5.2.3.1. Rats. In a sub-chronic inhalation study, groups of F344/N rats (10/sex/group) were exposed to atmospheres of 0, 25, 50, 100, 200, or 400 ppm of α-pinene for 6 h/day, 5 days/week for 14 weeks (NTP, 2006a). Based on an absorption rate of 50%, these inhalation concentrations correspond to estimated daily intakes of 0, 21, 42, 85, 170, or 340 mg/kg bw/day (Fassett, 1978). The animals were observed twice per day and weighed weekly. A complete histopathologic evaluation inclusive of treatment-related gross lesions were performed on all early death animals regardless of dose group, all control animals, all animals, and all animals in the highest treatment group with at least 60% survivors at the time of sacrifice plus all animals in higher treatment groups. Treatment-related lesions (target organs) were identified and these organs plus gross lesions were examined to a no-effect level.

All of the exposed males showed a non-statistically significant decrease in body weight gain (~⩾96% of controls) when compared to controls while the females exposed to 200 ppm or less showed a slight non-statistically significant increase in body weight gain when compared to controls. Females exposed to 400 ppm showed a statistically significant decrease in body weight gain (81.7% of controls) when compared to controls. Six female rats of the 400 ppm group were found dead during the study and three female rats of the same high exposure group displayed mild tremors. Absolute and relative liver weights were significantly increased in males at 200 ppm and greater and relative and absolute kidney weights were significantly increased in males at 100 ppm and greater. In females, relative and absolute liver weights were significantly increased at levels of 50–200 ppm, but there were no increases in either hepatic enzymes or any evidence of histopathological changes at any of these dose levels. Females showed significantly decreased absolute and relative thymus weights, increased relative kidney weight, and increased relative lung weight at the 400 ppm level.

Males showed significant reductions in SDH activity at 400 ppm, ALT activity at levels = 50 ppm, and alkaline phosphatase activity at levels = 100 ppm. Females showed significant reductions in ALT activity at levels = 200 ppm, and alkaline phosphatase activity at the 400 ppm. There were significant decreases at lower levels of exposure for females but these changes were not dose dependent. None of these changes in enzyme activity were related to organ weight changes or evidence of histopathology. Examination of the male kidneys at all dose levels revealed lesions including granular casts and hyaline droplets typical of α_{2u}-globulin nephropathy. It has been concluded that α_{2u}-globulin nephropathy is specific to the male rat and is not relevant to human health assessments (EPA, 1991; Capen et al., 1999). In females, there was no evidence of histopathology in any organ at any dose level. There was no evidence of histopathological changes to the clitoris, ovaries, uterus, epididymis, preputial gland, seminal vesicles, and testes any of the control or test groups of animals. Based on these observations, the NOAEL for male rats was 25 ppm or 21 mg/kg bw/day and the NOAEL for female rats was 200 ppm or 170 mg/kg bw/day.

The principal *in vivo* metabolite of α-pinene is verbenone, which is excreted as verbenol. A 28-day OECD guideline study has been performed with verbenone (Jones, 2003). Groups of male and female Sprague–Dawley rats were administered verbenone via gavage for 28 consecutive days, at a single dose level of 10 mg/kg bw/day. A control group of 10 males and 10 females was dosed with vehicle alone. Clinical signs, bodyweight development and food and water consumption were monitored throughout the study. Hematology and blood chemistry were evaluated for all animals at the end of the study. At study termination, gross necropsies were performed on all of the animals. Histopathological evaluations were conducted on selected tissues from all of the animals. No clinically observable signs of toxicity were reported. There were no adverse effects on body weight, survival, food consumption, water consumption, haematological or blood chemistry parameters. Organ weights for the test animals were comparable to controls. No treatment-related macroscopic effects were reported. Histopathological examination revealed globular accumulations of eosinophilic material in the tubular epithelium of male rats treated at 10 mg/kg bw/day. This finding is consistent with the presence of hydrocarbon nephropathy, which results from the excessive accumulation of $\alpha_{2\mu}$-globulin in renal proximal tubular epithelial cells. $\alpha_{2\mu}$-Globulin is found only in the proximal tubular epithelium of adult male rats (Capen et al., 1999). There was no toxicologically significant difference in incidence or distribution of severity grades of this condition between animals administered verbenone, or nootkatone, a structurally related ketone, which was administered in a parallel study. Oral administration of verbenone to rats for a period of 28 consecutive days at a single dose level of 10 mg/kg bw/day did not result in any toxicologically significant effects.

5.2.3.2. Mice. In a sub-chronic inhalation study, B6C3F$_1$ mice (10/sex/group) were exposed to atmospheres of 0, 25, 50, 100, 200, or 400 ppm of α-pinene for 6 h/day, 5 days/week for 14 weeks (NTP, 2006b). Based on an absorption rate of 50%, these inhalation concentrations correspond to estimated daily intakes of 0, 36, 72, 144, 288, and 576 mg/kg bw/day (Fassett, 1978). The study protocol for mice was the same as for the 14-week rat study.

All mice survived until the study was completed. Body weight gain was comparable for all test animals when compared to controls. Absolute liver weights were significantly increased for both sexes at the 400 ppm and relative and absolute liver weights were significantly increased for both sexes at 200 and 400 ppm. The 400 ppm male group showed significantly decreased absolute and relative thymus weight. No gross or microscopic lesions were associated with these organ weight findings.

Histopathological examination of male and female mice exposed to atmospheres of ⩾100 ppm of α-pinene revealed evidence of hyperplasia of the transitional epithelium of the urinary bladder. However, there was no evidence of histopathological changes to the clitoris, ovaries, uterus, epididymis, preputial gland, seminal vesicles, and testes any of the control or test groups of animals. Based on these observations, a NOAEL for both male and female mice was concluded to be 50 ppm. This dose is estimated to be approximately equal to 72 mg/kg bw/day for both male and female mice.

5.2.4. β-Pinene (No. 13) and 1,3,5-undecatriene (No. 1)

5.2.4.1. Rats. In a 14-day feeding study, 10 Sprague–Dawley rats (five males, five females) were fed diets calculated to provide 10 mg Galbelica/kg bw/day (Shapiro, 1988). Galbelica is a solution composed of 80% β-pinene and 20% 1,3,5-undecatriene in a Pinene, Beta Supra vehicle. For control, two other groups of 10 animals (five males, five females) were maintained on a basal diet with or without Pinene, Beta Supra vehicle only. Animals were fed *ad libitum* and daily observations for signs of gross toxicity and mortality were made. Body weight measurements of all animals were

made at 0, 7, and 14 days, and food consumption was recorded on days 7 and 14. All animals were sacrificed and necropsied on the 14th day. All the animals survived and appeared to be active and healthy throughout the study without signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior in any of the Galbelica-treated rats. Food consumption and body weights were not significantly different between groups. Absolute liver and kidney weights of treated rats were similar to controls, and no signs of gross toxicity were observed at necropsy.

5.2.5. Camphene (No. 14)

5.2.5.1. Rats. A 28-day repeat-dose study was performed with camphene according to OECD Guideline 407 in both sexes of Wistar rats (Hoechst, 1991). Groups of animals (5/sex/group) were given daily doses of 0, 62.5, 250, or 1000 mg/kg bw via gavage for 28 days. Weekly measurement of body weight and food intake revealed no significant differences between test and control animals. Animals of both sexes at the 1000 mg/kg bw/day dose exhibited vacuolisation of hepatocytes and increased liver weights (statistical significance not stated). Male rats also exhibited α_{2u}-globulin-type nephrotoxicity at all dose levels. The renal pathology reported in F344N male rats is a sex- and species-specific phenomenon (see Section 5.3) and was not considered relevant to the human health risk assessment. Based on liver toxicity, the NOAEL for camphene was considered to be 250 mg/kg bw/day.

5.3. Long term studies of toxicity and carcinogenicity

The results of long-term studies of toxicity and carcinogenicity with representative aliphatic and aromatic terpene hydrocarbons are summarized in Table 2 and are described below.

5.3.1. β-Myrcene (No. 2)

5.3.1.1. Mice. Groups of B6C3F$_1$ mice (50/sex/group) were administered 0, 250, 500, or 1000 mg β-myrcene/kg bw/day in corn oil by gavage once per day, 5 days/week for 104 or 105 weeks (NTP, 2010). Animals were housed five/cage and fed *ad libitum*. The animals were observed twice per day and weighed weekly for 12 weeks and monthly thereafter. Necropsies were performed on all animals. Histological examinations were performed on all animals dying during the study, all vehicle controls, and all dosed animals. Tissues from all major organs were examined. Body weight gain was reduced (statistical significance not stated) for all test groups when compared to the control group. Survival of the high-dose group was reduced for both males ($P = 0.008$) and females ($P < 0.001$).

The primary target organ in males and females was the liver. Male mice administered 250 and 500 mg/kg bw/day exhibited a significant ($p < 0.001$) increase in hepatocellular adenomas (41/50, 82% and 43/50, 86%, respectively) compared to that of control males (26/50, 52%). There was also a significant increase in the incidence of hepatocellular carcinomas (0 mg/kg, 14/50, 28%; 250 mg/kg, 20/50, 40%; 500 mg/kg, 28/50, 56%), hepatoblastomas (0 mg/kg, 4/50; 8%; 250 mg/kg, 6/50, 12%; 500 mg/kg, 11/50, 22%), and combined hepatocellular adenomas or carcinomas (0 mg/kg, 33/50, 66%; 250 mg/kg, 44/50, 88%; 500 mg/kg, 48/50, 96%).

In female mice, significantly increased incidences of hepatocellular adenoma (0 mg/kg, 6/50, 12%; 250 mg/kg, 13/50, 26%; 500 mg/kg, 6/50, 12%), hepatocellular carcinoma (0 mg/kg, 1/50, 2%; 250 mg/kg, 7/50, 14%; 500 mg/kg, 2/50, 4%), and hepatocellular adenoma or carcinoma (combined) (0 mg/kg, 7/50, 14%; 250 mg/kg, 18/50, 36%; 500 mg/kg, 8/50, 16%) occurred in the 250 mg/kg group compared to those in the vehicle controls. However, there was no statistically significant difference in the incidence of liver neoplasms between the 500 mg/kg and control female groups. Liver hypertrophy was observed to increase with dose (significant at the mid dose in both sexes), incidences of fatty change were significantly lower in mid-dose animals, and incidences of chronic active inflammation was significantly lower in low-dose females.

Significant findings reported in other tissues included increased incidence of bone marrow atrophy (mid-dose females), increased incidence of lymphoid follicle atrophy of the spleen (mid-dose females), increased incidence of atrophy in the mandibular lymph node (mid-dose females), increased incidence of inflammation and epithelial hyperplasia of the forestomach (mid-dose females), decreased incidence of pancreatic islet hyperplasia (mid-dose males), and decreased incidence of uterine endometrial hyperplasia (low- and mid-dose females).

Based on these observations the NTP concluded "Under the conditions of these 2-year gavage studies, there was *clear evidence of carcinogenic activity* of β-myrcene in male B6C3F$_1$ mice based on increased incidences of liver neoplasms. There was *equivocal evidence of carcinogenic activity* of β-myrcene in female B6C3F$_1$ mice based on marginally increased incidences of hepatocellular neoplasms".

The primary neoplastic effects reported in the 2-year NTP gavage study were associated with the liver of control and treated male and, to a lesser extent, female mice. The high incidence of hepatocellular adenomas, carcinomas and adenomas and carcinomas (combined) in both control and treated groups of male mice is indicative of the sensitivity of the B6C3F$_1$ male mouse liver to toxicity and eventually neoplastic changes. The incidence of hepatocellular adenomas (52%), carcinomas (28%) and ademonas and carcinomas (combined) (66%) indicates the spontaneous nature of these types of neoplasms in control B6C3F$_1$ male mice. This pattern of neoplastic responses is consistent with the historically high levels of background hepatocellular neoplasms in male B6C3F$_1$ mice (Maronpot et al., 1987). The historical spontaneous incidence of liver neoplasms in control male B6C3F$_1$ mice has revealed background incidences of combined hepatocellular adenoma and carcinomas of 32.4% for males with a range of 20–47% (NTP, 2006b). A higher incidence was reported in the control group of the myrcene study (66%). It is generally well accepted that male and female B6C3F$_1$ mouse liver tumors that arise in 2-year bioassays with various agents (e.g., chloroform[6]) are an indirect result of dose-related chronic toxicity and resulting cellular proliferation. In the absence of this chronic toxicity in humans, these tumors are not considered to represent a risk for humans (Cohen et al., 2004).

There is substantial evidence that the appearance of male B6C3F$_1$ mouse liver tumors is not relevant to a human risk assessment. First, there was no statistical significant difference in the incidence of liver neoplasms between the high dose and control females groups and no evidence of an increased incidence of hepatic tumors in male or female rats related to administration of β-myrcene. Second, all dose groups of male B6C3F$_1$ mice suffered chronic hepatic toxicity prior to the development of either liver adenomas or carcinomas, as evidenced by the results of the 90-day and 2-year studies. Hepatocellular adenomas and

[6] In court action (Chlorine Chemistry Council vs. Environmental Protection Agency, 2000 WL 301187, 206 F.3d 1285; D.C. Cir. 2000) related to the exposure to chloroform as a drinking water contaminant, the US EPA used the results of NTP bioassays showing increased liver tumors in rodents to set a maximum contaminant level goal (MCLG) for chloroform of zero. This action was in direct opposition to the recommendation of their own scientific advisory board, which recommended a non-zero MCLG and noted that the rodent liver tumours were the result of chronic high-dose liver toxicity, and as such did not pose a carcinogenic risk to humans. The Court held that US EPA's 1998 rule adopting a MCLG of zero for chloroform was arbitrary and capricious, beyond statutory authority and void. The Court stated that, "In promulgating a zero MCLG for chloroform EPA openly overrode the 'best available' scientific evidence, which suggested that chloroform is a threshold carcinogen" (2000 WL 301187).

carcinomas also occurred late in the life span of males and females. From a biological perspective, the increase in the incidence of tumors in control male $B6C3F_1$ mice reflects the impact of damage to an organ already prone to spontaneous development of neoplasms (Haseman et al., 1985, 1986). The continued use of this strain of mouse as an endpoint for evaluating the hepatocarcinogenic potential of chemical agents in humans is not recommended.

Therefore, it can be concluded that the carcinogenic potential in this sensitive breed and sex of laboratory rodent is a secondary biological response to dose-dependent hepatotoxicity, and is not relevant to humans who consume β-myrcene at low non-toxic levels (<1 mg per day) from intended use as a flavoring ingredient. These levels of intake are at least four orders of magnitude less than those used in the NTP study. The estimated daily *per capita* intake of β-myrcene as a flavoring agent in the US (3 μg/kg bw/day) is more than 83,300 times lower than the lowest dose level in the NTP study. The occurrence of these neoplasms in the present study is considered a high-dose phenomenon without any relevance for assessing the potential cancer risk from the use of β-myrcene as a food flavor ingredient.

5.3.1.2. Rats. A chronic 2-year bioassay on β-myrcene using the standardized NTP protocol with F344/N rats was conducted (NTP 2010). Doses were determined from the results of the prior 13-week subchronic studies. Groups of F344/N rats (50/sex/group) were administered 0, 250, 500, or 1000 mg β-myrcene/kg bw/day in corn oil by gavage, 5 days/week for 104 weeks. The most common clinical signs were thin appearance, ruffled fur and discharge from eyes and nose. Notable (no statistical analysis reported) mean body weight changes in the last half of the study included increased body weight in low- and mid-dose males (102–106% of controls), but decreased body weight in high-dose males (80% of controls); and decreased body weight in high-dose females (87% of controls). Survival rates of females were comparable across all control and dose groups; however, no males in the high-dose group survived past 83 weeks of the study. Males of the low- and mid-dose groups survived at levels similar to the controls.

Histopathological data from high-dose males were not evaluated due to early mortality. In the kidney of F344 rats, renal tubule hyperplasia, adenoma, and carcinoma represent a continuum of renal tubule proliferative lesions. Male rats showed a dose-related increase in levels of papillary mineralization (0 mg/kg, 1/50, 2%; 250 mg/kg, 48/50, 96%; 500 mg/kg, 40/50, 80%), hyperplasia (0 mg/kg, 0/50, 0%; 250 mg/kg, 0/50, 0%; 500 mg/kg, 2/50, 4%) and nephrosis (0 mg/kg, 0/50, 0%; 250 mg/kg, 42/50, 84%; 500 mg/kg, 46/50, 92%). Analysis of single and step sections (combined) revealed a significant increased incidence of renal adenomas (0 mg/kg, 0/50, 0%; 250 mg/kg, 12/50, 24%; 500 mg/kg, 13/50, 26%), but no statistically significant increase in the incidence of carcinomas (0 mg/kg, 0/50, 0%; 250 mg/kg, 3/50, 6%; 500 mg/kg, 1/50, 2%). In females, the incidence and severity of renal effects were less pronounced than in the male. Analysis of single and step sections (combined) in females revealed a non-statistically significant ($p < 0.05$) increase in adenomas (0 mg/kg, 0/50, 0%; 250 mg/kg, 2/50, 4%; 500 mg/kg, 1/50, 2%; 1000 mg/kg, 3/50, 6%). Time to first incidence of tumors was 551 days in males and 689 in females. This pattern of renal response in males and females F344 rats is indicative of the chronic nephropathy associated with aging rats. This type of renal mineralization reported in males in this study is a chronic manifestation of α_{2u}-globulin nephropathy (Hard et al., 1993).

Significant findings in the incidences of neoplasms and/or non-neoplastic lesions noted in other tissues include decreases in basophilic focus and mixed cell focus of the liver (low- and mid-dose males), decreased basophilic focus of the liver (high-dose females), increased eosinophilic focus of the liver (mid- and high-dose females), decreased chronic inflammation of the liver (mid-dose males), increased chronic inflammation of the nose (mid-dose males), increased chronic active inflammation of the forestomach (mid-dose males), increased thyroid gland C-cell adenoma (low-dose females), and increased cystic endometrial hyperplasia of the uterus (high-dose females). Inflammation and ulceration are common observances in rodents administered materials via gavage (Adams et al., 2008; Haseman et al., 1984; NTP, 2003).

Based on the reported renal effects, the NTP concluded "Under the conditions of these 2-year gavage studies, there was *clear evidence of carcinogenic activity* of β-myrcene in male F344/N rats based on increased incidences of renal tubule neoplasms. There was *equivocal evidence of carcinogenic activity* of β-myrcene in female F344/N rats based on increased incidences of renal tubule adenoma". Clearly, the renal neoplastic effects in males were more pronounced than in females.

The renal pathology reported in the 2-year bioassay for β-myrcene in the male F344/N rat mimic that of other hydrocarbons in NTP studies (i.e., pinene and D-limonene). The classic profile of results involves poor survival, mean body weight changes, chronic nephropathy, and associated renal toxicity that are specific to this strain and sex of rat. Analysis by NTP researchers (Haseman et al., 1998) have shown the survival rates of feeding study and control F344 male rats have decreased significantly over the last decade (66% and <50%, respectively). One of the major causes of death is severe chronic nephropathy that has been increasing in incidence in more recent control groups (Eustis et al., 1994). This species- and sex-specific phenomenon, in all probability, reflects the sensitivity of the male rat kidney to chronic progressive nephropathy, focal tubular and pelvic transitional urothelial hyperplasia, and specific tumorigenic responses. The interaction of test substances with spontaneous, age-related renal disease in laboratory rats has recently been reviewed (Hard, 1998; Lock and Hard, 2004). Based on a comprehensive review of renal tumors of all types reported in NTP bioassays, it seems that the interaction of chemical agents and spontaneous chronic progressive nephropathy occurs at two levels; (1) to exacerbate the rate of chronic progressive nephropathy, and (2) to stimulate tubule hyperplasia into foci of atypical hyperplasia eventually leading to adenomas. The induction of tumors via this pathway normally produces a minimal response in male rats leading to a low incidence of tumors of relatively small size and low grade that develop late in life.

In the β-myrcene study, poor survival, especially in control and high-dose animals, severely reduced the sensitivity of the study for detecting the presence of a carcinogenic response in chemically-exposed groups of male rats. Excessive mortality in the control that occurred primarily during the last quarter of the study limited the ability to detect the renal effects resulting from chronic nephropathy. Mean body weights of both control and test males peaked long before study termination (week 76 for control males to week 56 for high dose males) suggesting that systemic changes related to chronic nephropathy occurred and the overall health of the animals was adversely affected. These weight changes are similar to those observed in numerous other bioassays for other substances (Hard, 1998). Nevertheless, the severity of the chronic nephropathy was significantly greater with increasing dose as seen by increased renal tubule hyperplasia, increased hyperplasia of the transitional epithelium of the pelvis, increased renal tubule adenoma in both single section evaluation and step section evaluation.

Also relevant is the difference between male and female rats in the transport of organic anions such as glucuronic acid conjugates in the proximal tubules. Although substances can pass into the cell from the lumen, they can also accumulate in tubule cells from the interstitial compartment by a variety of transporters. Uptake of

substances from the peritubular plasma across the basolateral membranes is mediated by an organic anion transporter (OAT) comprised of approximately 550 amino acids (Tojo et al., 1999; Kojima et al., 2002). Although several isoforms have been identified, OAT1 has been detected exclusively in the S1, S2, and S3 sections of the proximal tubules. Messenger OAT1 RNA expression is significantly higher in male Sprague–Dawley rats compared to female rats (Buist et al., 2002). Therefore, the male rat is expected to experience greater glucuronide loads in the proximal tubules. This may be of little consequence when the organic moiety is relatively polar. However, when the conjugate is bound to a hydrophobic moiety (such as a hydrocarbon), increased renal tubule toxicity is anticipated. The fact that the pattern of renal pathology for the 2-year bioassay for α-methylbenzyl alcohol mirrors that of β-myrcene in male and female rats supports the conclusion that dose-dependent loading in the proximal tubule is a part of the basis for understanding the origin of the difference in renal pathology between the male and female F344/N rat, in addition to the normally present sex differences in urinary protein and aging chronic nephropathy.

Aging chronic nephropathy (Hard and Khan, 2004; Hard and Seely, 2005; Haseman et al., 2003; Seely et al., 2002) is considerably more severe in males compared to female rats in most strains, including the F344. Although the reason for this difference is not completely understood, the much higher urinary concentration of protein, primarily because of α_{2u}-globulin, in male rats is considered a major contributing factor.

Several modes of action for renal carcinogenesis in rats and mice have recently been summarized (Lock and Hard, 2004). One of these modes is a marked increase in the severity of the aging chronic nephropathy associated with an increase of tumors, mostly adenomas. One of the characteristics of aging chronic nephropathy is increased renal tubular degeneration and regenerative hyperplasia. Under normal circumstances, the degree of tubular proliferation is insufficient to generate atypical hyperplastic foci or adenomas. However, if the chronic nephropathy increases in severity, as in the 2-year rat study, there is considerably more tubular degeneration, but more importantly, an increase in tubular proliferation, hyperplastic foci, atypical hyperplastic foci and an increase in the incidence of adenomas. The findings of the 2-year rat study support a conclusion that increased severity of aging chronic nephropathy is an explanation for the renal tubular effects produced by β-myrcene, with a correlating dose–response change in tubular proliferation response, a predominant (or exclusive) effect in males compared to females, the lack of other changes (i.e., tubular necrosis, α_{2u}-globulin, increased apoptosis) that could explain the effect, and the lack of such effects in the mouse. Thus, in the 2-year rat study with β-myrcene, the increasing severity of the aging chronic nephropathy can be concluded to be largely responsible for the renal tubular proliferation in the male rat. This mode of action is not relevant to human renal carcinogenesis, and thus, these rat tumors indicate no renal carcinogenic risk in humans (Hard et al., 2009).

The dose-related increased incidences of renal pelvis transitional (urothelial) cell hyperplasia seen in this study can also be explained by the increasing severity of aging chronic nephropathy with dose. One of the manifestations of aging chronic nephropathy is marked deposits of calcium salts (especially calcium oxalate) in the medulla, cortico–medullary junction, and at the fornices of the renal pelvis. The amount of mineralization increases, both in incidence and severity, with increasing severity of the aging chronic nephropathy. The calcium deposition at the renal fornices extends along the renal pelvis and is associated with transitional cell proliferation (hyperplasia). Under exceptional circumstances, the degree of transitional cell proliferation can lead to a papilloma or carcinoma of the kidney pelvis.

The increasing incidences of transitional cell hyperplasia seen in this study were attributed to the increasing severity of the aging chronic nephropathy by the NTP itself. Supporting this conclusion is the lack of a hyperplastic effect in the urinary bladder. If the chemical itself was directly producing the transitional cell proliferation effect, rather than indirectly by increasing the severity of the aging chronic nephropathy, the effect would be expected to be greater in the bladder than in the kidney pelvis in the absence of urinary tract obstruction (Hanai et al., 2002). In addition, the effect was not seen in female rats and was not present in mice of either sex.

Thus, the kidney pelvis transitional cell hyperplasia seen in rats administered β-myrcene is due to increased severity of the aging chronic nephropathy and is not indicative of a risk to humans.

Excessive mortality, late-stage reduction in body weights, the presence of chronic nephropathy in all control and test groups, and the dose-dependent exacerbation of renal tubule hyperplasia by the test agent or more likely, the principal metabolite, may eventually lead to an increase in the incidence of renal tubule adenomas in male rats. Since the carcinogenic and hyperplastic effects are secondary to renal toxicity and specific to the male F344/N rat, the results have no relevance to the safety of β-myrcene in humans.

5.3.2. D-Limonene (No. 5)

5.3.2.1. Mice. Based on the standardized NTP protocol, groups of B6C3F$_1$ mice (50/sex/group) were administered 0, 250, or 500 mg D-limonene/kg bw/day (males) or 0, 500, or 1000 mg D-limonene/kg bw/day (females) in corn oil by gavage, 5 days/week for 103 weeks (NTP, 1990). Final mean body weights for of high-dose female mice administered (1000 mg/kg bw/day) D-limonene were generally 5–15% lower than vehicle controls from week 28 to study termination. No compound-related clinical signs were reported for the duration of the study. Results of survival data were not-dose related. Although survival of the low-dose male group (250 mg/kg bw/day) was significantly ($p = 0.048$) lower than that of the vehicle controls by study termination, survival of the high-dose (500 mg/kg bw/day) male mice was comparable to vehicle controls. High-dose male mice exhibited a significantly increased incidence of multinucleated and cytomegalic hepatocytes compared to controls. However, the incidences of hepatocellular adenomas or carcinomas (combined) in D-limonene treated mice were not significantly different from vehicle controls. In fact, no compound-related neoplasms were reported in any of the mice administered D-limonene. The authors concluded that under the conditions of the 2-year gavage study, there was no evidence of carcinogenic activity of D-limonene for male or female B6C3F$_1$ mice at the dose levels tested.

5.3.2.2. Rats. A chronic 2-year bioassay on D-limonene using the standardized NTP protocol with F344/N rats was conducted (NTP, 1990). Doses were determined from the results of the prior 13-week subchronic studies. Groups of F344/N rats (50/sex/group) were administered 0, 75, or 150 mg D-limonene/kg bw/day (males) or 0, 300, or 600 mg D-limonene/kg bw/day (females) in corn oil by gavage, 5 days/week for 103 weeks. Mean body weights of high-dose for male rats administered (150 mg/kg bw/day) of D-limonene were generally 4–7% lower than vehicle controls from week 2 to study termination. Mean body weights of high-dose females (600 mg/kg bw/day) were generally 4–7% lower than vehicle controls from week 28 to study termination. No compound-related clinical signs were reported for the duration of the study. Survival of the high-dose male group was significantly greater than that of the vehicle controls after week 81. Survival of the high-dose female group was significantly lower than that of the vehicle controls after week 39. In the kidneys of male rats, dose-related increases were

observed in the incidence of mineralization of the renal papilla and focal hyperplasia of the transitional epithelium overlying the papilla. A dose-related increase in the severity of nephropathy was reported in male rats administered D-limonene. In addition, increased incidences of tubular cell hyperplasia and neoplasia were also reported in dosed male rats. The incidences of tubular cell adenoma incidence in high-dose male rats and incidence of tubular cell adenoma or tubular cell adenocarcinomas (combined) in dosed male rats were significantly greater than vehicle controls. Dosed male rats also exhibited a significant dose-related increase in α_{2u}-globulin in the kidney when compared with vehicle controls. No such increase was observed in dosed female rats. No compound-related lesions of the uterus, testis, hematopoietic system, skin, or eye were noted in any of the rats dosed with D-limonene. The authors concluded that under the conditions of the 2-year gavage study, there was clear evidence of carcinogenic activity of D-limonene for male F344/N rats, as shown by the increased incidences of tubular cell hyperplasia, adenomas, and adenocarcinomas of the kidney. However, there was no evidence of carcinogenic activity of D-limonene for female rats receiving 300 or 600 mg/kg bw/day.

It has been demonstrated that renal lesions, which were observed in the NTP study, resulted from the accumulation of aggregates of α_{2u}-globulin (a low molecular-weight protein synthesized in the liver) and D-limonene metabolites, probably the 1,2-epoxide, in the P2 segment of the renal proximal tubule (Capen et al., 1999). These aggregates prevent lysosomal degradation, which leads to accumulation in the cytoplasm of the protein or the protein–chemical complex, which leads to single cell necrosis, regenerative tubular proliferation and ultimately, renal neoplasia (Lehman-McKeeman et al., 1990; Hildebrand et al., 1997). This phenomenon has only been observed in the male rat and is not relevant to humans (Strasser et al., 1988; Borghoff et al., 1990; Capen et al., 1999).

The gene that encodes α_{2u}-globulin has been isolated and the amino acid sequence deduced (Untermann et al., 1981). These proteins are expressed in the male rat liver under hormonal control (Roy and Neuhaus, 1967; Wang and Hodgetts, 1998). α_{2u}-Globulin belongs to a large superfamily of proteins that are characterized by a unique hydrophobic binding pocket, which serves as a carrier for small lipophilic molecules.

The nephrotic lesions observed in male F344/N rats do not develop in the female F344/N rat or in humans (Bucher et al., 1986). Subsequent investigations have shown that the α_{2u}-globulin nephropathy found in the F344/N male rat only develops in mammals that express the hepatic form of α_{2u}-globulin (Swenberg et al., 1989). Therefore, α_{2u}-globulin nephropathy has not been observed in some strains of rats (Dietrich and Swenberg, 1991), in any mice (Bucher et al., 1986; Lehman-McKeeman and Caudill, 1994) or dogs (Webb et al., 1990). The analogous protein in male mice, major urinary protein (MUP), does not bind D-limonene or its 1,2-epoxide, and no hyaline droplet formation occurs in the proximal tubules.

Transgenic mice that express rat α_{2u}-globulin were tested for their ability to form hyaline droplets and develop nephropathies similar to their adult male rat counterparts (Lehman-McKeeman and Caudill, 1994). This study involved male F344 rats as the positive control, transgenic C57BL/6J mice as the experimental group and native C57BL/6 mice as the negative controls. The animals at age 70–75 days were placed in metabolic cages and received 150 mg/kg bw/day of D-limonene in corn oil by gavage for 3 days. D-Limonene was used to induce renal nephropathy in adult male rats, as it was shown to be a potent inducer in the NTP studies (NTP, 1990; EPA, 1991). Twenty-four hours after the last dose, the animals were killed and the kidneys were analysed for evidence of nephropathy. Hyaline droplet formation was evaluated on a subjective scale, assessing droplet size and intensity (graded 0–4), which were subsequently multiplied by tubular loading of droplets graded (0–3), for an overall scale of 0–12 (with 12 being the most severe). Prior to D-limonene treatment, the control group transgenic mice and control rats showed hyaline droplet scores of 1 ± 0 and 6 ± 0.5, respectively. Transgenic mice and control rats showed hyaline droplet score of 2.5 ± 0.3 and 11 ± 1.3, respectively, upon dosing with D-limonene. The native mice (negative controls) developed no signs of hyaline droplet formation and tested negative for presence of α_{2u}-globulin in their urine. In contrast, urinary excretion of α_{2u}-globulin in transgenic mice was about 30% of that seen in control rats. Additionally, the urinary excretion of MUP in transgenic mice was significantly lower (approximately 40%) than in native mice, although the excretion of total urinary protein did not significantly differ between the two groups. Binding of D-limonene to transgenic mouse kidney proteins also was not observed in native mice. The authors asserted that based on the data presented, "α_{2u}-globulin is the only protein that is involved in the etiology of hyaline droplet nephropathy".

An increase in the α_{2u}-globulin was seen in the urine of male Sprague–Dawley rats when these animals were administered >30 mg/kg bw/day of D-limonene for 714 days by gavage (Saito et al., 1996). The increases in the urinary α_{2u}-globulin were dose-dependent and paralleled elevated accumulation in the kidney cells.

While humans produce low molecular weight serum proteins, which are reabsorbed by the kidney, there is no evidence that α_{2u}-globulin is produced (Olson et al., 1990), nor do any of the analogous proteins in humans bind limonene-1,2-epoxide (Lehman-McKeeman and Caudill, 1994). None of the analogous proteins in humans are present at concentrations anywhere near the concentration of α_{2u}-globulin in male rats (generally 4–6 orders of magnitude lower in concentration). Urine collected from adult male F344 rats and humans revealed no evidence indicative of α_{2u}-globulin production in humans (Olson et al., 1990).

It is unknown whether any human serum proteins possess a binding site similar to that of α_{2u}-globulin. Although this is a possibility, it appears remote, since female rats and both sexes of mice do not show the renal changes noted in male rats exposed to limonene. It should be noted that there is a class of human proteins referred to as the α_{2u}-globulin-related proteins. They appear to have no functional relationship to the adult male rat urine proteins. The human protein has a higher molecular weight (25 kDa), and is a component of a neutrophil gelatinase complex (Triebel et al., 1992; Kjeldsen et al., 2000). An extensive review of the current scientific literature and genome databases reveals no native protein or biological entity that acts as a nephrotoxic agent like mature male rat α_{2u}-globulin. The weight of evidence indicates that it is the unique nature of the high levels of α_{2u}-globulin in the male rat that allows D-limonene to interfere with renal processing of the male-specific α_{2u}-globulin. The 1,2-epoxide of D-limonene is expected to bind only to α_{2u}-globulin, not to any of the analogous proteins in rats, mice or humans. Therefore, this process is not predictive of human carcinogenicity. In a comprehensive review of α_{2u}-globulin nephropathy associated renal tubule tumors secondary to hyaline droplet formation in the male F344/N rat exposed to limonene and other simple chemical substances (e.g., isophorone, decalin and methyl isobutyl ketone), it was concluded that the male F344/N rat is not an appropriate model for assessing human renal carcinogenic risk by this mode of action (EPA, 1991). After careful review, it has been concluded that the mechanisms leading to the renal carcinogenic findings in the male F344/N rat are known and strongly indicate that the nephropathy associated with D-limonene have no significance for human risk assessment (Burdock et al., 1990). The International Agency for Research on Cancer (IARC) reiterates this conclusion by stating "the mechanism

by which D-limonene increases the incidence of renal tubular tumors in male rats is not relevant to humans" (Capen et al., 1999).

5.4. Genotoxicity and mutagenicity studies

Genotoxicity and mutagenicity testing has been performed on 10 representative aliphatic and aromatic terpene hydrocarbons. The results of these tests are summarized in Table 3 and described below.[7]

5.4.1. In vitro

No evidence of mutagenicity was observed when camphene (No. 14), β-caryophellene (No. 16), *p*-cymene (No. 17), D-limonene (No. 5), α-pinene (No. 12), β-pinene (No. 13), *p*-mentha-1,3-diene (No. 7), *p*-mentha-1,4-diene (No. 8), or β-myrcene (No. 2) were incubated with *Salmonella typhimurium* strains TA97, TA98, TA100, TA1535, TA1537, TA1538, and/or TA102, UTH8413, or UTH8414 with and without S9 metabolic activation at concentrations up to 150,000 μg/plate (Connor et al., 1985; DeGraff, 1983; Florin et al., 1980; Haworth et al., 1983; Heck et al., 1989; Jagannath, 1984a,b; Muller, 1993; NTP, 2010; Rockwell and Raw, 1979). β-Myrcene was also negative in *Escherichia coli* strain WP2 *uvrA*/pKM101 with and without S9 (NTP, 2010). In a study comparing the mutagenic potential of different terpene hydrocarbons, β-myrcene (No. 2), α-terpinene (*p*-mentha-1,3-diene, No. 7), or (+)- and (−)-α-pinene (No. 12) were incubated with *S. typhimurium* strains TA98, TA100, TA1535, and TA97a tester strains with and without S9 metabolic activation at concentrations up to 5000 μg/plate. There was no evidence of mutagenicity in any of these assays (Gomes-Carneiro et al., 2005).

Without metabolic activation, 3-carene tested positive in the plate incorporation Ames assay, in *S. typhimurium* strains TA100 and TA102, at concentrations between 2.5–5 μl/plate (1075–4300 μg/plate), but was negative in both strains with metabolic activation (Kurttio et al., 1990). 3-Carene also tested negative in TA98 with and without metabolic activation (Kurttio et al., 1990).

In an *in vivo–in vitro* study designed to investigate the mutagenicity of the metabolites of α-pinene and camphene, Sprague–Dawley rats were administered a single dose of 0.5 ml of α-pinene (approximately 1716 mg/kg bw) or camphene (approximately 1690 mg/kg bw) by gavage and the urine was collected for 24 h (Rockwell and Raw, 1979). Prior to their administration to rats, 0.05–100 μl samples of α-pinene or camphene were each assayed for mutagenicity in a reverse mutation assay using *S. typhimurium* strains TA98 and TA100 in the presence of metabolic activation. Both compounds produced negative results. To assess the genotoxic potential of urinary metabolites, the urine was directly assayed or extracted with ether following dilution in a phosphate buffer and treatment with β-glucuronidase for hydrolysis of glucuronide conjugates. The 24-h urine samples (500 μl), the ether extracts of the urine, and the aqueous fractions of the urine–ether extracts were then separately incubated with *S. typhimurium* strains TA98 and TA100 with S9 activation. The urinary solutions (i.e., direct urine sample, urine–ether extract, and the aqueous fraction of the urine–ether extract) isolated from the rats administered 0.5 ml of α-pinene did not show any evidence of mutagenicity in either TA98 or TA100 with metabolic activation. Assays of the ether extract of the urine from animals administered 0.5 ml camphene showed a weak response in TA100 with metabolic activation, but the aqueous fraction of the urine-ether extract did not (Rockwell and Raw, 1979). In a more standard assay with camphene itself, there were no effects on several strains of *S. typhimurium*, including TA100, with or without activation (Connor et al., 1985). Similarly, α-pinene, β-caryophellene and *p*-mentha-1,4-diene did not induce unscheduled DNA synthesis in rat hepatocytes at concentrations of up to 10,000 μg/mL (Heck et al., 1989).

D-Limonene did not induce chromosomal aberrations when incubated with Chinese hamster ovary (CHO) cells at a concentration of 10–500 μg/mL (Anderson et al., 1990), nor did it, β-pinene, camphene, β-caryophyllene, or α-phellandrene (No. 9) induce sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells (Anderson et al., 1990; Kauderer et al., 1991; Sasaki et al., 1989). In a mouse lymphoma forward mutation assay (MLA), D-limonene produced negative results in L5178Y cells with and without S9 metabolic activation, up to a maximum concentration of 100 μg/mL (Heck et al., 1989; Myhr et al., 1990).

In an *in vitro* chromosome aberration test and SCE tests with human lymphocytes, myrcene did not induce chromosomal aberrations at concentrations of up to 1000 μg/mL with and without metabolic activation (Kauderer et al., 1991). Additionally, myrcene tested negative when incubated with V79 and hepatic tumor (HPT) Chinese hamster cells at concentrations of up to 500 μg/ml in SCE assays (Roscheisen et al., 1991). While a slight, reproducible increase in SCE induction was noted in the HPT cell line, it was reported to be not dose-dependent (Roscheisen et al., 1991). In fact, myrcene reduced the SCE-inducing effect of S9 mix-activated cyclophosphamide in human lymphocytes and CHO cells, as well as of aflatoxin B1 in V79 and HTC Chinese hamster cells in a dose-dependent manner (Kauderer et al., 1991; Roscheisen et al., 1991).

5.4.2. In vivo

In an *in vivo* mammalian spot test, no evidence of mutagenicity was reported when 126 mouse embryos of C57BL/6JHan and T-stock crossed animals were treated *in utero* by intraperitoneal injection with 215 mg/kg bw/day of D-limonene on days 9–11 of gestation (Fahrig, 1984).

β-Myrcene (100, 500, or 1000 mg/kg) was orally administered via gavage to male and female Wistar rats (2 or 4/sex/group) (Zamith et al., 1993). Corn oil was used as the negative control, while cyclophosphamide (30 mg/kg bw via intraperitoneal injection) was used as the positive control. A mitotic inhibitor, colchicine (5 mg/kg bw via intraperioneal injection), was administered 1 h before sacrifice. At 24 or 48 h, the animals were sacrificed and their bone marrow cells were harvested. Evaluations included the mitotic index and frequency of chromosomal aberrations. A dose-related increase in the mitotic index in bone marrow cells was reported for rats administered the test substance, demonstrating that it was present at a sufficient dose in the target tissues. No significant increases in chromosomal aberrations were reported in treated animals upon examination at either 24 or 48 h. The authors concluded that β-myrcene was not clastogenic to the rat when orally administered at dose levels of up to 1000 mg/kg bw.

In an *in vivo* mouse micronucleus assay, groups of male and female mice (5/sex/group) were given a single oral dose of 0 or 4000 mg/kg bw of camphene by gavage (Hoechst, 1991). There was no evidence of micronucleated polychromatic erythrocytes for treated or control groups. β-Myrcene tested negative in an *in vivo* mouse peripheral blood micronucleus assay in which a single oral dose of 0, 250, 500, 1000, or 2000 mg/kg bw of β-myrcene via gavage (5 or 2/sex/group) was given to male and female mice (NTP, 2010).

5.4.3. Genotoxicity and mutagenicity conclusions

Nine representative substances of this group had consistently negative results in the Ames assay. Only 1 agent, 3-carene, produced positive results in this assay; specifically in strains TA100 and TA102 and only without metabolic activation. 3-Carene produced no increase in the number of revertants in strain TA98. In

[7] For conversions used, see Table 3.

Table 3
In vitro genotoxicity studies on aliphatic and aromatic terpene hydrocarbon derivatives.

#	Substance Name	Test system *in vitro*	Test object	Maximum concentration of substance	Result	Reference
2	β-Myrcene	Reverse mutation	*Salmonella typhimurium* TA97, TA98, TA100, and TA1535	Up to 10,0000 μg/plate	Negative[a]	NTP (2010)
2	β-Myrcene	Reverse mutation	*Escherichia coli* WP2 *uvrA* pKM101	Up to 10,0000 μg/plate	Negative[a]	NTP (2010)
2	Myrcene	Sister chromatid exchange	Human lymphocytes	100–1000 μg/ml	Negative[a]	Kauderer et al. (1991)
2	Myrcene	Sister chromatid exchange	Chinese hamster ovary cells and hepatic tumor cell line	100–500 μg/ml	Negative[a,b]	Roscheisen et al. (1991)
2	Myrcene	Chromosomal aberration	Human lymphocytes	100–1000 μg/ml	Negative[a]	Kauderer et al. (1991)
2	Myrcene	Gene mutation	Chinese hamster ovary V79 cells	100–1000 μg/ml	Negative[a]	Kauderer et al. (1991)
5	D-Limonene	Reverse mutation	*Salmonella typhimurium* TA100, TA98, TA1535 and TA1537	Up to 30 μmol/plate (4087.2 μg/plate)[c,d]	Negative[a,e]	Florin et al. (1980)
5	D-Limonene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98 TA1537 and TA1538	150,000 μg/plate	Negative[a]	Heck et al. (1989)
5	D-Limonene	Reverse mutation	*Salmonella typhimurium* TA102	Up to 5000 μg/plate[c]	Negative[a]	Muller (1993)
5	D-Limonene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98 and TA1537	0.3–3333 μg/plate	Negative[a]	Haworth et al. (1983)
5	D-Limonene	Reverse mutation	*Salmonella typhimurium* TA100, TA98, UTH8414 and UTH8413	10–500 μg/plate	Negative[a]	Connor et al. (1985)
5	D-Limonene	Forward Mutation	L5178Y Mouse Lymphoma	100 μg/ml	Negative[a]	Heck et al. (1989)
5	D-Limonene	Forward mutation	L5178Y Mouse Lymphoma	Up to 100 nl/ml (84.1 μg/ml)[f]	Negative[a]	Myhr et al. (1990)
5	D-Limonene	Sister chromatid exchange	Chinese hamster ovary cells	15–162 μg/ml	Negative[a]	Anderson et al. (1990)
5	D-Limonene	Sister chromatid exchange	Chinese hamster ovary cells	10–1000 μM (1.4–136.2 μg/ml)[g]	Negative[l]	Sasaki et al. (1989)
5	D-Limonene	Chromosomal aberration	Chinese hamster ovary cells	10–500 μg/ml	Negative[a]	Anderson et al. (1990)
8	*p*-Mentha-1,4-diene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98, TA1537 and TA1538	50,000 μg/plate	Negative[a]	Heck et al. (1989)
8	*p*-Mentha-1,4-diene	Unscheduled DNA synthesis	Rat hepatocytes	30 μg/ml	Negative	Heck et al. (1989)
9	α-Phellandrene	Sister chromatid exchange	Chinese hamster ovary cells	33.3–1000 μM (4.5–136.2 μg/ml)[h]	Negative	Sasaki et al. (1989)
11	3-Carene	Reverse mutation	*Salmonella typhimurium* TA100, TA98 and TA102	1.25–5 μl/plate (1075–4300 μg/plate)[i]	Positive[j] Negative[k]	Kurttio et al. (1990)
12	α-Pinene	Reverse mutation	*Salmonella typhimurium* TA100 and TA98	0.05–100 μl/plate (42.9–85,800 μg/plate)[l]	Negative[k]	Rockwell and Raw (1979)
12	α-Pinene	Reverse mutation	*Salmonella typhimurium* TA100, TA98, TA1535 and TA1537	Up to 30 μmol/plate (4087.2 μg/plate)[m]	Negative[a,e]	Florin et al. (1980)
12	α-Pinene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98 TA1537 and TA1538	25,000 μg/plate	Negative[a]	Heck et al. (1989)
12	α-Pinene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98, TA1537 and TA1538	0.1–25 μl/plat (85.8–21,450 μg/plate)[l,e]	Negative[a]	Jagannath (1984a)
12	α-Pinene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98, TA1537 and TA1538	0.01–5 μl/plate (8.6–4290 μg/plate)[l]	Negative[a]	DeGraff (1983)
12	α-Pinene	Reverse mutation	*Salmonella typhimurium* TA100, TA98, UTH8414 and UTH8413	10–500 μg/plate	Negative[a]	Connor et al. (1985)
12	α-Pinene	Unscheduled DNA synthesis	Rat hepatocytes	10,000 μg/ml	Negative	Heck et al. (1989)
13	β-Pinene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98, TA1537 and TA1538	5000 μg/plate	Negative[a]	Heck et al. (1989)
13	β-Pinene	Reverse mutation	*Salmonella typhimurium* TA100, TA98, TA1535 and TA1537	Up to 30 μmoles/plate (4087.2 μg/plate)[n]	Negative[a,o]	Florin et al. (1980)
13	β-Pinene	Sister chromatid exchange	Chinese hamster ovary cells	33–1000 μM (4.5–136.2 μg/ml)	Negative	Sasaki et al. (1989)
14	Camphene	Reverse mutation	*Salmonella typhimurium* TA100 and TA98	0.05–100 μl/plate (42–84,500 μg/plate)[p]	Negative[k]	Rockwell and Raw (1979)
14	Camphene	Reverse mutation	*Salmonella typhimurium* TA100, TA98, UTH8414 and UTH8413	10–1000 μg/plate	Negative[a]	Connor et al. (1985)
14	Camphene	Sister chromatid exchange	Chinese hamster ovary cells	10–1000 μM (1.4–136.2 μg/ml)[p]	Negative[q]	Sasaki et al. (1989)
16	β-Caryophyllene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98, TA1537 and TA1538	150,000 μg/plate	Negative[a]	Heck et al. (1989)
16	β-Caryophyllene	Reverse mutation	*Salmonella typhimurium* TA1535, TA100, TA98, TA1537 and TA1538	0.1–150 μl/plate	Negative[a]	Jagannath (1984b)
16	β-Caryophyllene	Sister chromatid exchange	Chinese hamster ovary cells	10–1000 μM (2.0–204.4 μg/ml)[r]	Negative[q]	Sasaki et al. (1989)
16	β-Caryophyllene	Unscheduled DNA synthesis	Rat hepatocytes	10,000 μg/ml	Negative	Heck et al. (1989)
17	*p*-Cymene	Reverse mutation	*Salmonella typhimurium* TA98, TA100	0.05–100 μl/plate (42.7–85,400 μg/plate)[c,s]	Negative[k]	Rockwell and Raw (1979)

(*continued on next page*)

Table 3 (*continued*)

#	Substance Name	Test system *in vitro*	Test object	Maximum concentration of substance	Result	Reference
In vivo genotoxicity studies on aliphatic and aromatic terpene hydrocarbons						
2	Myrcene	Chromosome aberrations	Rat bone marrow cells	100–1000 mg/kg bw[t]	Negative	Zamith et al. (1993)
2	Myrcene	Micronucleus formation	Mouse peripheral blood	250–2000 mg/kg bw[u]	Negative	NTP (2010)
5	D-Limonene	Mammalian spot test	Mouse embryos from C57BL/6JHan x T stocks	215 mg/kg bw[t]	Negative	Fahrig (1984)
14	Camphene	Micronucleus formation	Mouse bone marrow cells	4000 mg/kg bw[u]	Negative	Hoechst (1991)

[a] With and without metabolic activation.
[b] Slight increase in sister chromatid exchange induction was noted in the hepatic tumor cell line, which was reproducible but not dose-dependent.
[c] Isomer not specified.
[d] Cytotoxicity observed at doses ⩾333 μM.
[e] Cytotoxicity and precipitation observed at doses >3 μmol/plate.
[f] Calculated using density of D-limonene = 0.837–0.841 g/ml (FCC, 1996).
[g] Calculated using molecular weight of D-limonene = 136.24.
[h] Calculated using molecular weight for α-phellandrene = 136.24.
[i] Calculated using density of 3-carene = 0.860 g/ml (Weast, 1973).
[j] Without metabolic activation in TA100 and TA102 only at doses ⩾2.5 ml/plate.
[k] With metabolic activation.
[l] Calculated using density of α-pinene = 0.858 g/ml (Weast, 1973).
[m] Calculated using molecular weight for α-pinene = 136.24.
[n] Calculated using molecular weight of β-pinene = 136.24.
[o] Cytotoxicity observed at doses >3 μmol/plate.
[p] Calculated using molecular weight of camphene = 136.24.
[q] Cytotoxicity observed at the highest dose tested (1000 μM).
[r] Calculated using molecular weight of β-caryophyllene = 204.36.
[s] Calculated using specific gravity of *p*-cymene = 0.853–0.855 g/ml (FCC, 1996).
[t] Administered via intraperitoneal injection.
[u] Administered via gavage.

mammalian cell systems, representative members of this group generally showed no mutagenic activity in SCE, chromosomal aberrations, or unscheduled DNA synthesis. The few positive findings were found at near toxic concentrations. *In vivo* studies examining micronucleus formations, chromosomal aberrations, or mammalian spot test were consistently negative. Based on the available evidence, it can be concluded that the 17 monoterpene aliphatic and aromatic hydrocarbons in this group provide no significant genotoxic potential.

5.5. Reproductive and developmental toxicity studies

D-Limonene was administered to pregnant Japanese white rabbits from days 6 to 18 of gestation at dose levels of 0, 250, 500, or 1000 mg/kg bw/day (Kodama et al., 1977a). Signs of maternal toxicity included increased mortality in the high-dose group (1000 mg/kg bw/day) and a significant, but temporary, decrease in body weight gain and food consumption in the mid- (500 mg/kg bw/day) and high-dose groups. No anomalies or behavioral changes were observed in dams of the low- (250 mg/kg bw/day) and mid-dose groups. The NOEL of D-limonene for maternal toxicity was determined to be 500 mg/kg bw/day. No abnormalities were noted in any of the fetuses upon external examination. In addition, none of the anomalies observed upon visceral and skeletal examination were considered to be attributable to D-limonene treatment, since they were not dose dependent and were restored to normal during postnatal development. The NOEL for offspring toxicity was determined to be greater than 1000 mg/kg bw/day.

In a related study, D-limonene was administered to pregnant ICR mice from days 7 to 12 of gestation at dose levels of 0, 591, or 2363 mg/kg bw/day (Kodama et al., 1977b). A significant reduction of maternal body weight was observed in the high-dose group (2363 mg/kg bw/day). Males born to dams in the high-dose group also showed a significant decrease in body weight. Compared to controls, significant increased incidences of lumbar and fused ribs were seen in offspring of the high-dose group, as well as delayed ossification of some bones. In the offspring of the low-dose group (591 mg/kg bw/day), a significant retardation of the ossification of the middle phalanx also was observed relative to controls. However, these retarded ossifications were restored to normal during post-natal development. With the exception of a significant decrease in relative thymus weights observed in low-dose male offspring, treatment with D-limonene produced no significant alterations in absolute and relative body and organ weights of male offspring. However, significant increases in absolute and relative weights of the liver and ovary were noted in high-dose female offspring. Relative heart weights of female offspring at both low- and high-dose levels also were significantly increased compared to controls. Additionally, absolute thyroid and adrenal weights were significantly decreased in high-dose female offspring, while relative thyroid weight also was significantly reduced in low- and high-dose female offspring in comparison to controls.

Three groups of 20 female Wistar rats were orally administered 0, 591, or 2869 mg/kg bw/day of D-limonene on days 9–15 of gestation (Tsuji et al., 1975c). Maternal toxicity was noted in the high-dose group (2869 mg/kg bw/day), including increased mortality (40%) and decreased body weight on gestation day 16 compared to controls; however, by gestation day 20, body weights of high-dose dams were not significantly different from controls. No effects were seen in the low-dose group (591 mg/kg bw/day). The offspring of dams in the high-dose group showed several signs of toxicity including significant decreases in body weight in males, absolute and relative weights of the thymus and spleen in males and females, and absolute and relative ovary weights in females. High-dose offspring also exhibited delayed ossification of the metacarpal bone and proximal phalanx. However, any retarded ossification returned to normal within several weeks of birth. In the offspring of low-dose dams, males were reported to have

significantly increased relative testes weights, while females exhibited significantly decreased absolute kidney weights compared to controls.

β-Myrcene, dissolved in corn oil, was administered via gavage to female Wistar rats on days 6–15 of pregnancy (Delgado et al., 1993a). Three treatment groups were used with each group receiving 250, 500, or 1200 mg β-myrcene/kg bw/day. Control rats received either the vehicle only, or no treatment at all. All animals were sacrificed on day 20 of pregnancy. Signs of maternal or fetal toxicity were observed only in the high-dose group (1200 mg/kg bw/day) and were limited to decreased maternal body weight gain during the first days of treatment, mortality in one dam, and an increased incidence of fetal skeletal malformations. The authors also reported that the number of visible implantation sites and the number of live fetuses were significantly decreased in the high-dose group compared to controls. In addition, individual fetal weights were significantly decreased in the high-dose group relative to controls. The authors reported the NOEL for both maternal and offspring toxicity to be 500 mg/kg bw/day.

In a follow-up study designed to test peri- and post-natal developmental toxicity in rats, β-myrcene (in corn oil) was administered via gavage to pregnant Wistar rats at dose levels of 250, 500, 1000, or 1500 mg/kg bw/day (Delgado et al., 1993b). Treatment began on day 15 of pregnancy, and concluded upon weaning of the offspring on postnatal day 21. Control animals received gavage treatment of the vehicle only. The authors reported a dose-related decrease in birth weight, an increase in perinatal and postnatal mortality, and delayed developmental landmarks for the three highest treatment groups (500, 1000, and 1500 mg/kg bw/day). In addition, female offspring from the two highest dose groups (1000 and 1500 mg/kg bw/day) displayed impaired fertility. Maternal toxicity was evident only in the high-dose group (1500 mg/kg bw/day), as five of the dams died within 4 days of treatment, and all 15 dams showed a weight deficit at term (day 20 of gestation), which persisted after delivery. Upon necropsy of the dams that died during the study, or those killed at weaning, hyperkeratosis in the forestomach was noted in most of the rats at the two highest dose groups. While the duration of pregnancy, litter size, and post-weaning mortality did not differ significantly between groups, labor duration was significantly greater in the two highest dose groups, and the number of stillbirths was significantly increased in the high-dose group compared to controls. The animals in the lowest dose group (250 mg/kg bw/day) showed none of these effects and the NOEL for this study was reported to be 250 mg/kg bw/day.

Groups of 60 rats (15 male, 45 female) were given β-myrcene via gavage in peanut oil at doses of 0, 100, 300, or 500 mg/kg bw/day (Paumgartten et al., 1998). Males were treated for 91 days prior to mating, as well as during mating, while females were treated continuously for 21 days prior to mating, until the offspring were weaned 21 days after birth. Males were sacrificed after the mating period, and one-third of the females in each group were sacrificed on day 21 of pregnancy. The remaining females were sacrificed after weaning (postnatal day 21). The weight of the gravid uterus from all sacrificed pregnant females was determined. Resorptions, living and dead fetuses, and implantation sites were counted and all living fetuses were weighed and examined for skeletal abnormalities. The remaining pregnant females were allowed to give birth and the numbers of viable and dead newborns were counted. The pups were sexed, weighed on days 1, 6, 11, 16 and 21 and examined for signs of physical development. The only significant difference observed between control and test animals was a slight increase in the relative and absolute liver and kidney weights of males in the high-dose group (500 mg/kg bw/day). Body weight and body weight gain were not significantly different between control and test animals. A significant increase in the resorption rate and a parallel decrease in the number of live fetuses per implantation site as well as slight increases in the frequency of skeletal malformations were noted in the high-dose group. The authors noted, however, that the types of malformations observed (e.g., fused os zygomatic, dislocated sternums, and extra lumbar ribs) were strain-specific, and occurred at elevated levels in the study control group, as well as historical controls. Delays were noted in eye opening, incisor eruption, and primary coat appearance of offspring of β-myrcene-treated dams; however, these observations were not dose-related. Due to the slight fetotoxic effects observed at 500 mg/kg bw/day, the authors established the NOEL for this study at 300 mg/kg bw/day.

Sprague–Dawley rats were given oral doses of 0.16, 0.80, or 1.60 ml/kg bw of Rowachol® once daily for a period of 6 days, from 9–14 days of gestation (Hasegawa and Toda, 1978). Rowachol® is a mixture of L-menthol (32%), α,β-pinene (17%), menthone (6%), borneol (5%), D-camphene (5%), eucalyptol (2%), and olive oil (33%). A control group of rats received oral doses of 0.80 ml/kg bw of the vehicle (olive oil). Autopsies were performed on the day 20 of pregnancy. There were no statistically significant differences in maternal body weight gain, number of implantations, placental weight, intrauterine mortality and fetal weight reported among the groups treated with 0.16 or 0.80 ml/kg bw of Rowachol® and the control group. In the high-dose group (1.60 ml/kg bw/day), there were significant reductions in maternal, placental, fetal and newborn body weight compared to controls. Although newborn body weights were significantly decreased at the high-dose group, such arrested development recovered after 1 week. Additionally, the fetuses of the high-dose group did not show any retarded ossification. There were no gross, visceral or skeletal anomalies in the high-dose group, nor were there any significant differences in the incidence of fetal malformations reported between Rowachol®-treated and control rats. No teratogenic effect was observed for Rowachol® at any dose level. In this study, the maternal and fetal NOEL for the mixture was determined to be 0.80 ml/kg bw.

In a developmental study, Sprague–Dawley rats were given daily oral doses of 0, 250, or 1000 mg/kg bw/day of camphene on days 6–15 of gestation (Hoechst, 1991). Temporary clinical signs in dams at the 1000 mg/kg bw level included reduced motor activity and salivation on days 1 and 2 of treatment. No teratogenic effects were reported in any offspring. The maternal and developmental NOEL were reported to be 250 and 1000 mg/kg bw/day, respectively.

In a developmental study with *p*-mentha-1,3-diene, Sprague-Dawley rats were given daily oral doses of 0, 30, 60, 125, or 250 mg/kg bw/day on days 6–15 of gestation (Araujo et al., 1996). Pups at 60 mg/kg bw/day showed delayed ossification and skeletal malformations. No teratogenic effects were reported in any offspring at 30 mg/kg bw/day. The developmental NOEL was reported to be 30 mg/kg bw/day.

Several studies on D-limonene, β-myrcene, α- and β-pinene, and camphene indicate that this group of aliphatic and aromatic terpene hydrocarbons has very low reproductive toxicological or teratogenic potential.

6. Conclusions

Upon evaluation of the scientific data relevant to the safety evaluation of the use of aliphatic and aromatic terpene hydrocarbons as flavoring ingredients, it was concluded that they present no safety issues to humans. Although members of this group have been shown to exhibit renal carcinogenic potential in the male F344N/rat, the mechanism leading to these findings is known and strongly indicates that the nephropathy associated with *monoterpene hydrocarbons* have no significance for human risk

assessment. The safe use of these ingredients is also supported by their self-limiting properties as flavoring substances in food resulting in low levels of use; their rapid absorption, metabolic detoxication, and excretion in humans and other animals; the wide margins of safety between the conservative estimates of intake and the no-observed-adverse effect levels determined from subchronic and chronic studies and the lack of significant genotoxic, developmental and teratology potentials.

The group of aliphatic and aromatic monoterpene hydrocarbons discussed here was determined to be generally recognized as safe (GRAS) under conditions of intended use as flavor ingredients by the FEMA Expert Panel in 1965 (Hall and Oser, 1965; Newberne et al., 1998; Oser and Ford, 1973, 1974, 1977; Smith and Ford, 1993). In 1978, the Panel evaluated the available data and affirmed the GRAS status of these flavor ingredients (GRASa). In 1993, the Panel initiated a comprehensive program to reevaluate the status of all FEMA GRAS flavor ingredients concurrent with a systematic revision of the FEMA Scientific Literature Reviews (SLRs). In 2010, this group was reaffirmed as GRAS (GRASr) based on knowledge concerning their rapid absorption, metabolic conversion, and excretion in humans and animals; their low levels of use as flavors in food; the wide margins of safety between the conservative estimates of intake and the NOAEL or NOEL determined from subchronic and chronic studies and the lack of significant genotoxic and mutagenic potential. The consistency of the results obtained from both subchronic and chronic studies in rodent models support the conclusion that consumption of terpene hydrocarbons as part of the food supply is not associated with any significant risk to human health.

Conflict of Interest

The authors declare that there are no conflicts of interest.

References

Adams, T.B., Gavin, C.L., Taylor, S.V., Waddell, W.J., Cohen, S.M., Feron, V.J., Goodman, J., Rietjens, I.M.C.M., Marnett, L.J., Portoghese, P.S., Smith, R.L., 2008. The FEMA GRAS assessment of α, β-unsaturated aldehydes and related substances used as flavor ingredients. Food Chem. Toxicol. 46, 2935–2967.

Ambrose, S., 1983. Induction of hepatic microsomal enzymes with beta-caryophyllene in rats and mice. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Anderson, B.E., Zeiger, E., Shelby, M.D., Resnick, M.A., Gulati, D.K., Ivett, J.L., Loveday, K.S., 1990. Chromosome aberration and Sister Chromatid Exchange Test Results with 42 chemicals. Environ. Mol. Mutagen. 16, 55–137.

Araujo, I.B., Souza, C.A.M., De-Carvalho, R.R., Kuriyama, S.N., Rodrigues, R.P., Vollmer, R.S., Alves, E.N., Paumgartten, F.J.R., 1996. Study of the embryofetotoxicity of α-terpinene in the rats. Food Chem. Toxicol. 34, 477–482.

Asakawa, Y., Taira, Z., Takemoto, T., Ishida, T., Kido, M., Ichikawa, Y., 1981. X-ray crystal structure analysis of 14-hydroxycaryophyllene oxide, a new metabolite of (−)-caryophyllene, in rabbits. J. Pharm. Sci. 70 (6), 710–711.

Asakawa, Y., Ishida, T., Toyota, M., Takemoto, T., 1986. Terpenoid biotransformation in mammals IV.Biotransformation of (+)-longifolene, (−)-caryophyllene, (−)-caryophyllene oxide,(−)-cyclocolorenone, (+)-nootkatone, (−)-elemol, (−)-abietic acid and (+)-dehydroabietic acid in rabbits. Xenobiotica 16 (8), 753–767.

Ashley, D.L., Prah, J.D., 1997. Time dependence of blood concentrations during and after exposure to a mixture of volatile organic compounds. Arch. Environ. Health 52 (1), 26–33.

Austin, C.A., Shephard, E.A., Pike, S.F., Rabin, B.R., Phillips, I.R., 1988. The effect of terpenoid compounds on cytochrome P-450 levels in rat liver. Biochem. Pharmacol. 37 (11), 2223–2229.

Bakke, O.M., Scheline, R.R., 1970. Hydroxylation of aromatic hydrocarbons in the rat. Toxicol. Appl. Pharmacol. 16, 691–700.

Borghoff, S.J., Short, B.G., Swenberg, J.A., 1990. Biochemical mechanisms and pathobiology of nephropathy. Annu. Rev. Pharmacol. Toxicol. 30, 349–367.

Boyle, R., McLean, S., Foley, W.J., Davies, N.W., 1999. Comparative metabolism of dietary terpene, p-cymene, in generalist and specialist folivorous marsupials. J. Chem. Ecol. 25 (9), 2109–2126.

Brownlee, G., 1940. A pharmacological examination of cineole and phellandrene. Q. J. Pharm. Pharmacol. 13, 130–137.

Bucher, J.R., Huff, J., Kluwe, W.M., 1986. Toxicology and carcinogenesis studies of isophorone in F344 rats and B6C3F1 mice. Toxicology 39 (2), 207–219.

Buist, S.C.N., Cherrington, N.J., Choudhuri, S., Hartley, D.P., Klaassen, C.D., 2002. Gender-specific and developmental influences on the expression of rat organic anion transporters. J. Pharmacol. Exp. Ther. 301 (1), 145–151.

Burdock, G.A., 2005. Fenaroli's Handbook of Flavor Ingredients, Fifth Edition. CRC Press, Boca Raton, Florida.

Burdock, G.A., Wagner, B.M., Smith, R.L., Munro, I.C., Newberne, P.M., 1990. Recent progress in the consideration of flavoring ingredients under the food additives amendment. 15. GRAS substances. Food Technol. 44 (2), 78–86.

Capen, C.C., Dybing, E., Rice, J.M., Wilbourn, J.D., 1999. IARC Consensus: Species differences in thyroid, kidney and urinary bladder carcinogensis. IARC Sci. Publ. 147. International Agency for Research on Cancer, Lyon, France (pp. 175–189).

Clark, C.R., Marshall, T.C., Merickel, B.S., Sanchez, A., Brownstein, D.G., Hobbs, C.H., 1979. Toxicological assessment of heat transfer fluids proposed for use in solar energy applications. Toxicol. Appl. Pharmacol. 51 (3), 529–535.

Cohen, S.M., Klaunig, J., Meek, M.E., Hill, R.N., Pastoor, T., Lehman-McKeeman, L., Bucher, J., Longfellow, D.G., Seed, J., Dellarco, V., Fenner-Crisp, P., Patton, D., 2004. Evaluating the human relevance of chemically induced animal tumors. Toxicol. Sci. 78 (2), 181–186.

Colalianni, L.J., 1967. Acute toxicity, eye and skin irritation test on aromatic compounds. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA.

Connor, T.H., Theiss, J., Hanna, H., Monteith, D., Matney, T., 1985. Genotoxicity of organic chemicals frequently found in the air of mobile homes. Toxicol. Lett. 23, 33–40.

Crowell, P., Elson, C.E., Bailey, H., Elegbede, A., Haag, J., Gould, M., 1994. Human metabolism of the experimental cancer therapeutic agent D-limonene. Cancer Chemother. Pharmacol. 35, 31–37.

DeGraff, W.G., 1983. Mutagenicity evaluation of B155 (beta-pinene) in the Ames *Salmonella*/microsome plate test Final Report. Genetics Assay No. 7021. Litton Bionetics, Inc., Kensington, MD. Unpublished report to the Flavor and Extract Manufacturers Association of the United States, Washington, DC, USA.

Del Toro-Arreola, S., Flores-Torales, E., Torres-Lozano, C., Del Toro-Arreola, A., Tostado-Pelayo, K., Guadeloupe Ramirez-Dueñas, M., Daneri-Navarro, A., 2005. Effect of D-limonene on immune response in BALB/c mice with lymphoma. Int. Immunopharmacol. 5, 829–838.

Delgado, I.F., Carvalho, R.R., Nogueira, A., Mattos, A.P., Figueiredo, L.H., Oliveira, S.H.P., Chahoud, I., Paumgartten, F.J.R., 1993a. Study on embryo-fetotoxicity of beta-myrcene in the rat. Food Chem. Toxicol. 31, 31–35.

Delgado, I.F., Nogueira, A.C., Souza, C.M., Costa, M.N., Figueiredo, L.H., Mattos, A.P., Chahoud, I., Paumgartten, F., 1993b. Peri- and postnatal developmental toxicity of beta-myrcene in the rat. Food Chem. Toxicol. 31, 623–628.

De-Oliveira, A., Ribeiro-Pinto, L., Otto, S., Goncalves, A., Paumgartten, F., 1997a. Induction of liver monooxygenase by β-myrcene. Toxicology 124, 135–140.

De-Oliveira, A., Ribeiro-Pinto, L., Otto, S., Paumgartten, F., 1997b. In vitro inhibition of CYP2B1 by β-myrcene and other monoterpenoid compounds. Toxicol. Lett. 92, 39–46.

Dietrich, D.R., Swenberg, J.A., 1991. NCI-Black-Reiter (NBR) male rats fail to develop renal disease following exposure to agents that induce α-2u-globulin nephropathy. Fundam. Appl. Toxicol. 16, 749–762.

Dogra, R.K.S., Khanna, S., Srivastava, S.N., Shukla, L.J., Shanker, R., 1989. Styrene-induced immunomodulation in mice. Int. J. Immunopharmacol. 11 (5), 577–586.

Environmental Protection Agency (EPA), 1991. Risk assessment forum. Alpha-2u-globulin: association with chemically induced renal toxicity and neoplasia in the male rat. Risk assessment forum US Environemntal Protection Agency, Washington, DC. Publication No. EPA/625/3-91019F.

Eriksson, K., Levin, J.O., 1990. Identification of *cis*- and *trans*-verbenol in human urine after occupational exposure to terpenes. Int. Arch. Occup. Environ. Health 62 (5), 379–383.

Eriksson, K., Levin, J.O., 1996. Gas chromatographic mass spectrometric identification of metabolites from alpha-pinene in human urine after occupational exposure to sawing fumes. J. Chromatogr. 677 (1), 85–98.

Eustis, S.L., Hailey, J.R., Boorman, G.A., Haseman, J.K., 1994. The utility of multiple-section sampling in the histopathological evaluation of the kidney for carcinogenicity studies. Toxicol. Pathol. 22, 457–472.

Fahrig, R., 1984. Genetic mode of action of carcinogens and tumor promoters in yeast and mice. Mol. Gen. Gene. 194, 7–14.

Falk, A., Gullstrand, E., Lof, A., Wigaeus-Hjelm, E., 1990a. Liquid/air partition coefficients of four terpenes. Br. J. Ind. Med. 47, 62–64.

Falk, A., Hagberg, M., Lof, A., Wigaeus-Hjelm, E., Wang, Z., 1990b. Uptake, distribution and elimination of alpha-pinene in man after exposure by inhalation. Scand. J. Work Environ. Health 16 (5), 372–378.

Falk, A., Lof, A., Hagberg, M., Hjelm, E., Wang, Z., 1991. Human exposure to 3-carene by inhalation: toxicokinetics, effects on pulmonary function and occurrence of irritative and CNS symptoms. Toxicol. Appl. Pharmacol. 110, 198–2055.

Falk-Fillipsson, A., Lof, A., Hagberg, M., Wigaeus, E., Wang, Z., 1993. D-Limonene exposure ot humans by inhalation : uptake, distribution, elimination, and effects on the pulmonary function. J. Toxicol. Environ. Health 38, 77–88.

Fassett, D., 1978. Conversion of inhalation dose in parts per million to a dose in mg/kg. Unpublished personal communication to the Flavor and Extract Manufacturers Association (FEMA).

Food and Chemicals Codex (FCC), 1996. Food Chemicals Codex/Committee on Food Chemicals Codex, Food and Nutrition Board, Institute of Medicine (fourth Ed.). <http://www.nap.edu>. National Academies Press, Washington, DC.

Florin, I., Rutberg, L., Curvall, M., Enzell, C.R., 1980. Screening of tobacco smoke constituents for mutagenicity using the Ames test. Toxicology 18, 219–232.

Gavin, C.L., Williams, M.C., Hallagan, J.B., 2008. Flavor and Extract Manufacturers Association of the United States 2005 Poundage and Technial Effects Update Survey, Washington DC: Flavor and Extract Manufacturers Association of the United States.

Gomes-Carneiro, M.R., Viana, M.E.S., Felzenszwalb, I., Paumgartten, F.J.R., 2005. Evaluation of β-myrcene, α-terpinene, or (+)- and (−)- α-pinene in the *Salmonella*/microsome assay. Food Chem. Toxicol. 43, 247–252.

Guenther, A., Geron, C., Pierce, T., Lamb, B., Harley, P., Fall, R., 2000. Natural emissions of non-methane volatile organic compounds, carbon monoxide, and oxides of nitrogen from North America. Atmos. Environ. 34, 2205–2230.

Hall, R.L., Oser, B.L., 1965. Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. Food Technol. 19 (2), 151–197.

Hanai, T., Ma, F.H., Matsumoto, S., Park, Y.C., Kurita, T., 2002. Partial outlet obstruction of the rat bladder induces a stimulatory response on proliferation of the bladder smooth muscle cells. Int. Urol. Nephrol. 34, 37–42.

Hard, G.C., 1998. Mechanisms of chemically induced renal carcinogenesis in the laboratory rodent. Toxicol. Pathol. 26, 104–112.

Hard, G.C., Khan, K.N., 2004. A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. Toxicol. Pathol. 32, 171–180.

Hard, G.C., Seely, J.C., 2005. Recommendations for the interpretation of renal tubule proliferative lesions occurring in rat kidneys with advanced chronic progressive nephropathy (CPN). Toxicol. Pathol. 33, 641–649.

Hard, G.C., Rodgers, I.S., Baetcke, K.P., Richards, W.L., McGaughy, R.E., Valcovic, L.R., 1993. Hazard evaluation of chemicals that cause accumulation of alpha2u-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ. Health Perspect. 99, 313–349.

Hard, G.C., Johnson, K.J., Cohen, S.M., 2009. A comparison of rat chronic progressive nephropathy with human renal disease-implications of human risk assessment. Crit. Rev. Toxicol. 39, 332–346.

Hart, E.R., Wong, L.C.K., 1971. Acute oral toxicity studies in rats, acute dermal toxicity and primary skin irritation studies in rabbits of fragrance materials. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, New Jersey, USA.

Hasegawa, M., Toda, T., 1978. Teratological studies on rowachol. Remedy for cholelithiasis effect of Rowachol administered to pregnant rats during organogenesis on pre- and post-natal development of their offspring. Oyo Yakuri 15 (7), 1109–1116.

Hasegawa, R., Nakaji, Y., Kurokawa, Y., Tobe, M., 1989. Acute toxicity tests on 113 environmental chemicals. Sci. Rep. Res. Inst. Tohoku Univ. [Med.] 36(1-4), 10-16.

Haseman, J.K., Huff, J., Boorman, G.A., 1984. Use of historical control data in carcinogenicity studies in rodents. Toxicol. Pathol. 12 (2), 126–135.

Haseman, J.K., Huff, J.E., Rao, G.N., Arnold, J.E., Boorman, G.A., McConnell, E.E., 1985. Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6n × C3H/HeN)F (B6C3F) mice. J. Natl. Cancer Inst. 75 (5), 975–984.

Haseman, J.K., Tharrington, E.C., Huff, J.E., McConnell, E.E., 1986. Comparison of site-specific and overall tumor incidence analyses for 81 recent National Toxicology Program Carcinogenicity Studies. Regul. Toxicol. Pharmacol. 6, 155–170.

Haseman, J.K., Hailey, J.R., Morris, R.W., 1998. Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F(1) mice in two-year carcinogenicity studies: a National Toxicology Program update. Toxicol. Pathol. 26 (3), 428–441.

Haseman, J.K., Ney, E., Nyska, A., Rao, G.N., 2003. Effect of diet and animal care/housing protocols on body weight, survival, tumor incidences, and nephropathy severity of F344 rats in chronic studies. Toxicol. Pathol. 31, 674–681.

Haworth, S., Lawlor, T., Mortelmans, K., Speck, W., Zeiger, E., 1983. *Salmonella* mutagenicity test results for 250 chemicals. Environ. Mutagen. Suppl. 1, 3–142.

Heck, J.D., Vollmuth, T.A., Cifone, M.A., Jagannath, D.R., Myhr, B., Curren, R.D., 1989. An evaluation of food flavoring ingredients in a genetic toxicity screening battery. Toxicologist 9 (1), 257.

Helmig, D., Klinger, L., Guenther, A., Vierling, L., Geron, C., Zimmerman, P., 1999a. Biogenic volatile organic compound emissions (BVOCs) I. Identifications from three continental sites in the US. Chemosphere 38(9), 2163–2187.

Helmig, D., Klinger, L., Guenther, A., Vierling, L., Geron, C., Zimmerman, P., 1999b. Biogenic volatile organic compound emissions (BVOCs) II. Landscape flux potentials from three continental sites in the U.S. Chemosphere 38(9), 2189-2204.

Hildebrand, H., Hartmann, E., Popp, A., Bomhard, E., 1997. Quantitation of α_2-microglobulin after administration of structurally divergent chemical compounds. Arch. Toxicol. 71, 351–359.

Hiroi, T., Miyazaki, Y., Kobayashi, Y., Imaoka, S., Funae, Y., 1995. Induction of hepatic P450s in rat by essential wood and leaf oils. Xenobiotica 25 (5), 457–467.

Hoechst, A.G., 1991. Unveröffentl. Unters. (Ber.-Nr. 91.0246). Unpublished report to the Flavor and Extract Manufacturers Association of the United States, Washington, DC, USA.

Igimi, H., Kodama, M., Ide, H., 1974. Studies on the metabolism of D-limonene (p-mentha-1, 8-diene) I. The absorption, distribution and excretion of D-limonene in rats. Xenobiotica 4 (2), 77–84.

Ishida, T., Asakawa, Y., Takemoto, I., Aratani, T., 1979. Terpene metabolites in rabbit urine. IV. Metabolism of pinene, carene, and myrcene. Koen Yoshishu-Koryo. Terupen 23, 39–41.

Ishida, T., Asakawa, Y., Takemoto, T., Aratani, T., 1981. Terpenoids Biotransformation in Mammals III: biotransformation of *alpha*-pinene, *beta*-pinene, 3-carene, carane, myrcene, and *p*-cymene in rabbits. J. Pharm. Sci. 70, 406–415.

Jagannath, D.R., 1984a. Mutagenicity evaluation of alpha-pinene. Unpublished report to the Flavor and Extract Manufacturers Association of the United States, Washington, DC, USA.

Jagannath, D.R., 1984b. Mutagenicity evaluation of beta-caryophyllene in the Ames *Salmonella*/microsome plate test. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L., Fitzhugh, O.G., 1964. Food flavorings and compounds of related structure I. Acute oral toxicity. Food Cosmet. Toxicol. 2 (3), 327–343.

Jones L J., 2003. Nootkatone, Verbenone: 28-Day repeated dose single dose toxicity study in the rat. Safepharm Project No. 1834/003. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Kanerva, R.L., Ridder, G.M., Lefever, F.R., Alden, C.L., 1987. Comparison of short-term renal effects due to oral administration of decalin or D-Limonene in young adult male Fischer-344 rats. Food Chem.Toxicol. 25 (5), 345–353.

Kauderer, B., Zamith, H., Paumgartten, J.R., Speit, G., 1991. Evaluation of the mutagenicity of beta-myrcene in mammalian cells in vitro. Environ. Mol. Mutagen. 18, 28–34.

Kawai, T., Yasugi, T., Mizunuma, K., 1992. The comparative evaluation of urinanalysis and blood analysis as means of detecting exposure to organic solvents at low concentration. Int. Arch. Occup. Environ. Health 64, 223–234.

Keating, J.W., 1972. Acute toxicity study in rats and rabbits. Foster D Snell, Inc. Biological Science Laboratories, Elizabeth, NJ. Unpublished report to the Research Institute for Fragrance Materials, Wooscliff Lake, NJ, USA.

Kjeldsen, L., Cowland, J.B., Borregaard, N., 2000. Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. Biochim. Biophys. Acta 1482 (1–2), 272–283.

Kodama, R., Noda, K., Ide, H., 1974. Studies on the metabolism of D-Limonene. II. The metabolic fate of D-limonene in rabbit. Xenobiotica 4, 85–95.

Kodama, R., Yano, T., Furukawa, K., Noda, K., Ide, H., 1976. Studies on the metabolism of D-Limonene. IV Isolation and characterization of new metabolites and species differences in metabolism. Xenobiotica 6, 377–389.

Kodama, R., Okubo, A., Sato, K., Araki, E., Noda, K., Ide, H., Ikeda, T., 1977a. Studies on D-limonene as a gallstone solubilizer: Effect on development of rabbit fetuses and offsprings. Oyo Yakuri 13 (6), 885–898.

Kodama, R., Okubo, A., Sato, K., Araki, E., Noda, K., Ide, H., Ikeda, T., 1977b. Studies on D-limonene as a gallstone solubilizer: effect on development of mouse fetuses and offsprings. Oyo Yakuri 13 (6), 885–898.

Kojima, R., Sekine, T., Kawachi, M., Cha, S.H., Suzuki, Y., Endou, H., 2002. Immunolocalization of multispecific organic anion transporters, OAT1 OAT2, and OAT3, in rat kidney. J. Am. Soc. Nephrol. 13, 848–857.

Koppel, C., Tenczer, J., Tonnesmann, U., Schirop, T., Ibe, K., 1981. Acute poisoning with pine oil-metabolism of monoterpenes. Arch. Toxicol. 9, 73–78.

Kurttio, P., Kalliokoske, P., Lampelo, S., Jantunen, M.J., 1990. Mutagenic compounds in wood-chip drying fumes. Mutat. Res. 242, 9–15.

Lee, E.J.D., Williams, K., Day, R., Graham, G., Champion, D., 1985. Stereoselective disposition of ibuprofen enantiomers in man. Br. J. Clin. Pharmacol. 19, 669–674.

Lehman-McKeeman, L.D., Caudill, D., 1994. D-Limonene induced hyaline droplet nephropathy in alpha-2μ-globulin transgenic mice. Fundam. Appl. Toxicol. 23, 562–568.

Lehman-McKeeman, L.D., Rodriguez, P.A., Takigiku, R., Caudill, D., Fey, M.L., 1989. D-Limonene-induced male rat-specific Nephrotoxicity: evaluation of the association between D-limonene and *alpha*-2μ-globulin. Toxicol. Appl. Pharmacol. 99, 250–259.

Lehman-McKeeman, L., Caudill, D., Takigiku, R., Schneider, R., Young, J.A., 1990. Comparative disposition of D-limonene in rats and mice. relevance to male-rat-specific nephrotoxicity. Toxicol. Lett. 53, 193–195.

Levenstein, I., 1975. Acute toxicity studies in rats, mice, and rabbits. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, New Jersey, USA.

Levin, J.O., Eriksson, K., Falk, A., Lof, A., 1992. Renal elimination of verbenols in man following experimental α-pinene inhalation exposure. Int. Arch. Occup. Environ. Health 63, 571–573.

Lock, E.A., Hard, G.C., 2004. Chemically induce renal tubule tumors in the laboratory rat and mouse: Review of the NCI/NTP database and categorization of renal carcinogens based on mechanistic information. Crit. Rev. Toxicol. 34, 211–299.

Madyastha, K.M., Srivatsan, V., 1987. Metabolism of β-myrcene *in vivo* and *in vitro*: its effects on rat-liver microsomal enzymes. Xenobiotica 17 (5), 539–549.

Maltzman, T., Christou, M., Gould, M., Jefcoate, R., 1991. Effects of monoterpenoids on in vivo DMBA-DNA adduct formation and on phase I hepatic metabolizing enzymes. Carcinogenesis 12 (11), 2081–2087.

Maronpot, R.R., Haseman, J.K., Boorman, G.A., Eustis, S.E., Rao, G.N., Huff, J.E., 1987. Liver lesions in B6C3F1 mice. The National Toxicology Program, experience and position. Arch. Toxicol. Suppl. 10, 10–26.

Matsumoto, T., Ishida, T., Yoshida, T., Terao, H., Takeda, Y., Asakawa, Y., 1992. The enantioselective metabolism of *p*-cymene in rabbits. Chem. Pharm. Bull. 40 (7), 1721–1726.

Miyazawa, M., Shindo, M., Shimada, T., 2002. Sex differences in the metabolism of (+)- and (−)-limonene enantiomers to carveol and perillyl alcohol derivatives by cytochrome P450 enzymes in rat liver microsomes. Chem. Res. Toxicol. 15, 15–20.

Moreno, O.M., 1972a. Acute oral toxicity studies of d-limonene in mice, rats and rabbits. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, New Jersey, USA.

Moreno, O.M., 1972b. Acute oral toxicity studies of myrcene in mice, rats and rabbits. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno, O.M., 1972c. Acute oral toxicity studies of alpha-phellandrene in mice, rats and rabbits. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno, O.M., 1972d. Acute oral toxicity studies of 3-Carene in mice, rats and rabbits. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno, O.M., 1972e. Acute oral toxicity studies of α-Pinene in rats. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USAMoreno, O.M., 1973a. Acute oral toxicity studies of p-Mentha-1,3-diene on rats. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno, O.M., 1973a. Acute toxicity studies of p-mentha-1,3-diene on rats. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno, O.M., 1973b. Acute toxicity studies of p-mentha-1,4-diene on rats. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno O.M., 1974b Acute toxicity studies on Camphene. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, New Jersey, USA. Submitted to WHO by Moreno, O. M, 1975. Acute dermal toxicity of *beta*-pinene in rabbits. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno, O.M., 1975. Acute toxicity studies β-Pinene on rats. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno O.M., 1976. Acute toxicity studies in rats, mice, rabbits and guinea pigs. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno, O.M., 1980. Acute toxicity studies. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Moreno O.M., 1974a Acute toxicity studies. Bisabolene on rats. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA.

Muller, W., 1993. Evaluation of mutagencity testing with *Salmonella typhimurium* TA102 in three different laboratories. Environ. Health Perspect. 101, 33–36.

Myhr, B., McGregor, D., Bowers, L., Riach, C., Brown, A.G., Edwards, I., McBride, D., Martin, R., Caspary, W.J., 1990. L5178Y mouse lymphoma cell mutation assay Results with 41 compounds. Environ. Mol. Mutagen. 16, 138–167.

National Toxicology Program (NTP), 1990. Carcinogenicity and toxicology studies of D-limonene in F344/N Rats and B6C3F1 mice. NTP-TR-347. US Dept of Health and Human Services. NIH Publication No. 90–2802. <http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr347.pdf>.

National Toxicology Program (NTP), 2003. NTP Technical Report on the Carcinogenesis Studies of 2,4-hexadienal (CAS No. 142-83-6) in F344/N Rats and B6C3F1. NTP TR 509. NIH Publication No. 04-4443. National Toxicology Program, Research Triangle Park, NC. <http://ntp.niehs.nih.gov/>.

National Toxicology Program (NTP), 2006a. 13-week Inhalation Toxicity Study of α-Pinene – Rats. Unpublished report.

National Toxicology Program (NTP), 2006b. 13-week Inhalation Toxicity Study of α-Pinene – Mice. Unpublished report.

National Toxicology Program (NTP), 2010. NTP Technical Report on the Carcinogenesis Studies of beta-Myrcene (CAS No. 123-35-3) in F344/N Rats and B6C3F1. NTP TR 557. NIH Publication No. 09-5898. National Toxicology Program, Research Triangle Park, NC. <http://.ntp.niehs.nih.gov/>.

Newberne, P., Smith, R.L., Doull, J., Goodman, J.I., Munro, I.C., Portoghese, P.S., Wagner, B.M., Weil, C.S., Woods, L.A., Adams, T.B., Hallagan, J.B., Ford, R.A., 1998. GRAS flavoring substances 18. Food Technol. 52 (9), 65–92.

Nijssen, B., van Ingen-Visscher, K., Donders, J., 2010. Volatile compounds in Food 9.1. Centraal Instituut Voor Voedingsonderzioek TNO. Zeist, The Netherlands. <http://www.voeding.tno.nl/vcf/VcfNavigate.cfm>.

Olson, M.J., Johnson, J.T., Reidy, C.A., 1990. A comparison of male rat and human urinary proteins: implications for human resistance to hyaline droplet nephropathy. Toxicol. Appl. Pharmacol. 102, 524–536.

Oser, B.L., Ford, R.A., 1973. Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment 6. GRAS substances. Food Technol. 27 (1), 64–67.

Oser, B.L., Ford, R.A., 1974. Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment 8. GRAS substances. Food Technol. 28 (9), 76–80.

Oser, B.L., Ford, R.A., 1977. Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment 10. GRAS substances. Food Technol. 31 (1), 65–74.

Paumgartten, F.J., De-Carvalho, R.R., Souza, C.C., Madi, K., Chahoud, I., 1998. Study of the effects of beta-Myrcene on rat fertility and general reproductive performance. Braz. J. Med. Biol. Res. 31 (7).

Pellmont, B., 1973. The acute toxicity of 1,3,5-undecatriene in rats and mice. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Poon, G., Vigushin, D., Griggs, L.J., Rowlands, M.G., Coombes, R.C., Jarman, M., 1996. Identification and characterization of limonene metabolites in patients with advanced cancer by liquid chromatography/mass spectrometry. Drug Metab.Dispos. 24, 565–571.

Research Triangle Institute (RTI), 1989. Metabolism, disposition and pharmacokinetics of cumene in F-344 rats following oral, iv. administration or nose-only inhalation exposure RTI study number 3543 as referenced in EPA (1997) Toxicological review of cumene. U.S. Environmental Protection Agency.

Rockwell, P., Raw, I., 1979. A mutagenic screening of various herbs, spices, and food additives. Nutr. Cancer 1 (4), 10–15.

Roscheisen, C., Zamith, H., Paumgartten, F., Speit, G., 1991. Influence of *beta*-myrcene on sister chromatid exchanges induced by mutagens in V79 and HTC cells. Mutat. Res. 264, 43–49.

Roy, A.K., Neuhaus, O.W., 1967. Androgenic control of a sex dependent protein in rat. Nature 214, 618–620.

Rudy, A.C., Knight, P.M., Brater, D.C., Hall, S.D., 1995. Enantioselective disposition of ibuprofen in elderly persons with and without renal impairment. J. Pharmacol. Exp. Ther. 273, 88–93.

Saito, K., Uwagawa, S., Kaneko, H., Shiba, K., Tomigahara, Y., Nakatsuka, I., 1996. A_{2u}-Globulins in the urine of male rats: a reliable indicator for α_{2u}-globulin accumulation in the kidney. Toxicology 106, 149–157.

Sasaki, Y.F., Imanishi, H., Ohta, T., Shirasu, Y., 1989. Modifying effects of components of plant essence on the induction of sister-chromatid exchanges in cultured Chinese hamster ovary cells. Mutat. Res. 226, 103–110.

Schafer Jr., E.W., Bowles Jr., W.A., 1985. Acute oral toxicity and repellency of 933 chemicals to house and deer mice. Arch. Environ. Contam. Toxicol. 14, 111–129.

Seely, J.C., Haseman, J.K., Nyska, A., Wolf, D.C., Everitt, J.I., Hailey, J.R., 2002. The effect of chronic progressive nephropathy on the incidence of renal tubule cell neoplasms in control male F344 rats. Toxicol. Pathol. 30, 681–686.

Shapiro, R., 1988. Acute toxicity feeding study in rats of a 20% solution of 1,3,5-undecatriene in 80% pinene supra vehicle. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Smith, R.L., Ford, R.A., 1993. Recent progress in consideration of flavoring ingredients under the Food Additives Amendment. 16. GRAS substances. Food Technol. 47, 104–117.

Stofberg, J., Grundschober, F., 1987. Consumption ratio and food predominance of flavoring materials. Perfumer Flavorist 12, 27.

Stofberg, J., Kirschman, J.C., 1985. The consumption ratio of flavoring materials: a mechanism for setting priorities for safety evaluation. Food Chem. Toxicol. 23, 857–860.

Strasser Jr., J., Charbonneau, M., Borghoff, S.J., Turner, M.J., Swenberg, J.A., 1988. Renal protein droplet formation in male Fischer 344 rats after isophorone (IPH) treatment. Toxicologist 8, 136.

Suri, A., Grundy, B.L., Derendorf, H., 1997. Pharmacokinetics and pharmacodynamics of enantiomers of ibuprofen and flurbiprofen after oral administration. J. Clin. Pharm. Ther. 35, 1–8.

Swenberg, J.A., Short, B., Borghoff, S., Strasser, J., Charbonneau, M., 1989. The comparative pathobiology of nephropathy. Toxicol. Appl. Pharmacol. 97, 35–46.

Tan, S.C., Patel, B.K., Jackson, S.H.D., Swift, C.G., Hutt, A.J., 2002. Stereoselectivity of ibuprofen metabolism and pharmacokinetics following the administration of the racemate to healthy volunteers. Xenobiotica 32, 683–697.

Tojo, A., Sekine, T., Nakajima, N., Hosoyamada, M., Kanai, Y., Kimura, K., Endou, H., 1999. Immunohistochemical localization of multispecific renal organic anion transporter 1 in rat kidney. J. Am. Soc. Nephrol. 10, 464–471.

Triebel, S., Blaeser, J., Reinke, H., Tschesche, H., 1992. A 25 kDa alpha 2-microglobulin-related protein is a component of the 125 kDa form of human gelatinase. FEBS Lett. 314 (3), 386–388.

Tsuji, M., Fujisaki, Y., Arikawa, Y., Masuda, S., Kinoshita, S., Okubo, A., Noda, K., Ide, H., Iwanaga, Y., 1975a. Studies on D-limonene as a gallstone solubilizer: acute and sub-acute toxicities. Oyo Yakuri 9, 387–401.

Tsuji, M., Fujisaki, Y., Arikawa, Y., Masuda, S., Tanaka, T., Sato, K., Noda, K., Ide, H., Kikuchi, M., 1975b. Studies on D-limonene, as gallstone solubilizer (IV): chronic toxicity in dogs. Oyo Yakuri 10, 179–186.

Tsuji, M., Fujiski, Y., Okubo, A., Arikawa, Y., Noda, K., Ide, H., Ikeda, T., 1975c. Studies on D-limonene as a gallstone solubilizer (V) effects on development of rat fetuses and offsprings. Oyo Yakuri 10, 179–186.

Untermann, R.D., Lynch, K.R., Nakhasi, H.L., Dolan, K.P., Hamilton, J.W., Cohn, D.V., Feigelson, P., 1981. Cloning and sequencing of several alpha-2-microglobulin cDNAs. Proc. Nat. Acad. Sci. 78, 3478–3482.

Vigushin, D., Poon, G.K., Boddy, A., English, J., Halbert, G.W., Pagonis, C., Jarman, M., Coombes, R.C., 1998. Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer. Cancer Chemother.Pharmacol. 42 (2), 111–117.

Walde, A., Ve, B., Scheline, R.R., 1983. *p*-Cymene metabolism in rats and guinea pigs. Xenobiotica 13 (8), 503–512.

Wang, K.S., Hodgetts, R.B., 1998. The analysis of regulatory sequences required for the high level induction of rat alpha-2-microglobulin gene expression by dexamethasone. J. Mol. Endocrin. 20 (1), 129–139.

Watabe, T., Hiratsuka, A., Ozawa, N., Isobe, M., 1981. A comparative study on the metabolism of D-limonene and 4-vinylcyclohex-1-ene by hepatic microsomes. Xenobiotica 11, 333–344.

Weast, R.C., 1973. Handbook of Chemistry and Physics: A ready reference book of chemical and physical data, Fifty-fourth ed. CRC Press, Cleveland, Ohio.

Webb, D.R., Ridder, M., Alden, C.L., 1989. Acute and subchronic nephrotoxicity of D-limonene in Fischer 344 rats. Food Chem. Toxicol. 27, 639–649.

Webb, D.R., Kanerva, R.L., Hysell, D.K., Alden, C.L., Lehman-McKeeman, L.D., 1990. Assessment of the subchronic oral toxicity of D-limonene in dogs. Food Chem. Toxicol. 28, 669–675.

White Jr., R.A., Agosin, M., 1980. Metabolism of Alpha-Pinene by Rat Liver Reconstituted Cytochrome P-450 Systems. In: Gustaffsson, J., Carlstedt-Duke, J., Rafter, J. (Eds.), Biochemistry, Biopysics and Regulation of Cytochrome P-450. Elsevier/North-Holland Press, New York, pp. 85–88.

Zamith, H.P., Vidal, M.N.P., Speit, G., Paumgartten, F.J.R., 1993. Absence of genotoxic activity of beta-myrcene in the in vivo cytogenetic bone marrow assay. Braz. J. Med. Biol. Res. 26, 93–98.

Zlatkis, A., Lichtenstein, H.A., Tishbee, A., Bertsch, W., Shunbo, F., Liebich, H.M., 1973. Concentration and analysis of volatile urinary metabolites. J. Chromatogr. Sci. 11, 299–302.