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FEMA GRAS assessment of natural flavor complexes: Mint, buchu, dill and caraway derived flavoring ingredients

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ABSTRACT

In 2015, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) initiated a re-evaluation of the safety of over 250 natural flavor complexes (NFCs) used as flavor ingredients. NFC flavor materials include a variety of essential oils and botanical extracts. The re-evaluation of NFCs is conducted based on a constituent-based procedure outlined in 2005 and updated in 2018 that evaluates the safety of NFCs for their intended use as flavor ingredients. This procedure is applied in the re-evaluation of the generally recognized as safe (GRAS) status of NFCs with constituent profiles that are dominated by alicyclic ketones such as menthone and carvone, secondary alcohols such as menthol and carveol, and related compounds. The FEMA Expert Panel affirmed the GRAS status of Peppermint Oil (FEMA 2848), Spearmint Oil (FEMA 3032), Spearmint Extract (FEMA 3031), Cornmint Oil (FEMA 4219), Erospicata Oil (FEMA 4777), Curly Mint Oil (FEMA 4778), Pennyroyal Oil (FEMA 2839), Buchu Leaves Oil (FEMA 2169), Caraway Oil (FEMA 2238) and Dill Oil (FEMA 2383) and determined FEMA GRAS status for Buchu Leaves Extract (FEMA 4923), Peppermint Oil, Terpeneless (FEMA 4924) and Spearmint Oil, Terpeneless (FEMA 4925).

1. Introduction

For more than 50 years the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) has served as the primary independent body evaluating the safety of more than 2800 flavor ingredients. The FEMA GRAS program was established in 1960 in response to the 1958 Food Additives Amendment to the Food, Drug and Cosmetic Act with the mission to evaluate whether substances nominated by the flavor industry can be considered "generally recognized as safe" (GRAS) under conditions of intended use as flavor ingredients (Hallagan and Hall, 1995, 2009). The FEMA GRAS program has continually evolved as the technology and science related to the safety evaluation of food and flavor ingredients has progressed. The FEMA Expert Panel continues to review and revise their criteria for determining GRAS status for both chemically defined flavor ingredients (Smith et al., 2005a) and natural flavor complexes (NFCs) (Cohen et al., 2018; Smith et al., 2005b). The procedure for the safety evaluation of NFCs begins with a review of the prioritization of the NFC based on its presence in food, organization of the chemical data into congeneric groups and Cramer et al. (1978) decision tree classes of toxicity, estimations of exposure and consideration of biochemical and toxicological data (Cohen et al., 2018; Smith et al., 2005b). The procedure is conservative by design, comparing the estimated intake for each constituent congeneric group to the thresholds established by the Threshold of Toxicological Concern (TTC) approach (Kroes et al., 2000; Munro et al., 1996). In addition, rigorous consideration is given to the unidentified constituents.

Since its inception, the FEMA GRAS program has systematically re-

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Abbreviations

ATP	Adenosine Triphosphate
BrdU	Bromodeoxyuridine
CF	Correction Factor
CHO	Chinese Hamster Ovary (cells)
CPN	Chronic Progressive Nephropathy
DMAPP	Dimethylallyl Diphosphate
DTC	Decision Tree Class
ECHA	European Chemicals Agency
EFFA	European Flavour Association
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FCC	Food Chemical Codex
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GRAS	Generally recognized as safe
GPS	Geranyl Diphosphate Synthase (enzyme)
HPLC	High Pressure Liquid Chromatography
IFEAT	International Federation of Essential Oils and Aroma Trades
IOFI	International Organization of the Flavor Industry
i.p.	Intraperitoneal Injection
IPP	Isopentyl Diphosphate
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JFFMA	Japan Fragrance and Flavor Materials Association
LC-MS	Liquid Chromatography-Mass Spectrometry
MLA	Mouse Lymphoma Assay
MoS	Margin of Safety
MSD	Mass Spectrometric Detector
MTD	Maximum Tolerated Dose
NAS	National Academy of Sciences
NCI	National Cancer Institute
NFC	Natural Flavoring Complexes
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
PDA	Photodiode Array Detector
PCI	Per Capita Daily Intake
PTH	Parathyroid Hormone (secretion)
SCE	Sister Chromatid Exchange
SEM	Scanning Electron Microscopy
TK	Toxicokinetic (study)
TTC	Threshold of Toxicological Concern
TRP	Transient Receptor Potential (channels)
TPRM8	TRP Melastatin Family Member 8 (receptor)
US-EPA	U.S. Environmental Protection Agency
WHO	World Health Organization

evaluated the chemically defined FEMA GRAS flavor ingredients, taking into consideration relevant new scientific data and/or their usage in food. The FEMA Expert Panel is expanding their re-evaluation program to include the more than 250 NFCs that have FEMA GRAS status. In a previous publication, the procedure was applied to the evaluation of approximately 50 *Citrus*-derived NFCs (Cohen et al., 2019). In 2016, the FEMA Expert Panel issued a call for data requesting complete chemical analyses and physical properties for thirteen (13) NFCs listed in Table 1. These materials include nine mint (*Mentha*)-derived NFCs: Peppermint Oil (FEMA 2848), Peppermint Oil Terpeneless (FEMA 4924), Spearmint Oil (FEMA 3032), Spearmint Extract (FEMA 3031), Spearmint Oil Terpeneless (FEMA 4925), Cornmint Oil (FEMA 4219), Erospicata Oil (FEMA 4777), Curly Mint Oil (FEMA 4778) and Pennyroyal Oil (FEMA 2839), and four other NFCs: Buchu Leaves Oil (FEMA 2169), Buchu Leaves Extract (FEMA 4923), Caraway Oil (FEMA 2238) and Dill Oil (FEMA 2383). The constituent profile for all the NFCs in this group is characterized by high percentages of menthol, menthone, and/or carvone. Members from the International Organization of the Flavor Industry (IOFI) including the Flavor and Extract Manufacturers Association (FEMA) of the United States, Japan Fragrance and Flavor Materials Association (JFFMA), and the European Flavour Association (EFFA), in addition to the International Federation of Essential Oils and Aroma Trades (IFEAT) responded, providing the constituent and physical data required for the safety evaluation of these NFCs.

Table 1
NFCs evaluated by the Expert Panel.

Name	FEMA No.	Estimated Intake µg/person/day[a]	Most recent annual volume (kg)[b]
Peppermint Oil (*Mentha piperita* L.), *Mentha* 'MP-11', *Mentha x piperita* 'MP-2', Blue Balsam Mint Oil	2848	3240	303,000
Peppermint Oil Terpeneless (*Mentha piperita* L.)	4924	180	1680
Spearmint Oil (*Mentha spicata* L.), Macho mint oil, Julep mint oil	3032	490	45,700
Spearmint Oil Terpeneless (*Mentha spicata* L.)	4925	1	13
Spearmint Extract (*Mentha spicata* L.),	3031	1380	12,900
Cornmint Oil (*Mentha arvensis* L.)	4219	2090	195,000
Erospicata Oil (*Mentha spicata* 'Erospicata'), *Mentha spicata* 'Erospicata' oil	4777	540	50,100
Curly Mint Oil (*Mentha spicata* var. *crispa*), *Mentha spicata* L. var. *crispa* oil	4778	2620	244,000
Pennyroyal Oil (*Hedeoma pulegioides* (L.) var Pers. (American), *Mentha pulegium* L. var. eriantha (European, N. African))	2839	3	27
Caraway Oil (*Carum carvi* L.)	2238	140	1330
Dill Oil (*Anethum graveolens* L.)	2383	390	3600
Buchu Leaves Oil (*Barosma betulina* Bartl. et Wendl., *B. crenulata* (L.) Hook, *B. serratifolia* Willd.)	2169	34	320
Buchu Leaves Extract (*Barosma betulina* Bartl. et Wendl., *B. crenulata* (L.) Hook, *B. serratifolia* Willd.)	4923	0.1	1[c]

[a] For high volume materials (greater than 22,700 kg/year), the PCI per capita is shown. For materials with a lower surveyed volume (less than 22,700 kg/year, PCI × 10 ("eaters only') calculation is shown.

[b] Harman, C.L., Murray, I.J. 2018. 2015 Poundage and Technical Effects Survey. Flavor and Extract Manufacturers Association of the United States (FEMA), Washington DC, USA.

[c] Harman, C.L., et al., 2013. 2010 Poundage and Technical Effects Survey. Flavor and Extract Manufacturers Association of the United States (FEMA), Washington DC, USA.

2. History of food use

Mint plants are members of the *Mentha* genus and part of the larger Lamiaceae family which includes several culinary herbs that have historically been commonly used in foods such as rosemary, oregano, thyme, sage and basil. The creation of the mint plant is described in a Greek myth: following an affair with Hades, Minthe was turned into a plant by Hades' jealous wife, Persephone. In response, Hades gave the plant its aromatic qualities. Like other culinary herbs, wild mint plants grow around the Mediterranean basin and are the likely origin of the spearmint plant (*M. spicata*) (Lawrence, 2007). Wild mint plants are also known to have grown for thousands of years on the north and south banks of the Yangtze river, near Jiujiang, China, and were used in foods and medicines over time (Guenther, 1949b). European pennyroyal (*M. pulegium* L. var. *eriantha*) can be found growing wild in the southwestern and central regions of Europe and is harvested from wild plants that proliferate in coastal Spain and Morrocco (Guenther, 1949b; Lawrence, 2007). American pennyroyal (*Hedeoma pulegioides)* is known to grow wild in the eastern, mid-western and southern United States (Guenther, 1949b).

Across many cultures, mint plants were valued for their aromatic, flavor and medicinal properties. By the 1700s, peppermint (*M. piperita*) was cultivated in Mitcham, England and was the source of the White Mitcham variety that was brought to the United States in the late 1700s to early 1800s. Around 1883, a hardier peppermint cultivar, Black Mitcham, was brought to the United States and remains the dominant variety grown for the production of peppermint oil (Lawrence, 2008). Information on the cultivation of spearmint is less precise but "Native" spearmint (*M. spicata*) plants were grown in the US in the late 1700's also arriving from England (Morris, 2007). The historical progression of mint production in the United States from its introduction to recent times has been reviewed (Lawrence, 2008). According to the US Mint Industry website (www.usmintindustry.com), by 1920 mint oils were used to flavor products such as candy canes, chewing gum, candies and toothpaste. The cultivation of cornmint (*M. arvensis*) for production of mint oil and menthol began in the early 20th century in China and in the late 19th century in Japan. Although cornmint originated in China, it was brought to Japan approximately 1700 years ago. Around 1870, cultivation of cornmint for mint oil production was underway in the Yamagata prefecture in northern Japan and the first lots of menthol were exported in 1883. The Japanese mint industry expanded up to World War II and mint oil and menthol became important exports, supplying a large part of the world's demand (Guenther, 1949b). Currently, cornmint is cultivated in India and China for production of natural menthol and dementholated cornmint oil.

The essential oil produced by *Mentha* plants is found in the plant's leaves and stem. The yield and quality of mint oils is dependent on the growing conditions, requiring long days and limited temperature fluctuations (Morris, 2007). While peppermint and spearmint are still grown in the Midwestern United States, the cultivation of these plants now primarily occurs in the northwestern states, specifically Idaho, Washington and Oregon. Perhaps the most advantageous conditions for *M. piperita* and *M. spicata* cultivation are found in Washington state, where two harvests, July and September, are collected each year (Chen et al., 2011; Lawrence, 2008). While peppermint and spearmint are predominately cultivated in the USA, cornmint (*M. arvensis*) is successfully cultivated in India, China, Brazil and Indonesia. Raw cornmint oil typically is more than 80% menthol. Upon cooling, menthol is crystallized from the oil and the dementholated oil is used as a flavor material.

There is a diversity of *Mentha* species found in nature, likely a result of natural hybridization. Plant hybridization experiments with *M. aquatica* and *M. spicata* suggest that *M. piperita* is a hybrid of these two species (Murray et al., 1972). Breeding programs have successfully developed new *M. piperita* and *M. spicata* varieties for improved disease resistance, oil yield and quality. Black Mitcham and varieties derived from it are currently used in US peppermint production (Morris, 2007). New varieties of *M. spicata*, erospicata oil ('Erospicata') and curly mint oil (var. *crispa*) are also now cultivated for their essential oils. Erospicata oil was developed in 1994 as a disease-resistant alternative to traditional peppermint varieties, and curly mint oil (*M. spicata* var. *crispa*) is a perennial variety of mint.

Because the constituent profile of buchu leaves oil is rich in pulegone, menthone, and isomenthone, common constituents of mint oils, this NFC is evaluated with the *Mentha* NFCs. Buchu leaves oil (*Barosma betulina* Bartl. et Wendl., *B. crenulata* (L.) Hook, *B. serratifolia* Willd.), is valued for its characteristic black currant aroma and flavor (Posthumus et al., 1996). Buchu plants are native to the Cape region of South Africa where it was traditionally used as medicine and natural insect repellant (Moolla and Viljoen, 2008). The two major species of buchu shrubs are *B. betulina*, characterized by a round leaf and *B. crenulata* which has a more ovular-shaped leaf. However, over time, a number of hybrids of these two species have emerged, complicating the identification of the plant based on leaf shape (Moolla and Viljoen, 2008). Although wild growing plants are harvested for production of buchu leaves oil and extract, buchu is also now cultivated in South Africa to create a sustainable supply (Williams and Kepe, 2008).

Dill oil (*Anethum graveolens* L.) and caraway oil (*Carum carvi* L.) are included in this group due to their high carvone constituent profiles. Both dill (*A. graveolens* L.) and caraway (*C. carvi* L.) are herbs of the parsley family (Apicaceae or Umbelliferae), are native to Europe and Asia and have a long history of use as food (Bailer et al., 2001). Dill and caraway, like many aromatic botanicals, were traditionally used as herbal medicines by the ancient Sumerians and Egyptians (Falodun, 2010). The essential oil of the herbs, found primarily in the plant's seeds but also in the leaves of dill, varies in yield and quality depending on growing conditions and regions. Currently, parts of Eastern Europe and the Northern United States produce much of the global dill supply. Europe produces most of the global supply of caraway seed although it is also cultivated in Canada (Spencer et al., 2016).

3. Current usage

Mint oils, characterized and valued for their minty, green and cooling/refreshing organoleptic profile, are extensively used as flavor ingredients in a variety of foods as reflected in the most recent annual volumes and per capita intakes listed in Table 1. Mint oils are a familiar ingredient in chewing gums and candy (hard and soft) as well as in baked goods, confectionary goods, frozen foods, and beverages (alcoholic and non-alcoholic). Usual use levels of Peppermint Oil (FEMA 2848) range from 6 ppm in meat products, 95 ppm in frozen dairy products to 8300 ppm in chewing gum. Based on the 2015 industry survey, the annual volumes of Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219) and Curly Mint Oil (FEMA 4778) each exceeded 100,000 kg. The annual volumes for Spearmint Oil (FEMA 3032) and Erospicata Oil (FEMA 4777) reported in the same survey were approximately 50,000 kg each (Harman and Murray, 2018). The per capita consumption of Peppermint Oil (FEMA 2848) is estimated to be 3.2 mg/person/day while that for Spearmint Oil (FEMA 3032) is 490 μg/person/day. In contrast, the per capita consumption for Pennyroyal Oil (FEMA 2839) and Buchu Leaves Oil (FEMA 2169) are much lower, 3 and 34 μg/person/day, respectively, reflecting lower usage of these NFCs.

In the 2015 industry survey, Caraway Oil (FEMA 2238) and Dill Oil (FEMA 2383) reported annual volumes of 1330 and 3600 kg, respectively, with corresponding per capita consumptions of 143 and 385 μg/person/day (Harman and Murray, 2018). Dill Oil is commonly used to flavor pickles, cheeses and snack foods. Caraway Oil is used in gelatins, baked goods and meat products.

In examining the industry survey data on the use of Peppermint Oil (FEMA 2848) as a flavor ingredient, usage was relatively flat, ranging from 300,000 to 400,000 kg per year between 1970 and 1987, before a

sharp increase was observed in the 1995 survey (Lucas et al., 1999; NAS, 1970, 1975, 1982, 1987). Survey data collected in 2005, 2010 and 2015 indicate a declining use of Peppermint Oil (FEMA 2848) as a flavor ingredient with usage at the level reported in the 1987 survey (Gavin et al., 2008a; Harman et al., 2013; Harman and Murray, 2018). Sheldon (2007) postulated that the recent decrease in peppermint oil usage is related to market conditions. During the mid–to-late 1990s, there was an over-production of peppermint oil reported. As the market corrected and the availability of other mint oils increased, the usage of peppermint oil has declined since 1995 (Sheldon, 2007). The declining use of Peppermint Oil (FEMA 2848) coincides with the increasing use of related mint oils, such as Cornmint Oil (FEMA 4219), Erospicata Oil (FEMA 4777) and Curly Mint Oil (FEMA 4778).

Although use levels of mint oils used for flavor in chewing gum are relatively high, research shows that less than half of the amount of the flavor ingredients in gums are released during the chewing process (Johnson and Tran, 2014). The percentage of flavor release from chewing gum is dependent on several factors, including the nature of the gum matrix and the rate and force of mastication. In addition, the release of each flavor ingredient varies depending on its chemical properties, particularly the relative hydrophilicity or hydrophobicity of a given constituent. To measure the amount of each major component of Peppermint Oil (FEMA 2848) released from chewing gum upon mastication, a study was performed in which a group of 5 individuals were given a 2.7 g portion of Peppermint Oil (FEMA 2848) flavored chewing gum to chew for a measured amount of time. At the end of the chewing time, the 5 gum samples were pooled, extracted into chloroform and analyzed by GC-MS. The experiment was performed at time points 0, 5, 10 and 20 min and the results are summarized in Table 2. Following 20 min of chewing, the percentage of release of key Peppermint Oil (FEMA 2848) constituents ranged from 11.1% for β-caryophellene to 41.7% for pulegone. For *l*-menthol and *l*-menthone, the most abundant constituents, only 28.1% was released during the 20-min chewing experiment. Thus, the intake of mint oils from chewing gum is estimated to be significantly lower than the intake calculated from the total concentration in the gum (Johnson and Tran, 2014).

4. Manufacturing methodology

Mentha plants are cultivated from root stock and are harvested at the onset of flowering for the optimal yield of a high-quality essential oil. Once cut, the plants are sun dried in the planting field for a few days then raked into windrows. The resulting hay is gathered by a harvester that chops the plants into smaller pieces and collects them in tubs in preparation for steam distillation. Peppermint, cornmint, erospicata, curly mint, spearmint and pennyroyal oils are isolated by steam distillation, typically at the growing site, and are further rectified using fractional distillation techniques to improve the aromatic and flavor qualities of the oil (Sheldon, 2007). Terpeneless oils are made by removal of the monoterpene hydrocarbon fraction by fractional distillation. Raw cornmint oil is characterized by a very high menthol content that will crystallize from the oil in cold storage. The menthol is separated from the oil and sold as natural menthol (Hopp and Lawrence, 2007). The remaining dementholized oil, cornmint oil, is used as a flavoring material. In addition, aqueous ethanolic extracts of the spearmint plant are also used as a flavor ingredient.

Buchu leaves oil is also collected by steam distillation of the leaves and stems hand-trimmed from the growing plants. Buchu leaves oil may be produced on-site in South Africa (Muller, 2015) while in the past, leaves were exported to Europe or the USA for distillation (Guenther, 1949a). Buchu leaves extract may be prepared by fractional distillation.

Dill produces different oils depending on the maturity of the plant upon harvesting and on whether the oil is extracted from the leaves or the seeds or both. In the production of Dill Oil (FEMA 2383) used for flavor, the plant is harvested at the stage at which the carvone and α-phellandrene content is considered optimal, typically before the seeds ripen (Guenther, 1950; Porter et al., 1983). The whole plant, either freshly cut or partially dried, is steam distilled to extract the essential oil (Callan et al., 2007; Tucker and DeBaggio, 2000).

Caraway's essential oil is exclusively located in the ducts of the seed's pericarp. The seeds, depending on the species and length of growing season, can contain between 2 and 7% oil by weight (Toxopeus and Bouwmeester, 1992). To avoid seed shattering, or seed dispersal, the plants are harvested once the oldest seeds reach maturity and partially dried while in storage. These partly dried seeds are crushed and steam distilled to collect the essential oil (Aćimović et al., 2014).

Table 2
Release of principal constituents of Peppermint oil (FEMA 2848) from chewing gum by mastication.

			% Release
Constituent	5 min	10 min	20 min
l-Menthol	12.1	17.1	28.1
l-Menthone	15.1	18.9	28.1
Isomenthone	14	23.3	32.6
Menthyl acetate	7	9.3	14
Limonene/1,8-Cineole	22.7	27.3	36.4
Menthofuran	17.9	21.4	28.6
beta-Caryophyllene	5.6	11.1	11.1
Pulegone	16.7	25	41.7

5. Chemical composition

Complete analyses of the flavor materials listed in Table 1 were collected. The flavor materials are characterized by their volatile constituents and are typically analyzed by gas chromatography (GC) using a mass spectrometric detector (MSD) to identify constituents by comparison to a standardized library and flame ionization detector (FID) for quantitation of each chromatographic peak. Identified and unidentified GC peaks are reported as the area % of the chromatogram. When appropriate, the analysis of non-volatile constituents was performed by high pressure liquid chromatography (HPLC) coupled to a photodiode array detector (PDA). Constituent data for each NFC were compiled and statistical summaries were prepared. The Cramer decision tree class is determined for each NFC constituent and each constituent is classified into a congeneric group based on the chemical structure and the functional groups present (Cohen et al., 2018; Cramer et al., 1978). The congeneric groups listed in Cohen et al., 2018 are consistent with the chemical groups used by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its evaluation of chemically defined flavor materials. From this analysis of collected data, the identity of each NFC under consideration is summarized (see Appendix A).

Gas chromatography-mass spectrometry (GC/MS) analyses of Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219), Erospicata Oil (FEMA 4777), Curly Mint Oil (FEMA 4778) and Pennyroyal Oil (FEMA 2839) demonstrate constituent profiles dominated by *l*-menthol ((−)-menthol) and related *p*-menthane-based constituents that are responsible for its characteristic flavor and cooling properties. Interestingly, the component profile of these mint oils appears to be directly determined by the *l*-menthol biosynthetic pathway. A biosynthetic pathway for *l*-menthol in peppermint has been elucidated and is shown in Fig. 1. In *Mentha* species, essential oil biosynthesis and storage is localized to the oil glands found on the aerial surfaces of the plant. The biosynthesis of menthol begins with the condensation of two isoprene compounds, isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by geranyl diphosphate synthase (GPS) to give geranyl diphosphate which then undergoes cyclization to form (−)-limonene. The stereospecific oxidation of (−)-limonene to form (−)-*trans*-isopiperitenol is followed by a dehydrogenation reaction to form (−)-isopiperitenone. (−)-Isopiperitenone is reduced to (+)-*cis*-isopulegone by a reductase. Next, (+)-*cis*-isopulegone undergoes

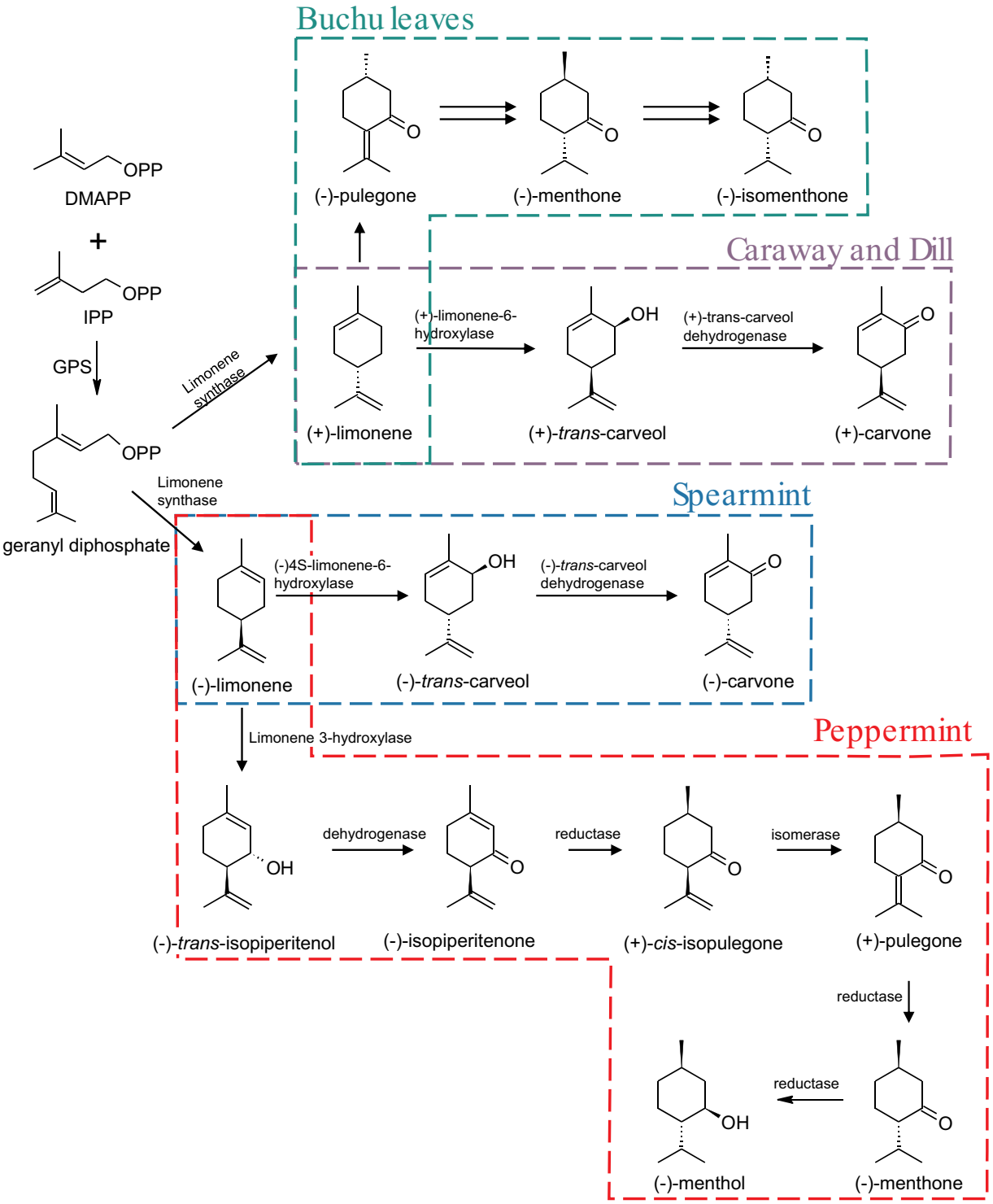


Fig. 1. Biosynthetic pathways elucidated for the production of *l*-menthol in peppermint plants, (−)-carvone in spearmint plants, (+)-carvone in caraway and dill plants and (−)-pulegone and (−)-menthone in buchu plants.

isomerization to (+)-pulegone. (+)-Pulegone is converted to menthone by a reductase and in the final step, menthone is reduced to *l*-menthol.

All the steps in the biosynthetic pathway are stereospecific except for the reduction of pulegone by pulegone reductase in which menthone is the major product but isomenthone is also formed. A scheme summarizing how the four major isomers of menthol are derived from pulegone in *M. piperita* is shown in Fig. 2 (Croteau et al., 2005). Two different enzymes have been isolated that catalyze the reduction of both menthone and isomenthone, accounting for the formation of the four stereoisomers of menthol detected in these mint oils, although the formation of *l*-menthol is clearly favored. Pulegone, a central intermediate in the pathway, is a major constituent of Pennyroyal Oil (FEMA 2839) and a minor constituent of Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219), Erospicata Oil (FEMA 4777), Curly Mint Oil (FEMA 4778) and Spearmint Oil (FEMA 3032). The early biosynthetic intermediates in the pathway, isopiperitenol, isopiperitenone and isopulegone are found in only low levels in these mint oils. Upon close examination, the *l*-menthol biosynthetic pathway is responsible for the

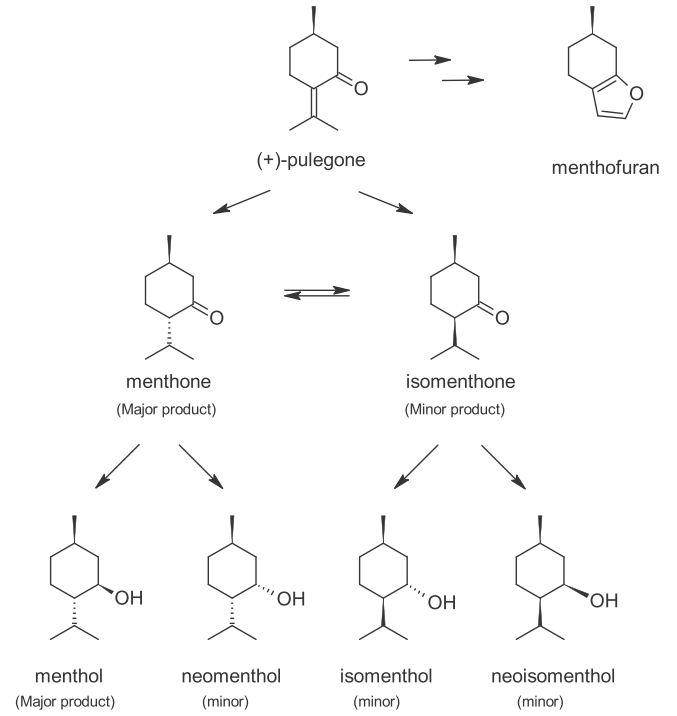


Fig. 2. In peppermint, the intermediate pulegone is reduced to menthone and isomenthone that is subsequently reduced, resulting in the four stereoisomers of menthol found in peppermint oil (Croteau et al., 2005).

characteristic constituent profile for several of the mint NFCs. *l*-Menthol is the most abundant component of both Peppermint Oil (FEMA 2848) and Cornmint Oil (FEMA 4219). For Curly Mint Oil (FEMA 4778), the average concentration of menthone is slightly higher than *l*-menthol and for Erospicata Oil (FEMA 4777) menthone is the most abundant constituent. Erospicata Oil (FEMA 4777) contains a relatively large percentage of isomenthone (~17%) while Peppermint Oil (FEMA 2848) and Curly Mint Oil (FEMA 4778) contain between 2 and 6% isomenthone. The most abundant constituent in Pennyroyal Oil (FEMA 2839) is pulegone with smaller percentages of menthone and piperitenone. The biosynthesis of pulegone, *d*-limonene, isomenthone, menthone and piperitenone in buchu leaves, is also shown in Fig. 1. The stereochemistry of menthone, isomethone, and pulegone from buchu was determined to be (1*S*)-configured whereas the (1*R*) configuration occurs in peppermint (Fuchs et al., 2001; Köpke et al., 1994). The minor constituents, approximately ~20% of the total composition, also contribute to the flavor profile including 1,8-cineole, octan-3-ol and 1-octen-3-ol. Two sulfur compounds, 3-oxo-*p*-menthane-8-thiol and 3-oxo-*p*-menthane-8-thiol acetate are prominent in Buchu Leaves Extract (FEMA 4923) and minor components of Buchu Leaves Oil (FEMA 2169) and are key contributors to the cassis-like flavor of this NFC. Several monoterpenes, including β-pinene and β-phellandrene and several sesquiterpenes including germacrene D and β-caryophellene are also consistently identified in mint oils. Constituent profiles for Peppermint Oil (FEMA 2848), Spearmint Oil (FEMA 3032) and related NFCs by congeneric group are shown in Fig. 3.

Gas chromatography-mass spectrometry (GC/MS) analyses of Spearmint Oil (FEMA 3032), Caraway Oil (FEMA 2238) and Dill Oil (FEMA 2383) give a constituent profile dominated by carvone and limonene that are responsible for their characteristic flavor. The constituent profiles of these oils are reflected in the biosynthetic pathway for carvone. The organoleptic response of carvone is dependent on the stereoisomer, (−)-carvone (*l*-carvone) for spearmint oils and (+)-carvone (*d*-carvone) for dill and caraway seed oils. The biosynthesis of (−)-carvone in spearmint and (+)-carvone in dill and caraway seed is shown in Fig. 1. Both pathways begin with the condensation of two isoprene units to form geranyl pyrophosphate which is cyclized by stereospecific limonene synthases forming predominately (−) *l*-limonene in spearmint and (+) *d*-limonene in caraway and dill. This stereochemistry remains in place for the hydroxylation step that forms (−)-*trans*-carveol and (+)-*trans*-carveol, respectively. In the final step, a dehydrogenation reaction yields (−)-carvone in spearmint and (+)-carvone in dill and caraway seeds (Bouwmeester et al., 1998; Gershenzon et al., 1989).

Upon close examination, the carvone biosynthetic pathways are responsible for much of the characteristic constituent profiles of Spearmint Oil (FEMA 3032) and Caraway Oil (FEMA 2238) depicted in Fig. 4. In these essential oils, carvone and limonene account for more than 80% of the constituent profile. Varying amounts of *trans*-carveol, dihydrocarvone, dihydrocarveol and dihydrocarvyl acetate account for much of the remaining constituents of the spearmint and caraway NFCs. Dill Oil (FEMA 2383) also contains relatively high amounts of (+)-carvone and limonene, but also has a significant amount of α-phellandrene and β-phellandrene (~20%) and dill ether (~7%) which give dill its characteristic flavor profile.

The ethanol and water contributions to constituent profile of Spearmint Extract are removed from this depiction and the remaining constituents were normalized.

6. Safety evaluation

The procedure for the safety evaluation for NFCs (Fig. 5) is guided by a set of criteria as outlined in two publications (Smith et al., 2004, 2005b) with a recent update (Cohen et al., 2018). Briefly, the NFC passes through a 14-Step process; Step 1 requires the gathering of data and assesses the consumption of the NFC as a flavor relative to the estimated intake from the natural source when consumed as food; Steps 2 through 6 evaluate the exposure and potential toxicity of the identified constituents by application of the threshold for toxicologic concern (TTC) approach and scientific data on metabolism and toxicity for each congeneric group; Steps 7-12 address the potential toxicity, including genotoxicity of the unidentified constituents; lastly in Steps 13 and 14 the overall safety is evaluated along with considerations of potential biologically relevant interactions among constituents.

The FEMA Expert Panel incorporated conservatism into the procedure at several steps. The calculation of intake for most NFCs in Step 1 uses the PCI × 10 approach which assumes that its annual usage is consumed by 10% of the population and applies a correction factor of 0.8 to account for possible unreported volumes of use. Also, in Step 1, a conservative decision tree class is assigned to each congeneric group in the assignment of the decision tree class of the constituent of the highest toxicological potential. In Step 5, the estimated intake for each congeneric group of the NFC is compared to the TTC thresholds which are based on the 5th percentiles of the NOAEL of each class with an additional 100-fold uncertainty factor, resulting in a highly conservative threshold for each class (Kroes et al., 2000; Munro et al., 1996). The TTC thresholds are also applied in the evaluation of the unidentified constituent fraction in Steps 10 and 11. Below, the safety evaluation is presented in which each step of the procedure (Cohen et al., 2018) (provided in italics), is considered and answered for the NFCs under consideration.

Step 1

To conduct a safety evaluation of an NFC, the Panel requires that comprehensive analytical data are provided. The analytical methodologies employed should reflect the expected composition of the NFC and provide data that identify, to the greatest extent possible, the constituents of the NFC and the levels (%) at which they are present. It is anticipated that GC-MS and LC-MS would be used for characterization of most NFCs, and that the chromatographic peaks based on peak area of total ion current will be almost completely identified. The percentage of unknowns should be low enough to not raise a safety concern. Other appropriate methods (e.g., Karl Fischer titration, amino acid analysis, etc.) should be employed as necessary. The

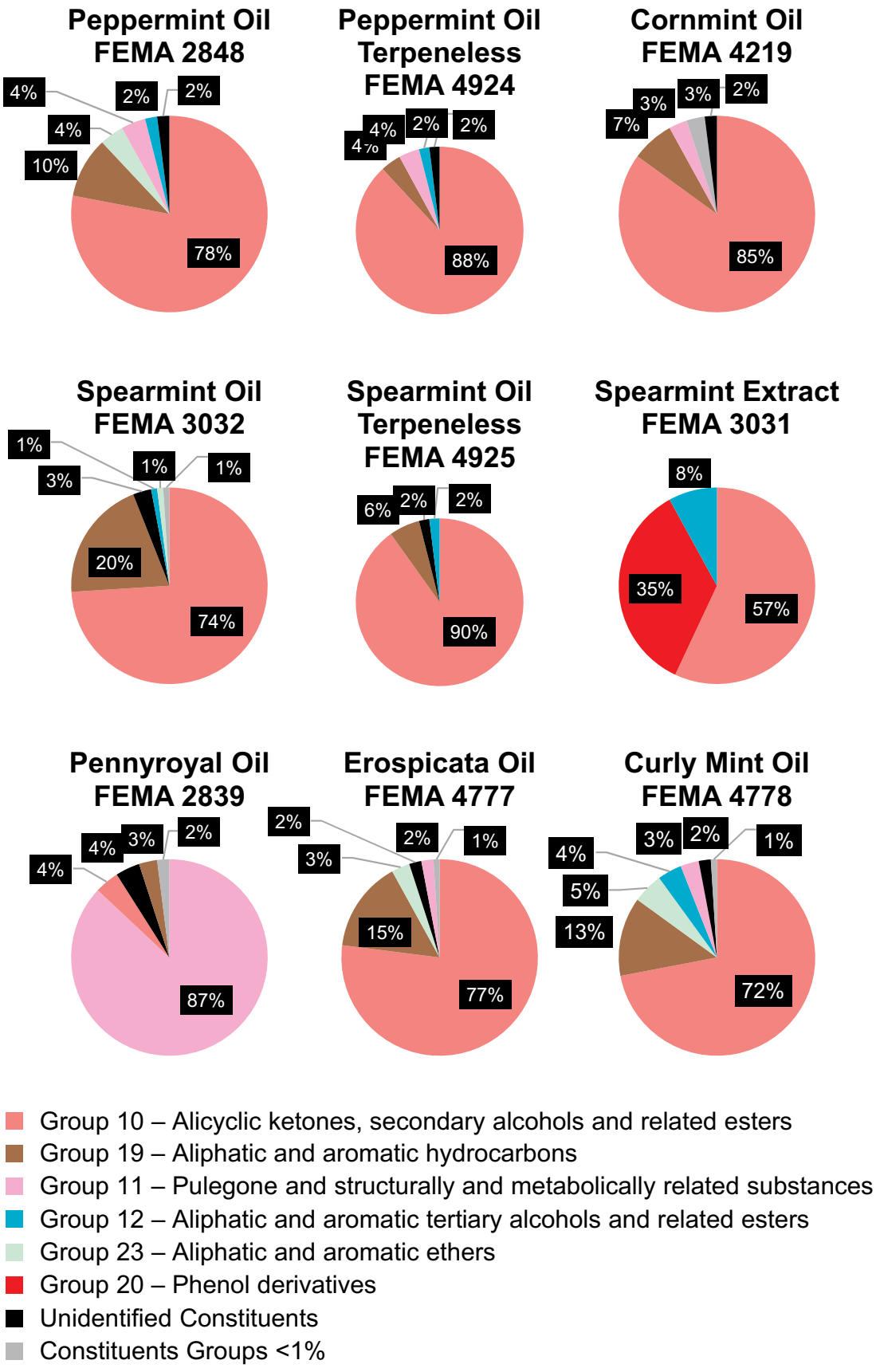


Fig. 3. Constituent profile of peppermint, spearmint and other mint NFCs by congeneric group. The ethanol and water contributions to constituent profile of Spearmint Extract are removed from this depiction and the remaining constituents were normalized.

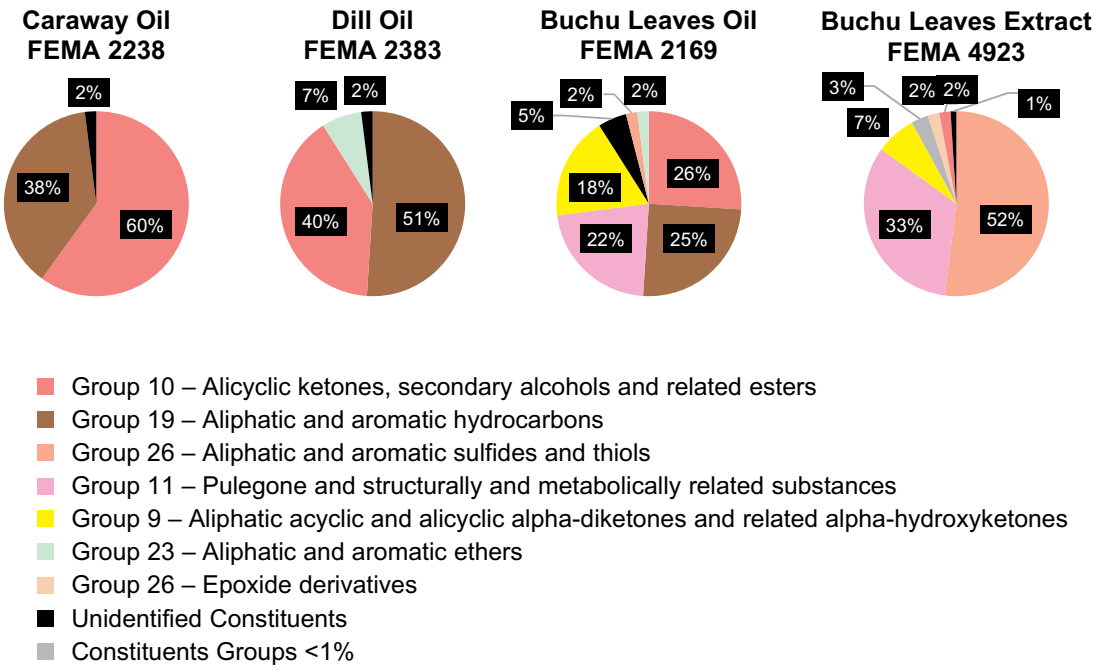
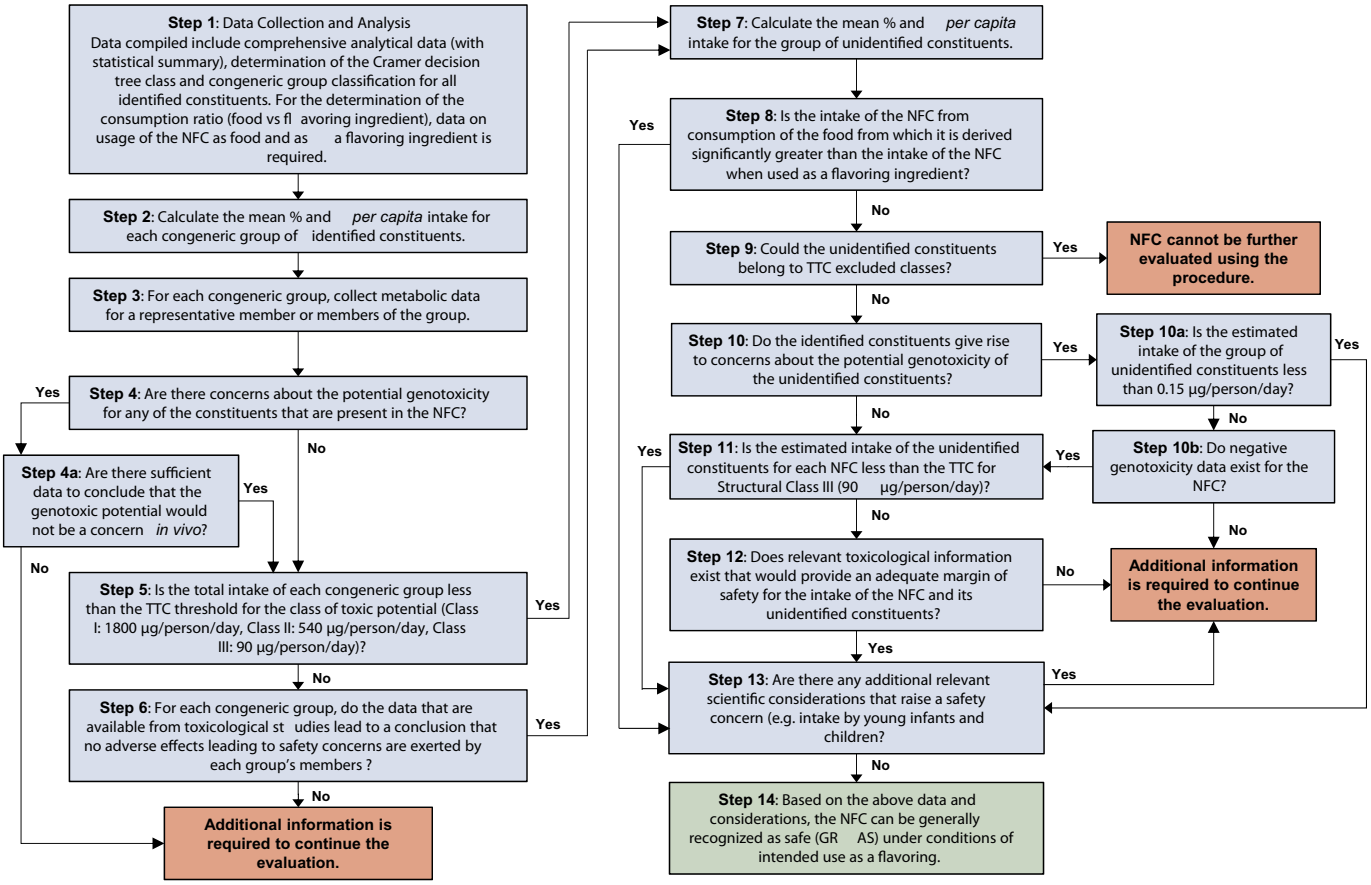


Fig. 4. Constituent profiles for Dill Oil, Caraway Oil, Buchu Leaves Oil and Buchu Leaves Extract.



This scheme presents a summary of the revised procedure for the evaluation of NFCs to give an overall structural view. When applying the procedure, the full procedure described in the manuscript should be followed.

Fig. 5. Procedure for the safety evaluation of NFCs (Cohen et al., 2018).

analytical parameters should be submitted for each type of analysis, including the method of quantitation for both identified and unidentified constituents and libraries, databases and methodology employed for the identification of analytes. The Panel requires data from multiple batches to understand the inherent variability of the NFC.

a. Consumption of foods from which the NFCs are derived

Calculate the per capita daily intake (PCI)[1] of the NFC based on the annual volume added to food.

For NFCs with a reported volume of use greater than 22,700 kg (50,000 lbs), the intake may be calculated by assuming that consumption of the NFC is spread among the entire population, on a case-by-case basis. In these cases, the PCI is calculated as follows:

$$PCI\ (\mu g/person/day) = \frac{annual\ volume\ in\ kg \times 10^9}{population \times CF \times 365\ days}$$

where:

The annual volume of use of NFCs currently used as flavorings for food is reported in flavor industry surveys (Gavin et al., 2008b; Harman et al., 2013; Harman and Murray, 2018; Lucas et al., 1999). A correction factor (CF) is used in the calculation to correct for possible incompleteness of the annual volume survey. For flavorings, including NFCs, that are undergoing GRAS re-evaluation, the CF, currently 0.8, is established based on the response rate from the most recently reported flavor industry volume-of-use surveys.

For new flavorings undergoing an initial GRAS evaluation the anticipated volume is used and a correction factor of 0.6 is applied which is a conservative assumption that only 60% of the total anticipated volume is reported.

For NFCs with a reported volume of use less than 22,700 kg (50,000 lbs), the eaters' population intake assumes that consumption of the NFC is distributed among only 10% of the entire population. In these cases, the per capita intake for assuming a 10% "eaters only" population (PCI × 10) is calculated as follows:

$$PCI \times 10\ (\mu g/person/day) = \frac{annual\ volume\ in\ kg \times 10^9}{population \times CF \times 365\ days} \times 10$$

If applicable, estimate the intake resulting from consumption of the commonly consumed food from which the NFC is derived. The aspect of food use is particularly important. It determines whether intake of the NFC occurs predominantly from the food of which it is derived, or from the NFC itself when it is added as a flavoring ingredient (Stofberg and Grundschober, 1987).[2] At this step, if the conditions of use[3] for the NFC result in levels that differ from intake of the same constituents in the food source, it should be reported.

The NFCs in this group are derived from commonly used culinary plants. In the production of mint and dill oils, the above ground parts of the plant are collected and allowed to partially dry. The dried product is then steam distilled to express the essential oil. In the case of caraway, the essential oil is collected from the crushing and steam distillation of the plant's seeds. Buchu leaves oil and extract are derived from steam distillation of the leaves of the buchu shrub. Later refinement of the crude essential oil by fractional distillation and blending are commonly practiced techniques used to improve the aromatic and flavor qualities of the NFC. Peppermint and spearmint plants are commonly grown in kitchen gardens and their leaves are used in teas and other foods. Peppermint and other mint teas are among the most popular herbal teas sold in the USA (Keating et al., 2015). Dill weed and caraway seeds are commonly available in American and European grocery stores and are used to season a variety of foods. Because of these uses, the intake of the essential oils of mint, buchu, dill and caraway from the consumption of the whole leaf or seed is expected to be significant. However, because quantitative data specific to consumption of these various plants were not available, a consumption ratio comparing intake from food to intake as added flavoring to food could not be calculated.

b. Identification of all known constituents and assignment of Cramer Decision Tree Class

In this step, the results of the complete chemical analyses for each NFC are examined, and where appropriate for each constituent the Cramer Decision Tree Class (DTC) is determined (Cramer et al., 1978).

In Appendix A, the congeneric groups with constituents with a mean % greater or equal to 1% of the NFC are listed in order of highest to lowest mean%. For each congeneric group listed, the constituents with a mean % equal or greater than 1% are also shown and the minor constituents (< 1%) are summed and reported.

c. Assignment of the constituents to congeneric groups; assignment of congeneric group DTC

In this step, the identified constituents are sorted by their structural features into congeneric groups. Each congeneric group should be expected, based on established data, to exhibit consistently similar rates and pathways of absorption, distribution, metabolism and excretion, and common toxicological endpoints (e.g. benzyl acetate, benzaldehyde, and benzoic acid are expected to have similar toxicological properties). The congeneric groups are listed in Appendix A.

Assign a decision tree structural class to each congeneric group. Within a congeneric group, when there are multiple decision tree structural classes for individual constituents, the class of highest toxicological concern is assigned to the group. In cases where constituents do not belong to a congeneric group, potential safety concerns would be addressed in Step 13.

Proceed to step 2

All reported constituents in the NFCs under consideration are organized by congeneric group and are shown in Appendix A. Appendix A lists the constituent congeneric groups in order of highest to lowest mean %. The DTC for each congeneric group is also provided.

Step 2

Determine (a) the mean percentage (%) of each congeneric group in NFCs, and (b) the daily per capita *intake[4] of each congeneric group. (a) is calculated by summing the mean percentage of each of the constituents within a congeneric group, and (b) is calculated from consumption of the NFC and the mean percentage.*

Calculation of PCI for each constituent congeneric group of the NFC

$$Intake\ of\ congeneric\ group\ (\mu g/person/day) = \frac{Mean\ \%\ congeneric\ group \times Intake\ of\ NFC\ (\mu g/person/day)}{100}$$

where:

The mean % is the mean percentage % of the congeneric group.

The intake of NFC (μg/person/day) is calculated using the PCI × 10 or PCI equation as appropriate.

Proceed to step 3

In the summary report for each NFC provided in Appendix A, the total mean% for each congeneric group is subtotaled and reported with

[1] See Smith et al., 2005a and Hall and Ford (1999) for a discussion on the use of PCI and PCI × 10 for exposure calculations in the procedure.

[2] See Stofberg and Grundschober, 1987 for data on the consumption of NFCs from commonly consumed foods.

[3] The focus throughout this evaluation sequence is on the intake of the constituents of the NFC. To the extent that processing conditions, for example, alter the intake of constituents, those conditions of use need to be noted, and their consequences evaluated in arriving at the safety judgments that are the purpose of this procedure.

[4] See Smith et al., 2005b for a discussion on the use of PCI × 10 for exposure calculations in the procedure.

the DTC and intake (PCI × 10 or PCI, as appropriate) for each congeneric group listed.

Step 3

For each congeneric group, collect metabolic data for a representative member or members of the group. Step 3 is critical in assessing whether the metabolism of the members of each congeneric group would require additional considerations in Step 13 of the procedure.

Proceed to step 4

For the mint, buchu, caraway and dill NFCs, Appendix A lists the constituent congeneric groups for each NFC. For each congeneric group, sufficient data on the metabolism of constituents of each congeneric group or related compounds exists to conclude that members of the respective groups are metabolized to innocuous products. The use of metabolic data in the safety evaluation of flavoring compounds and a summary of the expected metabolism of flavoring compounds by congeneric group is described in a recent FEMA Expert Panel publication (Smith et al., 2018). The FEMA Expert Panel has reviewed the major representative congeneric groups, Group 10 (Alicyclic ketones, secondary alcohols and related esters) and Group 11 (Pulegone and structurally and metabolically related substances) and published their safety evaluation (Adams et al., 1996). In addition, minor constituent groups, Group 19 (Aliphatic and aromatic hydrocarbons) and Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) have also been reviewed (Adams et al., 2011; Marnett et al., 2014).

Step 4

Are there concerns about potential genotoxicity for any of the constituents that are present in the NFC?

If Yes, proceed to Step 4a.

If No, proceed to Step 5.

No. The potential genotoxicity of pulegone, a major constituent of Pennyroyal oil (FEMA 2839), Buchu Leaves oil (FEMA 2169) and Buchu Leaves Extract (FEMA 4923) and a minor constituent of Peppermint oil (FEMA 2848), Cornmint oil (FEMA 4219), Erospicata oil (FEMA 4777), Curly mint oil (FEMA 4778) and Spearmint oil (FEMA 3032), has been unclear due to conflicting Ames assay results reported by the NTP (NTP, 2011). The toxicology and potential genotoxicity of pulegone is evaluated later in this manuscript and the results of two new OECD-compliant Ames assays conducted on pulegone and peppermint oil which were negative for mutagenicity are presented. Based on the weight of evidence, it is the conclusion of the FEMA Expert Panel that pulegone is not of genotoxic concern.

Step 4a

Are there sufficient data to conclude that the genotoxic potential would not be a concern in vivo?

If Yes, proceed to Step 5.

If No, additional information is required to continue the evaluation.

Not required.

Step 5

Is the total intake of the congeneric group less than the TTC for the class of toxic potential assigned to the group (i.e., Class I: 1800 μg/person/day, Class II: 540 μg/person/day, Class III: 90 μg/person/day) (Kroes et al., 2000; Munro et al., 1996)? For congeneric groups that contain members of different structural classes, the class of highest toxicological concern is selected.

If Yes, proceed to Step 7.

If No, proceed to Step 6.

The estimated intake for each of the congeneric groups present in Dill Oil (FEMA 2383), Caraway Oil (FEMA 2238), Peppermint Oil Terpeneless (FEMA 4924), Spearmint Oil (FEMA 3032), Spearmint Extract (FEMA 3031), Spearmint Oil Terpeneless (FEMA 4925), Erospicata Oil (FEMA 4777), Pennyroyal Oil (FEMA 2839), Buchu Leaves Oil (FEMA 2169) and Buchu Leaves Extract (FEMA 4923) is below the corresponding TTC for the group. These NFC materials proceed to Step 7 of the evaluation procedure. For the remaining NFCs, Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219) and Curly Mint Oil (FEMA 4778), the estimated intake of Group 10 constituents (Alicyclic ketones, secondary alcohols and related esters) exceeds the relevant TTC. In Peppermint Oil (FEMA 2848), the estimated intake of Group 11 constituents (Pulegone and structurally and metabolically related substances) also exceeds the relevant TTC. Data on these groups are summarized in Table 3. These NFC materials proceed to Step 6 of the evaluation procedure.

Step 6

For each congeneric group, do the data that are available from toxicological studies lead to a conclusion that no adverse effects leading to safety concerns are exerted by each group's members?

This question can commonly be answered by considering the database of relevant metabolic and toxicological data that exist for a representative member or members of the congeneric group, or the NFC itself. A comprehensive safety evaluation of the congeneric group and a sufficient margin of safety (MoS) based on the data available is to be determined on a case-by-case basis. Examples of factors that contribute to the determination of a safety margin include 1) species differences, 2) inter-individual variation, 3) the extent of natural occurrence of each of the constituents of the congeneric group throughout the food supply, 4) the nature and concentration of constituents in related botanical genera and species. Although natural occurrence is no guarantee of safety, if exposure to the intentionally added constituent is trivial compared to intake of the constituent from consumption of food, then this should be taken into consideration in the safety evaluation (Kroes et al., 2000).

If Yes, proceed to Step 7.

If No, additional information is required to continue the evaluation.

For Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219) and Curly Mint Oil (FEMA 4778) the estimated intake of Group 10 constituents, (Alicyclic ketones, secondary alcohols and related esters) exceeds the relevant TTC. The FEMA Expert Panel has previously evaluated Group 10 and 11 flavoring ingredients (Adams et al., 1996) and updated its review in a later section of this manuscript. Upon review of the toxicological literature on Group 10 constituents, a well conducted 103 week oral gavage study of *d,l*-menthol in rats (NCI, 1979) was selected for the calculation of a MoS for Group 10 constituents in Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219) and Curly Mint Oil (FEMA 4778). As shown in Table 3, the NOAEL for this study was 375 mg/kg bw/day for *d,l*-menthol (NCI, 1979) and an adequate MoS for each NFC with an estimated intake above TTC for Group 10 was calculated.

For the calculation of a MoS for Group 11 constituents in Peppermint Oil (FEMA 2848), a similar approach was taken in which a review of the toxicology of *d*-pulegone, menthofuran and other Group 11 constituents was performed to select an appropriate study for the

Table 3
Consideration of Congeneric groups for NFCs where the Estimated Intake > TTC for the Congeneric Group.

Name (FEMA No.)	DTC	Estimated Intake for CG (mg/person/day)	Estimated Intake for CG (mg/kg bw/day)	NOAEL (mg/kg bw/day)	MoS
Congeneric Group 10 - Alicyclic ketones, secondary alcohols and related esters					
Peppermint oil (FEMA 2848)	II	2.5	0.042	375	> 8000
Cornmint oil (FEMA 4219)	II	1.8	0.03	375	> 12,000
Curly mint oil FEMA (4778)	II	1.9	0.032	375	> 11,000
Congeneric Group 11 - Pulegone and structurally and metabolically related substances					
Peppermint oil (FEMA 2848)	III	0.13	0.0022	9.375	> 4000

calculation of a MoS. For Group 11, a 14-week oral gavage study of pulegone in rats conducted by the National Toxicology Program was chosen as the most relevant study for the calculation of MoS for this group (NTP, 2011). This study with a NOAEL of 9.375 mg/kg bw/day provides an adequate MoS for the NFCs with an estimated intake above TTC for Group 11 (see Table 3). A detailed review of relevant toxicological studies is presented later in this manuscript. Proceed to Step 7.

Step 7

Calculate the mean percentage (%) for the group of unidentified constituents of unknown structure in each NFC (as noted in Step 1) and determine the daily per capita intake (PCI or PCI × 10) for this group.

Proceed to step 8

The mean concentration and the estimated daily per capita intake for the group of unidentified constituents was calculated and the estimated daily per capita intake is listed for each NFC in Table 4 and Appendix A.

Step 8

Using the data from Step 1, is the intake of the NFC from consumption of the food[5] from which it is derived significantly greater than the intake of the NFC when used as a flavoring ingredient?

If Yes, proceed to Step 13.

If No, proceed to Step 9.

No. As discussed in Step 1, the estimated intake of the essential oils derived from mint, dill and caraway from the consumption of the whole leaf or seed added to food is expected to be significant. However, quantitative data for the consumption of these botanicals as the whole leaf or seed are unavailable. Proceed to Step 9.

Step 9

Could the unidentified constituents belong to TTC excluded classes?[6] The excluded classes are defined as high potency carcinogens, certain inorganic substances, metals and organometallics, certain proteins, steroids known or predicted bio-accumulators, nanomaterials, and radioactive materials (EFSA; WHO, 2016; Kroes et al., 2004).

If Yes, the NFC is not appropriate for consideration via this procedure.

If No, proceed to Step 10.

All the NFCs in this group are harvested from the botanical material by steam distillation and further rectified by fractional distillation. The oil is primarily composed of low molecular weight terpenoids (monoterpene hydrocarbons, alcohols and esters) derived from the isoprene pathway. Based on the identified constituents, production process and current literature, members of the TTC excluded classes are not present in these oils. Proceed to Step 10.

Step 10

Do the identified constituents give rise to concerns about the potential genotoxicity of the unidentified constituents?

If Yes, proceed to Step 10a.

If No, proceed to Step 11.

No. The mint, buchu, dill and caraway NFCs are primarily composed of menthol, menthone, menthyl esters, carvone, monoterpene hydrocarbons, and minor amounts of terpenoids that are intermediates in the menthol or carvone biosynthetic pathways. The unidentified substances in these NFCs are also expected to be among these classes, which generally do not exhibit genotoxic properties. A review of relevant genotoxicity studies is presented later in the manuscript. Proceed to Step 11.

Step 10a

Is the estimated intake of the group of unidentified constituents less than 0.15 μg/person/day (Koster et al., 2011; Rulis, 1989)? A TTC of 0.15 μg/person/day has been proposed for potentially genotoxic substances that are not from the TTC excluded classes (Kroes et al., 2004).

If Yes, proceed to Step 13.

If No, proceed to Step 10b.

This step is not required.

Step 10b

Do negative genotoxicity data exist for the NFC?

If Yes, proceed to Step 11.

If No, retain for further evaluation, which would include the collecting of data from appropriate genotoxicity tests, obtaining further analytical data to reduce the fraction of unidentified constituents, and/or considering toxicity data for other NFCs having a similar composition. When additional data are available, the NFC could be reconsidered for further evaluation.

This step is not required.

Step 11

Is the estimated intake of the unidentified constituents (calculated in Step 7) less than the TTC (Kroes et al., 2000; Munro et al., 1996) for structural Class III (90 μg/person/day)?[7]

If Yes, proceed to Step 13.

If No, proceed to Step 12.

Yes. For the fourteen NFCs re-evaluated (see Table 4), none have estimated intake levels for the unidentified constituents that exceed the TTC at structural Class III, 90 μg/person/day. Proceed to Step 13.

Step 12

Does relevant toxicological information exist that would provide an adequate margin of safety for the intake of the NFC and its unidentified constituents?

This question may be addressed by considering data for the NFC or an NFC with similar composition. It may have to be considered further on a case-by-case basis, particularly for NFCs with primarily non-volatile constituents.

If Yes, proceed to Step 13.

If No, perform appropriate toxicity tests or obtain further analytical data

Table 4
Estimated intake of unidentified constituents.

Name	FEMA No.	Estimated Intake μg/person/day
Peppermint Oil	2848	57
Peppermint Oil Terpeneless	4924	3
Spearmint Oil	3032	14
Spearmint Extract	3031	43
Spearmint Oil Terpeneless	4925	0.03
Cornmint Oil	4219	54
Erospicata Oil	4777	11
Curly Mint Oil	4778	58
Pennyroyal Oil	2839	0.1
Caraway Oil	2238	2
Dill Oil	2383	8
Buchu Leaves Oil	2169	1
Buchu Leaves extract	4923	0.001

[5] Provided the intake of the unidentified constituents is greater from consumption of the food itself, the intake of unidentified constituents from the added NFC is considered trivial.

[6] This can be based on arguments including: Expert judgement; Nature of the identified ingredients; Knowledge on the production/extraction process (see also Koster et al. (2011); EFSA; WHO, 2016).

[7] The human exposure threshold of 90 μg/person/day is determined from a database of NOAELs obtained from 448 subchronic and chronic studies of substances of the highest toxic potential (structural class III) mainly herbicides, pesticides and pharmacologically active substances (Munro et al.,1996). The 5th percentile NOAEL (lowest 5%) was determined to be 0.15 mg/kg bw/day which upon incorporation of a 100-fold safety factor for a 60 kg person yielded a human exposure threshold of the 90 μg/person/day. However, no flavoring substance or food additive in this structural class exhibited a NOAEL less than 25 mg/kg bw/d. Therefore the 90 μg/person/day threshold is an extremely conservative threshold for the types of substances expected in natural flavoring complexes. Additional data on other specific toxic endpoints (*e.g.,* neurotoxicity, reproductive and endocrine disruption) support the use of this threshold value (Kroes et al., 2000).

to reduce the fraction of unidentified constituents. Resubmit for further evaluation.

This step is not required for this set of NFCs.

Step 13

Are there any additional relevant scientific considerations that raise a safety concern (e.g. intake by young infants and children)?

If Yes, acquire and evaluate additional data required to address the concern before proceeding to Step 14.

If No, proceed to Step 14.

A further evaluation to consider possible exposure to children and infants, given their lower body weights and the potential for differences in toxicokinetics and toxicodynamics as compared to adults, was conducted. Table 3 lists the congeneric groups that exceed TTC threshold and in each instance, the margin of safety remains > 100 using a body weight of 20 kg. A review of the estimated intake of congeneric groups for each NFC shows found no others close to the TTC threshold. Table 4 lists the estimated intake of the unknown constituent fraction, none of which is close to or exceeding the TTC thresholds for Class III. Proceed to Step 14.

Step 14

Based on the above data and considerations, the NFC can be generally recognized as safe (GRAS) under conditions of intended use as a flavoring ingredient.

Yes, Peppermint Oil (FEMA 2848), Spearmint Oil (FEMA 3032), Spearmint Extract (FEMA 3031), Cornmint Oil (FEMA 4219), Erospicata Oil (FEMA 4777), Curly Mint Oil (FEMA 4778), Pennyroyal Oil (FEMA 2839), Buchu Leaves Oil (FEMA 2169), Caraway Oil (FEMA 2238) and Dill Oil (FEMA 2383) are affirmed as GRAS under conditions of intended use as flavor ingredients based on the above assessment and the application of the judgment of the FEMA Expert Panel. In addition, Buchu Leaves Extract (FEMA 4923), Peppermint Oil, Terpeneless (FEMA 4924) and Spearmint Oil, Terpeneless (FEMA 4925) are determined to be GRAS under conditions of intended use as flavor ingredients.

7. Biochemical and toxicological supporting information relevant to the safety evaluation

As chemical analyses of the mint, buchu leaves, dill and caraway NFCs (see Table 1) have demonstrated, Group 10 (Alicyclic ketones, secondary alcohols and related esters) and Group 11 (Pulegone and structurally and metabolically related substances) are the two primary congeneric groups that account for the majority of the NFC composition (Appendix A). The estimated intake of Group 10 constituents exceeds the TTC for the Cramer decision tree class for Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219) and Curly Mint Oil (FEMA 4778). Also, for Peppermint Oil (FEMA 2848), the estimated intake of Group 11 constituents exceeds the TTC threshold. The FEMA Expert Panel has reviewed the safety of flavoring ingredients of these groups (Adams et al., 1996) was well as the flavoring ingredients of Group 19 (Aliphatic and aromatic hydrocarbons) and Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) flavoring ingredients which also contribute to the constituent profile (Adams et al., 2011; Marnett et al., 2014). The additional information presented here includes studies on the NFCs themselves, studies on the principal constituents of these NFCs and newly available studies on constituents not considered within the reviews mentioned above.

7.1. Peppermint (Mentha piperita) oil

7.1.1. Short-term studies of toxicity

An analyzed (GC/FID) sample of peppermint oil was determined to contain 46.8% menthol, 21.81% menthone, 5.11% menthyl acetate, 1.98% menthofuran, 1.20% pulegone and other identified constituents that account for 95.01% of the composition (Vollmuth, 1989). This peppermint oil sample was administered to groups (10/sex/group) of male and female Sprague-Dawley rats at dose levels of 0, 100, 200, or 400 mg/kg bw by gavage in corn oil (10 ml/kg) daily for 29 or 30 days (Serota, 1990). Clinical signs were monitored twice weekly and body weights and food consumption were measured weekly. At the initiation of the study, 10 animals were randomly selected from the pool of animals not selected for the study. They were fasted overnight and blood samples were drawn and analyzed for baseline clinical chemistry and hematology parameters. Prior to termination, animals were injected with ketamine and blood samples were drawn for clinical chemistry and hematology. At necropsy, organ weights (brain, spleen, liver, heart, kidneys, testes with epididymis, adrenals, ovaries, and pituitary) were measured, and tissues (26) were preserved in 10% formalin. All tissues from the control and high-dose groups and tissues from the heart, liver, kidneys, and gross lesions from the low- and mid-dose group were embedded in paraffin, stained with hematoxylin and eosin, and examined microscopically.

All animals survived to study termination with high dose males showing increased incidence of urine staining during clinical observations. Except for a non-statistically significant decrease in mean body weight in high-dose males, there were no statistically significant differences in body weight or food consumption between treated and control groups. A significant decrease in serum glucose levels was reported in the mid- and high-dose males that the authors, in part, attribute to change in nutritional status as revealed by decreased body weights in the high-dose group. A treatment-related increase in alkaline phosphatase was reported in high-dose males. Measurement of body weight, food consumption, hematology and clinical chemistry parameters revealed no significant changes between test and control female rats. There were statistically significant increases in relative kidney weights in males treated with a high dose. Histopathological findings revealed renal tubule protein droplets in all treated male rats. The authors of this review considered these findings related to the lysosomal handling of α_{2u}-globulin, a protein specific to the male rat and of no toxicological relevance to humans (Capen et al., 1999; EPA, 1991; Flamm and Lehman-McKeeman, 1991; Swenberg and Lehman-McKeeman, 1999). Absolute and relative liver weights in high-dose females also were significantly increased but these changes were not associated with histopathological alterations. Based exclusively on the renal pathology reported in all dosed groups of male rats, the authors concluded that the no-observed-adverse effect level (NOAEL) for peppermint oil is less than 200 mg/kg bw per day in male rats and female rats (Serota, 1990). Since the renal pathology is not relevant to humans, the Expert Panel interprets the NOAEL as 200 mg/kg bw/day.

In another study showing similar kidney effects, peppermint oil was administered by oral gavage in soybean oil to male and female Wistar rats (14/sex/dose) at daily doses of 0, 10, 40 or 100 mg/kg bw (Spindler and Madsen, 1992). Body weights and food and water consumption measured weekly revealed no differences between test and control animals. Hematological examinations and blood chemical determinations performed on 10 animals of each sex on days 30 or 86 of dosing gave normal values. There were no effects in either the low or mid-dose animals, however, at the high dose, nephropathy in the form of hyaline droplets was reported in male rats. The authors interpreted these results as an early manifestation of sex and species-specific α_{2u}-globulin nephropathy in male rats. Cyst-like spaces in the cerebellum, judged to be identical to those seen in other studies from the same laboratory (Madsen et al., 1986; Thorup et al., 1983a, b), were also reported in this study in the high dose animals but there were no other signs of encephalopathy nor was there evidence of an aggravation in the extension of cyst-like spaces (Spindler and Madsen, 1992). Later analysis of the histology slides of the brain tissue concluded that the rat cerebellar “cyst-like spaces” were likely artifacts that resulted from inadequate tissue fixation procedures (Adams et al., 1996). A subsequent study also concluded that the cyst like spaces may be artifacts arising from inadequate tissue fixation procedures and that discrepancies in the cerebellar findings may have been caused by test

article impurities or a change in the genetic constitution of the animals (Mølck et al., 1998).

Peppermint oil was administered to groups of 10 male and 10 female Wistar SPF rats by gavage in daily doses of 0, 10, 40 or 100 mg/kg bw for 28 days (Thorup et al., 1983a). There were no significant differences in body weights and food consumption between test and control animals and a slight non-significant increase in water consumption in all test groups. Hematological examinations, blood chemical determinations and urine analysis revealed normal values. The only significant histopathological change was the appearance of cyst-like spaces in the white matter of the cerebellum at the 40 and 100 mg/kg doses which were later determined to be an artifact of the tissue fixation procedure (Adams et al., 1996; Mølck et al., 1998).

Peppermint oil was administered by gavage to groups of 3 males and 3 female beagle dogs at daily doses of 25 or 125 mg/kg bw and to groups of 12 male Wistar rats at daily doses of 20, 150 or 500 mg/kg bw each for 5 weeks. The animals were inspected daily for clinical signs, records taken weekly for body weight and food consumption, hematological, blood biochemical and urinary parameters were measured prior to treatment and during the 5th week, and histological examination conducted after termination. The rats showed no effects on general health, behavior, or body weight and all the hematological and urinary parameters were normal. The histological examination revealed no specific toxic lesions. A reduction in triglyceride levels in the high dose male rats was attributed to decreased food consumption. The results were similar for the dogs except for a slight increase in alkaline phosphatase and blood urea nitrogen levels in the high dose males. These increases were not statistically significant and not thought to be of toxicological relevance (Mengs and Stotzem, 1989).

7.1.2. Reproductive and developmental toxicity

Four groups of 10 virgin Crl CD rats were administered oral dose levels of 0, 150, 750 or 1500 mg/kg bw of peppermint oil by gavage once daily, 7 days prior to cohabitation, through cohabitation (maximum of 7 days), gestation, delivery and a 4-day post-parturition period. The duration of the study was 39 days (Hoberman, 1989). The composition of the oil was identical to that used in the 28-day study (Vollmuth, 1989). Maternal indices monitored included twice-daily clinical observation, measurement of body weights, food consumption, duration of gestation and fertility parameters (mating and fertility index, gestation index, number of offspring per litter). Offspring indices monitored included daily observation, clinical signs, examination for gross external malformations and measurement of mortality (number of stillborns), viability (pups dying on days 1–4), body weight and body weight gain.

Deaths or moribund sacrifice were reported in 2/10 females at 750 mg/kg bw per day and 5/10 females at 1500 mg/kg bw per day. Additional clinical observations included decreased motor activity, ataxia, dysnea, rales, un-groomed coat and thin appearance at the 750 and 1500 mg/kg bw per day dose levels. Urine-stained fur and excess salivation were observed at all dose levels. Significant ($p \leq 0.05$) decreases in body weight and food consumption were reported during the pre-mating period in the 750 and 1500 mg/kg bw per day groups compared to those for control group. A non-statistically significant decrease in maternal body weight gain was reported in the 750 mg/kg bw per day group compared to the control group. The single dam that delivered a litter in the high-dose group also showed less weight gain. Absolute and relative feed consumption were comparable between the low, middle and control groups.

On day 1 of lactation, the average body weight of dams in the mid-dose group and the single dam in the high-dose group was significantly ($p \leq 0.01$) less than in the control group. During lactation, dams in the mid-dose group gained weight while the weight gain in the single dam in the high-dose group was comparable to that for the control group. Compared to control animals, feed consumption in the mid- and high-dose group decreased significantly ($p \leq 0.01$) during pre-mating but was increased significantly ($p \leq 0.01$ to 0.05) during lactation. Of the rats surviving the cohabitation period 4 of 5 became pregnant at the highest dose level (1500 mg/kg bw per day).

Live litters were reported for 9/19, 8/10, 5/6, and 1/4 pregnant females in the control, 150, 750 and 1500 mg/kg bw per day groups, respectively. Increased number of dams with stillborn pups, stillborn pups, and late resorptions in utero were reported in the 750 mg/kg bw per day group. At 1500 mg/kg bw per day, two rats had only resorptions in utero when found dead on gestation day 23 and one rat possessed only empty implantation sites in utero on day 25 of presumed gestation.

On day 1 post-parturition, litters of dams in the 750 and 1500 mg/kg bw per day groups showed non-statistically significant decreases in pup weight which by day 4 were comparable to controls in the mid-dose group, but less than the control value in the high dose group. On day 4 post-parturition, significant ($p \leq 0.01$) increases in pup mortality were reported in the mid- and high-dose groups compared to controls. However, even at the highest dose level, there was no evidence of an effect of the test article on implantation, duration of gestation, pup sex ratio, or gross morphology of pups.

Based on these results the authors concluded that the maternal NOAEL for reproductive effects was 150 mg/kg bw per day and the offspring NOAEL for developmental effects is higher than 150 mg/kg bw per day, but less than 750 mg/kg bw/day (Hoberman, 1989).

7.1.3. Genotoxicity studies

Following a preliminary toxicity study in which toxicity was observed at plate concentrations equal to and greater than 8.5 μg/plate, concentrations of 0.005–9.0 μg of peppermint oil in DMSO were incubated with plates cultured with *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, or TA1538 in the presence or absence of metabolic activation, Aroclor 1254-induced rat liver preparations. There was no evidence of mutagenicity in any of the assays conducted. Under the conditions of the assays, peppermint oil was not mutagenic (DeGraff, 1983). In a separate OECD-compliant study, no evidence of mutagenicity was observed when concentrations of Peppermint oil (FEMA 2848) up to 5000 μg/plate were incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA without metabolic activation or with metabolic activation by S9 liver homogenate prepared from Aroclor 1254-treated male Sprague-Dawley rats (Dakoulas, 2017a). Chromatographic analysis of the peppermint oil used in this study showed a composition of 30.8% menthol, 27.7% menthone, 5.6% eucalyptol, 4.4% *d*-isomenthone, 3.4% neomenthol, 2.1% pulegone and other identified constituents that account for 89.7% of the composition.

In a mouse lymphoma assay (MLA), concentrations of 5.6–180 μg/ml of peppermint oil[8] were incubated with mouse lymphoma cell line, L5178Y TK+/-, in the presence of 5-bromo-2′-deoxyuridine. In the absence of metabolic activation, a wide range of cytotoxicity (% relative growth 99.6%–15.6%) was observed over this dose range. There was no evidence of an increase in the frequency of forward mutations at any test concentration. In the presence of metabolic activation induced by liver preparations of Aroclor-treated rats, concentrations in the range from 7.0 to 113 μg/ml resulted in low to moderate toxicity (% relative growth, 68.8%–25.6%). In this dose range only the 113 μg/ml concentration induced an increase in mutational frequency. Therefore, the study was repeated over the concentration range from 22.5 to 135 μg/ml. The concentration range induced a wide range of toxicity (% relative growth, 115.1%–10.4%). There was no evidence of an increase in the frequency of mutation at any dose level. It was the conclusion of the authors that the test material was not mutagenic either in the presence or absence of metabolic activation (Cifone, 1982).

To better assess the genotoxic potential of the essential oil derived

[8] Based on the average density of peppermint oil = 0.902 g/mL (FCC, 2019).

from peppermint leaves, human peripheral lymphocytes were incubated in the presence of 0.1, 0.15, 0.20, 0.25 and 0.30 µl/ml of peppermint oil. These concentrations correspond to 90, 135, 180, 227 and 271 µg/ml of peppermint oil.[8] The peppermint oil used in this study was reported to be composed of 59.17% menthol, 18.78% menthone, 5.16% limonene, 3.55% isomenthone, 2.93% β-caryophellene, 1.73% germacrene, 1.67% caryophellene oxide, 1.08% bornyl acetate, 0.61% caraway aldehyde, 0.61% β-pinene, 0.56% α-pinene, 0.34% myrcene, 0.32% eucalyptol, 0.32% isoeugenol, 0.27% methyl cinnamate, 0.26% sabinene and 0.25% ocymene. Although a statistically significant concentration-dependent increase in chromosomal aberrations was observed, a concomitant inhibition of the cell mitotic activity (≥70%) was observed at all treatment levels indicating that this effect is related to the cytotoxicity of the test substance. When the same peppermint oil was tested in a sister chromatid exchange (SCE) assay, a small increase in SCE was observed at concentrations ranging from 90 to 271 µg/ml but there was no dose-response relationship (Lazutka et al., 2001). To assess the ability of peppermint oil to induce somatic and recombination mutations in *Drosphila melanogaster*, larvae were treated with 0.10, 0.25, 0.75, 1.00 and 1.50% dissolved in a 3% aqueous acetone solution. Progeny were examined for spots on their wings. Peppermint oil exposed groups showed a small, non-dose related increase in wing spots with increased exposure to peppermint oil (Lazutka et al., 2001).

7.2. Buchu leaves (Barosma crenulata (L.) Hook) oil

In an OECD-compliant study, no evidence of mutagenicity was observed when concentrations of Buchu Leaves Oil (FEMA 2169) up to 5000 µg/plate were incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA without metabolic activation or with metabolic activation by S9 liver homogenate prepared from Aroclor 1254 treated male Sprague-Dawley rats (Mee, 2017).

7.3. Erospicata (Mentha spicata 'erospicata') oil

In an OECD-compliant study, no evidence of mutagenicity was observed when concentrations of erospicata oil up to 5000 µg/plate were incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvrA* using both plate incorporation and pre-incubation protocols, without metabolic activation or with metabolic activation by S9 liver homogenate prepared from phenobarbital/β-naphthoflavone treated rats (Chang, 2016).

7.4. Dill oil (Anethum graveolens L.)

No evidence of mutagenicity was observed in a GLP study when concentrations of dillweed oil were incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 up to the limit of cytotoxicity. Concentrations up to 22,250 µg/plate were tested. Cytotoxicity was observed in strain TA100 at 1040 µg/plate (appearance of microcolonies). No evidence of mutagenicity was observed using the plate incorporation method either in the presence or absence of S9 metabolic activating system prepared from the liver of Aroclor 1254-treated male Sprague-Dawley rats (Jagannath, 1984).

A sample of dill essential oil (chemical composition and part(s) of the botanical from which the sample was derived were not specified) was reported to be positive in strains TA1535 and TA1538 at a concentration of 4 ng/plate[9] and in strain TA1537 at a concentration of 9 ng/plate (Sivaswamy et al., 1991). Because this study did not specify the part(s) of the plant used to prepare the sample or provide the chemical composition of the tested dill oil sample, did not demonstrate a dose response, did not evaluate the cytotoxicity of the test substance and used test concentrations that were unusually low for this assay and inconsistent with OECD guidance (OECD, 1997), the results of this study are not considered relevant to the safety evaluation of the NFCs under consideration. The GLP-compliant Ames study on dill weed oil is considered the most valid of the studies described here.

Dill weed oil was negative in the unscheduled DNA synthesis (UDS) assay in rat hepatocytes at 60 µg/mL, the only concentration tested (Heck et al., 1989). A sample of commercial *Anethum graveolens* dill (herb) oil (chemical composition provided by manufacturer α-phellandrene 36.33%, limonene 31.57%, 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran ("dill ether"), 21.46%, carvone 6.66% and smaller amounts of α-pinene, myrcene, *trans*-dihydrocarvone and *cis*-dihydrocarvone) was reported to induce an increase in a chromosomal aberrations at the two highest concentrations tested, 0.2 to 0.25 µL/mL, in human lymphocytes. However, at these concentrations, there was also a significant decrease in the mitotic index to less than 45% of the control, the threshold for cytotoxicity in human lymphocytes indicated in the OECD guidance for this assay (OECD, 2016). A sister chromatid exchange (SCE) assay on dill oil in human peripheral lymphocytes was also positive when tested at concentrations of 0.1–0.25 µl/mL (Lazutka et al., 2001). Currently, there is no correlation between SCE frequencies and cancer risk (Mateuca et al., 2012) and the OECD no longer has a guideline for this assay (OECD, 2015). An *in vivo* micronucleus study in C_{57}Bl/6 and CBA mice found that dill seed oil given at 1 g/kg i.p. did not induce micronuclei in polychromatic erythrocytes in the bone marrow (Mortkunas, 2002).

7.5. Spearmint oil (Mentha spicata L.)

No evidence of mutagenicity was observed when spearmint oil was incubated with *S. typhimurium* strains TA98 and TA100 at 2 and 7 mg/plate, in both the presence and absence of a rat liver supernatant (S-13) metabolic activation system (Marcus and Lichtenstein, 1982). Similar results were reported for spearmint oil in these strains when tested in the presence and absence of liver S9 metabolic activation derived from Aroclor 1254-treated SD rats (Crebelli et al., 1990). No evidence of mutagenicity was observed when spearmint oil collected from wild growing *M. spicata* plants in Greece was incubated with *S. typhimurium* strains TA97, TA98, TA100 and TA102 at concentrations up to 2000 ppm. GC-MS analysis yielded the following composition: 59.12% carvone, 6.27% dihydrocarveol, 5.07% limonene, 5.42% 1,8-cineole and other minor components (Adam et al., 1998). A sample of spearmint essential oil (chemical composition and part(s) of the botanical from which the sample was derived were not specified) was tested in *S. typhimurium* strains TA98, TA1535, TA1537 and TA1538 and was reported to be positive in strains TA98 and TA1538 at a concentration of 10 ng/plate[10] (Sivaswamy et al., 1991). Similar to the results reported for dill oil discussed above, this study did not demonstrate a dose response, did not evaluate the cytotoxicity of the test substance and chose test concentrations that are very low for this assay and inconsistent with OECD guidance (OECD, 1997), such that the results of the study are not considered relevant to the safety evaluation of spearmint oil.

Spearmint oil (composition and origin not reported) was negative in the chromosomal aberration assay at concentrations up to 0.125 mg/mL (Ishidate et al., 1984). In the spore rec assay, conducted at a concentration of 10 mg/disk, equivocal results were obtained in the absence of S9 metabolic activation and toxicity was observed in the presence of metabolic activation (Ueno et al., 1984). In an *in vivo* micronucleus assay in male ddY mice (6 animals/group), spearmint oil was not found to induce the formation of micronuclei in polychromatic erythrocytes of the femoral bone marrow when tested at doses of 0, 200, 400 and 800 mg/kg, administered by i.p. injection (Hayashi et al., 1988).

[9] Based on density of dill oil, 0.884 g/mL (FCC, 2019).

[10] Based on density of spearmint oil, 0.92 g/mL (FCC, 2019).

7.6. Caraway oil (Carum carvi L.)

Mutagenic activity was not observed when caraway oil (composition not reported) was incubated with *S. typhimurium* strains TA98 and TA100 at 2 and 7 mg/plate, either in the presence or absence of a rat liver supernatant (S-13) metabolic activation system (Marcus and Lichtenstein, 1982). In addition, aqueous, methanolic and hexane extractions (uncharacterized) of up to 75 mg of caraway seed (results reported are based on the corresponding original weight of caraway seed extracted) were not found to be mutagenic in *S. typhimurium* strains TA98 and TA100 in the presence and absence of rat liver S9 metabolic activation (Higashimoto et al., 1993). In a reverse mutation assay in *S. typhimurium* strains TA98 and TA102, 10 mg of a concentrated ethanolic extract of caraway seed was found to be mutagenic in strain TA102 but non-mutagenic in strain TA98. The extract was prepared by the cold percolation of 50 g ground seed with 95% ethanol, concentrated to yield a syrupy residue. No further analytical characterization was performed (Mahmoud et al., 1992). No mutagenic activity was observed when 100 μL of a water extract of caraway seed (concentration not provided) was incubated with *S. typhimurium* strains TA97a, TA98, TA100 and TA102 using the plate incorporation protocol without metabolic activation (Al-Bataina et al., 2003).

7.7. Carvone, carveol and dihydrocarveol

d-Carvone is a major constituent of the spearmint-derived NFCs and *l*-carvone is a major constituent of caraway and dill oils. Studies related to the genotoxicity of carvone and related compounds carveol and dihydrocarveol support the conclusion of a lack of genotoxic potential for carvone-rich NFCs Spearmint Oil (FEMA 3032), Spearmint Oil Terpeneless (FEMA 4925), Caraway Oil (FEMA 2238) and Dill Oil (FEMA 2383).

d-Carvone showed no increase in mutagenicity in several reverse mutation assays. In an OECD-compliant assay, no increase in mutagenicity was observed when *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2*uvr*A were incubated with *d*-carvone at concentrations up to 5000 μg/plate, in the presence and absence of phenobarbitone/*β*-naphthoflavone-induced rat liver S9 metabolic activation. Both the plate incorporation and pre-incubation methods were performed (ECHA, 2018a). In an earlier report, no increase in mutagenicity was observed when *d*-carvone was incubated with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 at concentrations up to 5000 μg/plate, in the presence or absence of Aroclor 1254-induced male rat liver S9 metabolic activation. Toxicity was observed at a concentration of 500 μg/plate for strain TA1537 both with and without metabolic activation and at a concentration of 5000 μg/plate for the other S. *typhimurium* strains (Glover, 1987). Carvone (enantiomer not specified) was negative in a reverse mutation assay in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 at a concentration of 466 μg/plate in the presence and absence of S9 metabolic system derived from the liver of Aroclor 1254-treated male rats (Florin et al., 1980). No mutagenicity was reported for *d*-carvone in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 at concentrations up to 333 μg/plate. *d*-Carvone was tested using the preincubation method in both the presence and absence of S9 mix from the liver of Aroclor 1254-treated male rats (Mortelmans et al., 1986; NTP, 1990). *d*-Carvone was negative for mutagenicity in *S. typhimurium* strains TA98 and TA100 at concentrations up to 3756 μg/plate, in the presence and absence of S9 metabolic activation (Stammati et al., 1999). For carveol, no mutagenicity was reported in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 at concentrations up to 560 μg/plate. Carveol was tested using the preincubation method in both the presence and absence of S9 mix from the liver of Aroclor-1254 treated male rats (Mortelmans et al., 1986). For dihydrocarveol, no increase in mutagenicity was observed in an OECD-compliant reverse mutation assay, when *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2*uvr*A were incubated with dihydrocarveol at concentrations up to 5000 μg/plate, in the presence and absence of a phenobarbitone/*β*-naphthoflavone-induced rat liver S9 metabolic activating system (Thompson, 2016).

d-Carvone was negative in an OECD-compliant *in vitro* mammalian cell chromosome aberration assay in which human lymphocytes were incubated with *d*-carvone in the presence and absence of a S9 metabolic activation system prepared from phenobarbitone/*β*-naphthoflavone-induced male rat liver. Based on a preliminary toxicity test, the dose ranges were 25–400 μg/mL for the 4 h exposure without S9, 50–800 μg/mL for the 4 h exposure with S9, and 12.5–400 μg/mL for the 24 h exposure without S9. For all exposure groups, *d*-carvone did not induce any biologically relevant increases in aberrations in either the presence of absence of S9 metabolic activation (ECHA, 2018b). In a previous chromosomal aberration assay in Chinese hamster ovary (CHO) cells, *d*-carvone was positive when tested in the absence of S9, although a dose-response relationship was not observed in the second of two trials and a chemical-induced delay in the cell cycle was observed. A clear dose-response was not seen in either of the two trials conducted in the presence of S9 (NTP, 1990).

In an SCE assay, an increase in SCEs was found when *d*-carvone was incubated with CHO cells at concentrations up to 20 μg/mL in the absence of S9 metabolic activation system and concentrations up to 502 μg/mL in the presence of S9 metabolic activation system. However, a dose-response relationship was not observed in any of the trials performed (NTP, 1990). In a DNA repair assay, *d*-carvone was incubated with *E. coli* WP2 *trpE65* and its isogenic DNA-repair deficient derivative CM871 *trpE65, uvrA155, recA56, lexA* in a filter disc assay. At a concentration range of 25–80 μmol *d*-carvone, a dose-dependent increase in the visible inhibition zone was observed in the repair-deficient strain, indicating the capacity of the test compound to induce DNA damage (Stammati et al., 1999). In another DNA repair assay, in which 1235 μg/mL carvone (enantiomer not specified) was incubated with *S. typhimurium* strain TA1535 containing plasmid pSK1002 carrying a fused *umuC'-'lac* gene *Z,* induction of the SOS response was reported in the presence of a phenobarbital/5,6-benzoflavone rat liver S9 metabolic activation system but not in the absence of metabolic activation. In this assay, the *umu* operon is induced by DNA-damaging agents and the intensity of DNA repair is measured by β-galactosidase activity produced from the fused gene (Ono et al., 1991).

In an SOS-Chromotest, no increase in β-galactosidase activity from induction of the SOS response was measured when *d*-carvone, at concentrations up to 0.25 μmol, was incubated with *E. coli* strain PQ37 containing the *sfiA* gene, a deletion in the *lac* region with a *uvrA* mutation and a *rfa* mutation that increases the permeability of the cells to chemical agents (Stammati et al., 1999). Carvone (isomer not specified) was negative in a *Bacillus subtilis* rec assay using strains H17 and M45, both in the presence and absence of S9 metabolic activation (Matsui et al., 1989).

d-Carvone was negative in an OECD-compliant mammalian cell forward mutation assay in L5178Y mouse lymphoma cells. Upon incubation with *d*-carvone at concentrations of 23–372 μg/mL for the 4 h exposure without S9, 25–300 μg/mL for the 4 h exposure with S9 and 3–100 μg/mL for the 24 h exposure without S9, no significant increases in mutant frequency were observed both in the presence and absence of phenobarbitone/*β*-naphthoflavone-induced male rat liver S9 metabolic activation (ECHA, 2018c).

In a separate OECD-compliant micronucleus assays, carveol and dihydrocarveol were not found to induce increases in the frequency of binucleated cells with micronuclei in cultured human peripheral blood lymphocytes (HPBL) in any treatment group. For carveol, the maximum dose levels selected for the micronucleus assay were 800 μg/mL for 4 h exposure groups tested both with and without phenobarbitone/*β*-naphthoflavone-induced male rat liver S9 metabolic activation and 720 μg/mL for the 24 h exposure group without S9 (Morris, 2014). For dihydrocarveol, concentrations up to 384 μg/mL for the 4 h exposure

group without phenobarbitone/β-naphthoflavone-induced male rat liver S9 metabolic activation, 480 μg/mL for the 4 h exposure group with S9, and 288 μg/mL for the 24 h exposure group without S9 were tested (Morris, 2016).

7.7.1. Conclusion about genotoxicity

Several reverse mutation studies reported no mutagenicity for carvone, including an OECD-compliant study. Positive results for carvone were only reported in non-standard assays and are inconsistent with the negative results for carvone in an OECD-compliant chromosome aberration assay in human lymphocytes and an OECD-compliant mammalian cell forward mutation assay in L5178Y mouse lymphoma cells. In addition, in reviewing the two-year carcinogenicity study in B6C3F1 mice conducted by the NTP (in which mice were administered *d*-carvone at 375 or 750 mg/kg bw/day 5 days a week), the NTP concluded that there was no evidence of carcinogenic activity (Adams et al., 1996; NTP, 1990) For the related compounds, carveol and dihydrocarveol, OECD-compliant micronucleus assays in human peripheral blood lymphocytes were negative and carveol was negative in an OECD-compliant Ames assay. In summary, the weight of evidence indicates a lack of genotoxic potential for carvone and the related compounds carveol and dihydrocarveol.

7.8. dl-Menthol and menthone

Menthol, menthone and their derivatives have characteristic organoleptic properties and comprise a large percentage of the identified Group 10 constituents of Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219) and Curly Mint Oil (FEMA 4778) where the estimated intake exceeds the TTC. The absorption, distribution, metabolism and excretion of compounds in this group has been reviewed in detail previously. (Adams et al., 1996). The following discussion reviews these and newer studies relevant to the safety of consumption of NFCs containing menthol and menthone.

The ability of menthol to provide a cooling sensation has been associated with the activation of sensory neurons known as transient receptor potential (TRP) channels (Farco and Grundmann, 2013). Menthol binds to TRP melastatin family member 8 (TPRM8) (Dragoni et al., 2006). Upon binding to this receptor, calcium flux through the channel increases creating the cooling sensation most commonly associated with peppermint.

7.8.1. Absorption, distribution, metabolism and excretion

Menthol is primarily metabolized by reaction with glucuronic acid followed by elimination in the urine or feces. The glucuronic acid conjugate of menthol was detected in the urine of 19 male and female volunteers following oral administration of a 180 mg dose of peppermint oil (Kaffenberger and Doyle, 1990).

In a toxicokinetic (TK) study, 16 healthy male volunteers received a dose of 100 mg caraway oil plus 180 mg peppermint oil in the form of 2 capsules of the enteric coated preparation or 5 capsules of the reference preparation. The peppermint oil fraction estimated to contain greater or equal to 44.0% free alcohols calculated as menthol. The capsules were administered with 250 mL water following a 10 h fast and blood samples were subsequently collected for analysis. The $C_{max,}$ total bioavailability ($AUC_{0-\infty}$) and terminal half-life ($t_{1/2}$) of menthol for both preparations were similar (within one standard deviation) for the enteric coated capsule (C_{max} = 1196 ng/mL, $AUC_{0-\infty}$ = 3272, $t_{½}$ = 3.5 h) and the non-enteric coated capsule (C_{max} = 1492 ng/mL, $AUC_{0-\infty}$ = 3226, $t_{½}$ = 4.4 h). The T_{max} was 3.0 and 1.7 h for the enteric and non-enteric

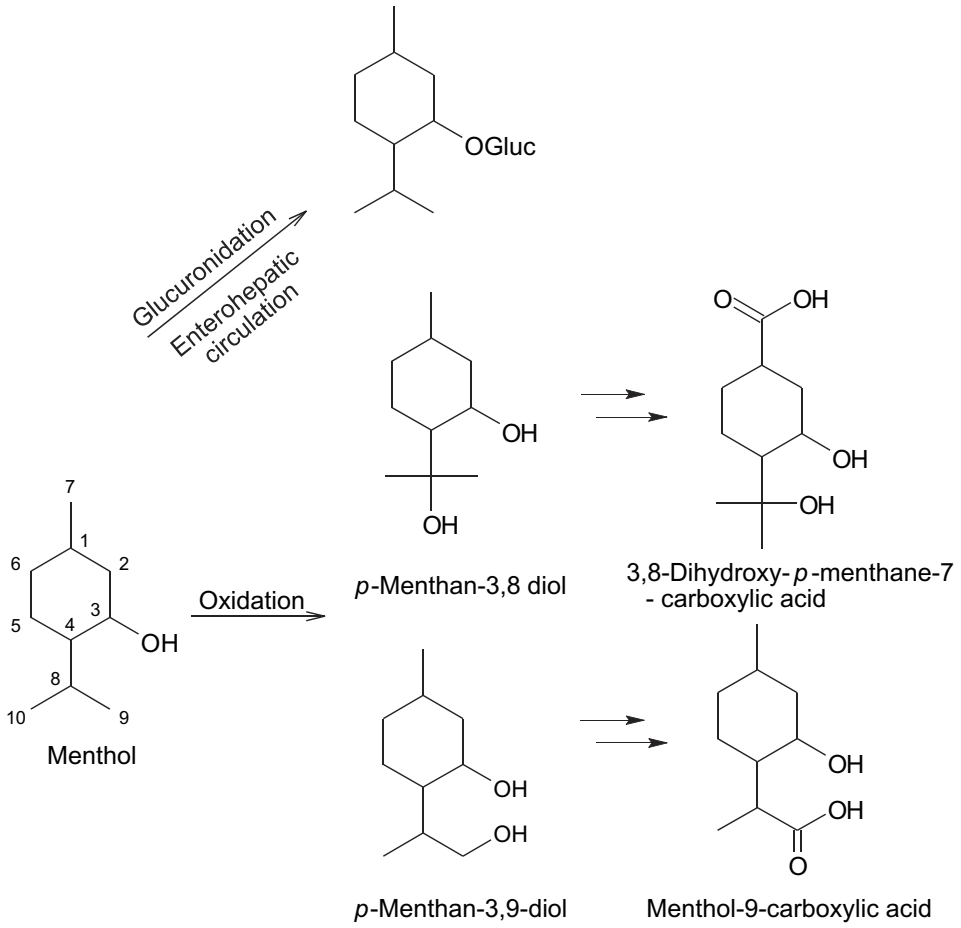


Fig. 6. Metabolic pathways of menthol.

preparations, respectively (Mascher et al., 2001).

In rats, exposure to menthol through oral routes shows that the majority is eliminated in either the urine or feces as the glucuronic acid conjugate or various oxidation products (Madyastha and Srivatsan, 1988; Yamaguchi et al., 1994). Non-cannulated and bile duct-cannulated male Fischer 344 rats were administered a single dose of 500 mg [^{3}H]-*l*-menthol/kg bw. In the non-cannulated rats, total recovery of the radiolabeled substance in the urine or feces was 71.7% with most of the dose (45.4%) recovered within the first 24 h. In the urine, 37.8% percent of the radioactivity was excreted with equal amounts for each 24 h period. In the feces, 33.9% of the radioactivity was recovered in the first 24 h (26.6%) (Yamaguchi et al., 1994). In the bile duct-cannulated rats, total recovery of the radiolabeled substance in the urine or bile was 74.2% with the majority recovered in the bile (66.9%). Menthol glucuronide was the major metabolite reported in the bile and a variety of oxidation products were reported in the urine (Yamaguchi et al., 1994).

Menthol glucuronide was also the major metabolite detected in a clinical study in which *l*-menthol was administered to healthy male human volunteers (6/group) at doses of 0, 80, 160 or 320 mg/person by the spraying of a 0.8% solution directly on to the gastric antrum with a standardized spraying catheter. In the 24 h post-dose period, 65–68% of the administered *l*-menthol was excreted in the urine as the glucuronide conjugate. Over 24 h, a total of 32 metabolites were identified from the analysis of plasma and urine samples from the 320 mg dose group. In plasma, menthol glucuronide, menthol sulfate conjugate and hydroxyl menthol glucuronide were detected. Pharmacokinetic analysis of sampled plasma showed that the concentration of menthol and menthol glucuronide rise rapidly, increase with increasing dose and attain a peak concentration within 1 h of dose administration. The median values of C_{max} and $AUC_{0-\infty}$ of menthol and menthol glucuronide increased with increasing dose. The T_{max} and $t_{1/2}$ of *l*-menthol were 0.54 h and 1.34 h, respectively (Hiki et al., 2011).

Menthol glucuronide formed in the liver passes into bile with subsequent elimination or entry into enterohepatic circulation where it undergoes various oxidation reactions upon each passage through the liver. Oxidation products of menthol include *p*-menthane-3,8-diol primarily, *p*-menthane-3,9-diol, and 3,8-dihydroxy-*p*-menthane-7-carboxylic acid (Madyastha and Srivatsan, 1988; Yamaguchi et al., 1994) (see Fig. 6). Additional oxidation metabolites have been identified including a primary alcohol, a triol, and hydroxy acids (Yamaguchi et al., 1994). The biliary route of menthol metabolism appears to be more important in rodents and dogs as compared to humans and rabbits.

In a recent metabolism study, menthol enantiomers (+) - (1*S*,3*S*,4*R*) and (−) - (1*R*,3*R*,4*S*)-menthol were incubated with human liver microsomes. The enantiomers were oxidized to their corresponding diols, (+)-(1*S*,3*S*,4*R*) and (−)-(1*R*,3*R*,4*S*)-*trans*-*p*-menthane-3,8-diol by human liver P450 microsomal enzymes. Through screening with a variety of recombinant human liver P450 enzymes it was determined that CYP2A6 was responsible for the oxidative metabolism (Miyazawa et al., 2011).

Results of an *in vitro* study using rat liver microsomes suggest that side chain oxidation of menthol is mediated by cytochrome P450s. Rats receiving repeated oral doses of 800 mg/kg bw/day *l*-menthol for three days exhibited increased activity of hepatic microsomal cytochromes P450 and NADPH-cytochromes P450 reductase by nearly 80%. Although further treatment over the following four days decreased the activity of hepatic microsomal cytochrome P450 and NADPH-cytochrome P450 reductase, the activity remained higher than that reported in control rats (Madyastha and Srivatsan, 1988).

In an *in vitro* study in human hepatocytes, 64.5–71.5% of menthyl acetate (initial concentrations of 20 and 100 μM) incubated in the cell culture was metabolized to menthol in a 4 h incubation. 64.5–71.5% (ECHA, 2017).

7.8.2. Short-term studies of toxicity

Groups of 10 female and 10 male Fischer 344 rats per group were maintained on diets containing *dl*-menthol at concentrations of 0, 930 ppm, 1870 ppm, 3750 ppm, 7500 ppm or 15,000 ppm for 13 weeks (NCI, 1979). Dietary concentrations were calculated (FDA, 1993) to provide corresponding average daily intake levels of 0, 46.5, 93.5, 187.5 or 375 mg *dl*-menthol/kg bw, respectively. Necropsies were performed on all animals at the end of the study. Histopathological examination was performed on tissues from the control animals, the highest dose group and selected tissues from the second highest dose group. Final mean body weights of the male and female rats at all dose levels were similar to controls. A slight increase in the incidence of interstitial nephritis (most likely chronic progressive nephropathy) was observed in male rats in the highest-dose group. No adverse effects were reported for male or female rats administered 93, 187, 375 or 750 mg *dl*-menthol/kg bw/day (NCI, 1979).

Groups of 10 male and 10 female B6C3F1 mice were maintained on diets containing *dl*-menthol at concentrations of 0, 930, 1870, 3750, 7500 or 15,000 ppm for 13 weeks (NCI, 1979). Dietary concentrations were calculated (FDA, 1993) to provide average daily intake levels of 0, 140, 281, 563, 1125 or 2250 mg *dl*-menthol/kg bw, respectively. Necropsies were performed on all animals at the end of the study. Histopathological examination was performed on tissues from the control animals, the 2250 mg/kg bw/day group, and selected tissues from the 1125 mg/kg bw/day group. Six mice (sex not specified) died during the study but these deaths could not be attributed to administration of the test substance. Significantly decreased body weights were observed for the high dose group in female mice. Final mean body weights of the male and female mice in the all other dose groups were not statistically different from the control group. A slight increase in the incidence of perivascular lymphoid hyperplasia and interstitial nephritis was reported in the female mice given the two highest dose levels. No adverse effects were reported for male or female mice administered 140, 281 or 563 mg *dl*-menthol/kg bw/day (NCI, 1979).

7.8.3. Long term studies of toxicity

Groups of 50 Fischer 344 rats of each sex were administered 0, 3750 or 7500 ppm *dl*-menthol in their feed daily for 103 weeks (NCI, 1979). Dietary concentrations were calculated (FDA, 1993) to provide corresponding average daily intake levels of approximately 0, 187 or 375 mg/kg bw, respectively. Standard NCI (predecessor to NTP) Bioassay protocols and procedures were followed.

The mean body weights of the male and female rats administered 187 or 375 mg/kg *dl*-menthol were slightly lower when compared to the controls. Survival of the high- and low-dose groups of male (controls, 31/50; low-dose, 33/50; high-dose, 34/50) and female (controls, 36/50; low-dose, 35/50; high-dose, 38/50) rats was similar to control animals. At the time of the study, the National Cancer Institute noted that chronic inflammation of the kidney observed in the dosed older males is commonly observed in aged male Fischer 344 rats. This effect was later considered to be due to chronic progressive nephropathy (CPN) which is observed in aged male Fischer 344 rats and is an effect that is often exacerbated by the administration of test substances (Hard, 1998; Hard et al., 2012, 2013; Lock and Hard, 2004; Travlos et al., 2011). CPN is considered not to have a counterpart in humans (Hard et al., 2009). There was no increase in the incidence of neoplasms of the kidney in treated females compared to that of control animals.

In the low-dose (10/49) and high-dose (7/49) female groups, fibroadenomas of the mammary glands occurred at a lower incidence than in the control group (20/50). Bonchiolar/alveolar adenomas or carcinomas were reported only for the female control rats (3/50). Under the conditions of this study, it was concluded that *dl*-menthol was neither carcinogenic nor toxic for either sex of Fischer 344 rats at dose levels of 187 or 375 mg dl-menthol/kg bw (NCI, 1979). This NOAEL value of 375 mg *dl*-menthol/kg bw was used to assess the MoS for Group 10 (Alicyclics ketones, secondary alcohols and related esters) constituents of Peppermint Oil (FEMA 2848), Cornmint Oil (FEMA 4219) and Curly Mint Oil (FEMA 4778) in Table 3 above.

A carcinogenicity study was conducted in which groups of 50 B6C3F1 mice of each sex were administered 0, 2000 or 4000 ppm *dl*-menthol in their feed daily for 103 weeks (NCI, 1979). Dietary concentrations were calculated (FDA, 1993) to provide corresponding average daily intake levels of 300 or 600 mg/kg bw, respectively. Standard NCI Bioassay protocols and procedures were followed.

The mean body weights of the male and female mice administered 300 or 600 mg/kg bw *dl*-menthol were slightly lower when compared to the controls. Survival of the high- and low-dose groups of male mice was similar to vehicle controls (controls, 32/50; low-dose, 32/50; high-dose, 35/50). Survival of the high-dose group of female mice was significantly less than that of the control animals (controls, 45/50; high-dose, 36/50). Decreased survival was not accompanied by correlated evidence of toxicity in the high-dose group. Survival of the low-dose female mice was similar to control animals (controls, 45/50; low-dose, 40/50). An increase in the incidence of hepatocellular carcinomas was observed in high-dose male mice (controls, 0/46; high-dose, 1/48), but was not statistically different from that observed historically in mice of that age and strain (Haseman et al., 1986). A low incidence of bronchiolar/alveolar adenomas of the lung was observed in both the low- and high-dose females but was not statistically different from the incidence of this neoplasm in historical control groups. Under the conditions of this study, it was concluded that *dl*-menthol was not carcinogenic and did not produce any organ-specific toxicity for either sex of $B6C3F_1$ mice at dose levels of 300 or 600 mg/kg bw (NCI, 1979).

7.8.4. Genotoxicity studies

Uniformly negative results for mutagenic activity of menthol were obtained in several studies using the standard Ames or preincubation protocol with *S. typhimurium* strains TA92, TA94, TA97, TA98, TA100, TA102, TA1535, TA1537, TA2537 and TA2637 with or without metabolic activation when tested at concentrations up to approximately 5000 μg menthol/plate (Andersen and Jensen, 1984; Gomes-Carneiro et al., 1998; Ishidate et al., 1984; Kirkland et al., 2016; Nohmi et al., 1985; Zeiger et al., 1988). Negative results for mutagenic activity were also reported for *dl*-isomenthol using the standard Ames and preincubation protocol with *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 with or without metabolic activation when tested at concentrations up to 1000 μg/plate (Flügge, 2010) Menthol also did not display mutagenic activity in the *Escherichia coli WP2 uvrA* mutation assay when tested at concentrations of 0.1–0.8 mg/plate (Yoo, 1986). In the rec assay using *Bacillus subtilis* strains H17 and M45, menthol was negative when tested up to 10 mg/disk (Oda et al., 1978; Yoo, 1986).

There was some evidence of potential genotoxicity for menthol in the alkaline elution assay. Primary rat hepatocytes were incubated with concentrations of 0.1–1.3 mM menthol for 3 h. Increased double strand breaks and cytotoxic effects were observed at concentrations of 0.7 mM and higher (Storer et al., 1996).

No indication of mutagenicity was evident in various cytogenetic assays in which menthol showed no evidence of increased sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells (Ivett et al., 1989), human lymphocytes (Murthy et al., 1991) or human embryonic lung cells without metabolic activation at concentrations of up to 1563 μg menthol/ml (Murthy et al., 1991). Furthermore, in chromosome aberration tests using Chinese hamster lung fibroblasts or ovary cells exposed at concentrations of up to 300 μg/ml, or in human lymphocytes exposed at concentrations of up to 10 mM, menthol also were negative (Ishidate et al., 1984; Ivett et al., 1989; Matsuoka et al., 1998; Murthy et al., 1991). In L5178Y mouse lymphoma cell mutation assays, menthol was negative at concentrations of 150–200 μg/ml, both in the presence and absence of metabolic activation (Myhr et al., 1991; Tennant et al., 1987). A micronucleus assay in mouse lymphocytes treated at concentrations up to 250 μg/mL *dl*-menthol in both the presence and absence of metabolic activation showed no increase in the frequency of binucleated cells with micronuclei (Olivo, 2016). No induction of DNA damage was observed in CHO cells treated with *dl*-menthol was at concentrations up to 100 μg/mL in a standard comet assay and up to 1000 μg/mL in a modified comet assay (Kiffe et al., 2003).

In a mouse micronucleus assay, *dl*-menthol was negative when groups of five male B6C3F1 mice were administered 0, 250, 500 or 1000 mg menthol (in corn oil)/kg body weight/day by intraperitoneal injection for three consecutive days (Shelby et al., 1993). In this study, bone marrow cells were harvested 24 h after the last dose of *dl*-menthol. No increase in the induction of micronuclei was observed up to the highest concentration.

Groups of 5 male Sprague-Dawley rats were administered 1.45, 14.5 or 145 mg menthol/kg body weight by oral gavage for one or five days. In a second test, groups of 10 male rats were administered either 500 or 3000 mg/kg body weight in a single dose or 1150 mg/kg body weight/day for 5 days. Additional groups (3 or 5/group) received the vehicle control by oral administration or a positive control (triethylene melamine) by intraperitoneal injection. Independent of concentration or duration of exposure, analysis of bone marrow smears showed no evidence of chromatid and chromosome gaps and breaks, other aberrations or an altered mitotic index (FDA, 1975).

In an alkaline Comet assay, *dl*-menthol was administered to male Sprague-Dawley [Crl: Cd(SD)] rats (5/group) at 0 (corn oil), 125, 200, 250, 500, 1000 or 2000 mg/kg bw per day for 3 days by oral gavage. Histopathological analysis of the liver and glandular stomach, performed for the control and highest dose groups, reported diffuse hepatocellular vacuolation and increased hepatocyte mitotic figures in the liver and ulcers and erosion in the pyloric mucosa of the glandular stomach in the 2000 mg/kg bw/day dose group. No significant difference in DNA damage between the treated and vehicle control groups in the liver and stomach tissues was detected in the comet assay (Uno et al., 2015). No DNA damage was reported in another alkaline Comet assay in liver and glandular stomach tissues from male Sprague-Dawley [Crl: Cd(SD)] rats (5/group) administered 0 (corn oil), 500, 1000 or 2000 mg *dl*-menthol/kg bw per day for 3 days by oral gavage (Uno et al., 2015).

Menthyl acetate was not mutagenic in an Ames assay when incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 at concentrations up to 5000 μg/plate in the presence and absence of Aroclor 1254 induced rat liver S9 metabolic activation (Bowles, 1999). Menthyl acetate did not increase the mutant frequency at the HPRT locus of V79 Chinese hamster cells, both in the presence and absence of metabolic activation in an *in vitro* mammalian cell gene mutation assay (Morris, 2013b). Menthyl acetate was also negative in an *in vitro* micronucleus assay in human peripheral blood lymphocytes in both the presence and absence of metabolic activation (Bohnenberger, 2013).

No mutagenic activity was observed when menthone was incubated with *S. typhimurium* strains TA98, TA100, or TA1535, at concentrations of up to 800 μg/plate, both with and without metabolic activation in a standard Ames assay (Andersen and Jensen, 1984). In *S. typhimurium* strain TA97, menthone was reported to exhibit mutagenic activity in the standard Ames test at concentrations of up to 160 μg/plate in the presence of S9 and at concentrations of up to 800 μg/plate in the absence of S9 (Andersen and Jensen, 1984). Menthone also was found positive in *S. typhimurium* strain TA1537 when tested at concentrations of 32 and 6.4 μg/plate in the absence of S9, but was negative at the two highest concentrations, 160 and 800 μg/plate (Andersen and Jensen, 1984). In contrast, no mutagenic activity was observed in two Ames assays conducted on menthone/isomenthone mixtures in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E.coli* WP2uvrA performed under the OECD guideline at concentrations up to 5000 μg/plate in both the presence and absence of S9 metabolic activation (Sokolowski, 2012a, b).

Menthone/isomenthone mixtures (84:16 and 76:24) were assessed for their ability to induce mutations at the HPRT locus using V79 Chinese hamster cells. In experiment 1 the cells were exposed to the test

material for 4 h with a 20 h recovery period in the presence and absence of metabolic activating system (S-9). In the second experiment, cells were continuously exposed to menthone/isomenthone for 24 h in the absence of S-9 and for 4 h with a 20 h recovery period in the presence of S-9. Menthone/isomenthone showed no statistically significant mutagenic ability (Morris, 2013a; Wollny, 2013).

There was no evidence of clastogenicity in femoral bone marrow harvested 24 h post dose when NMRI mice (6/sex/dose) were administered 500, 1000 or 2000 mg of menthone/kg bw via corn oil gavage. There was an additional group that received 2000 mg/kg bw of menthone in which the femoral bone marrow was harvested 48 h post dose. There were no significant differences in the numbers of polychromatic erythrocytes in any of the test groups when compared to the vehicle control group (Honarvar, 2009; Scognamiglio et al., 2010).

Although menthol gave a positive result in the alkaline elution rat hepatocyte assay at concentrations of 0.7 mM and higher, *in vivo* micronucleus assays in mice and rats were negative for genotoxicity. Other assays in a variety of cell systems all were negative, including the L5178Y mouse lymphoma test, *in vitro* micronucleus test in mouse lymphocytes and comet assays in CHO cells. For menthone, sporadic positive results in Ames assays were limited to specific (non-standard) strains and/or were not concentration dependent. When tested in OECD-compliant Ames assays in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA, menthone/isomenthone mixtures were negative up to 5000 μg/plate. They were also negative in the HPRT test in V79 Chinese hamster cells and did not induce chromosomal damage in NMRI mice up to 2000 mg/kg. Thus, the overall weight of evidence shows menthol and menthone to be devoid of genotoxic potential.

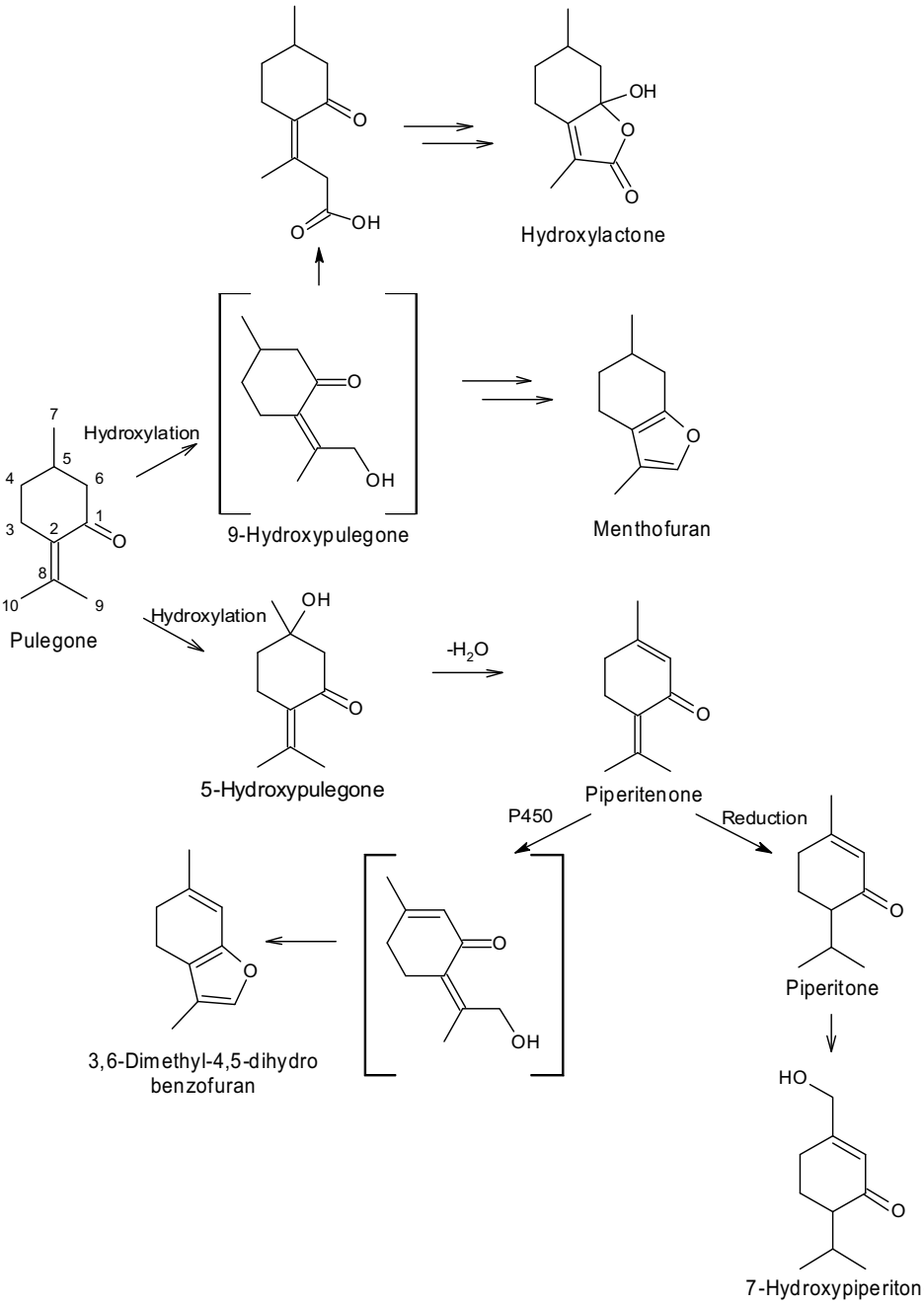


Fig. 7. Metabolism of R-(+)-pulegone and S-(−)-pulegone observed in rats (Madyastha and Gaikwad, 1998; Madyastha and Raj, 1993).

7.9. Pulegone and menthofuran

Pulegone, an intermediate in the biosynthesis of menthol, is produced in several members of the Lamiaceae family, and is a known major constituent of pennyroyal oil. The toxic properties associated with the ingestion of large quantities of pennyroyal oil are historically well documented (Anderson, 1996) and were the basis for further studies into the toxicology of pulegone. A summary of studies on the toxicology of pulegone relevant to the safety of the use of pulegone as a flavor ingredient was published in 1996 (Adams et al., 1996). Since that time, additional studies have become available, including subchronic and chronic toxicity assays of pulegone in F344N rats and B6C3F1 mice and Ames assays conducted by the NTP and a recent OECD-compliant study. In addition, new metabolic studies of pulegone in both rats and humans were published. The following discussion reviews these and other studies relevant to the safety of consumption of NFCs containing pulegone.

7.9.1. Absorption, distribution, metabolism, and excretion

Studies on the metabolism of S-(−)-pulegone (Madyastha and Gaikwad, 1998) and R-(+)-pulegone (Madyastha and Raj, 1993) in rats indicated 2 major metabolic pathways for these compounds, as summarized in Fig. 7. In the first pathway, pulegone is hydroxylated forming 9-hydroxypulegone which can then cyclize to yield menthofuran or undergo further oxidation and cyclization to form the hydroxylactone 3,6-dimethyl-7a-hydroxy-5,6,7,7a-tetrahydro-2(4H)-benzofuranone, which was detected in an earlier study (Moorthy et al., 1989b). In the second major pathway, pulegone is hydroxylated forming 5-hydroxypulegone which may be further metabolized to form piperitenone, piperitone, 7-hydroxypiperitone and 3,6-dimethyl-4,5-

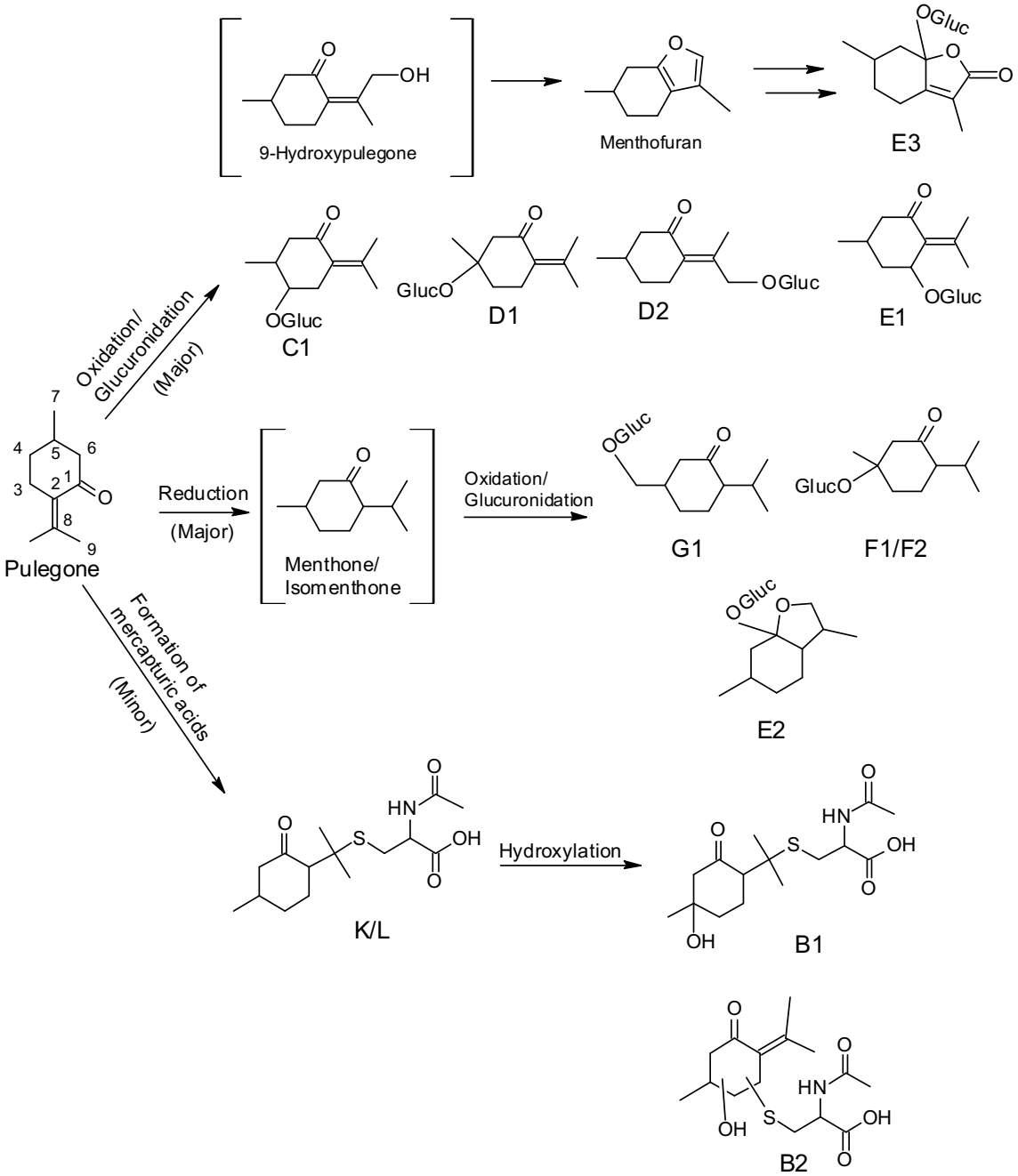


Fig. 8. Metabolic scheme for R-(+)-pulegone in rats based on the determination of conjugated metabolites.

dihydrobenzofuran metabolites. Piperitenone was the major metabolite observed in an 18 h urine collection at the end of a 6-week study in rats administered pulegone at 75 or 150 mg kg bw/day. Piperitone, pulegone and menthofuran were also detected in both treatment groups (Da Rocha et al., 2012). The reduction of R-(+)-pulegone to pulegol has also been observed in rats (Moorthy et al., 1989b). While these major metabolites were observed for both enantiomers of pulegone, the relative amounts differed. When S-(−)-pulegone was administered, higher amounts of pulegone, piperitone and benzoic acid and lower amounts of menthofuran were present in the urine, compared to the urine of R-(+)-pulegone treated rats (Madyastha and Gaikwad, 1998). The formation of a γ-ketoenal intermediate from the oxidiation of menthofuran that binds to cellular proteins has been proposed to be related to the observed heptatotoxicity of pulegone (Madyastha and Raj, 1990; McClanahan et al., 1989; Nelson et al., 1992; Thomassen et al., 1992). The higher levels of menthofuran observed in the administration of R-versus S- pulegone was proposed to be correlated with the higher hepatotoxicity of the R-entantiomer observed in mice (Gordon et al., 1982). In nature, the R-entantiomer of pulegone predominates the S-entantiomer.

The hydroxylated and reduced metabolites of pulegone are expected to undergo further detoxification reactions, including conjugation with glucuronic and/or glutathione prior to excretion. In the rat, the concentration of glutathione measured in the liver and plasma was reduced with the administration of pulegone, indicating that pulegone is detoxified by a pathway requiring reduced glutathione (Thomassen et al., 1990). Using tandem mass spectrometry, glucuronide and glutathione conjugates of hydroxypulegone and hydroxylated reduced pulegone were detected in the bile of male rats upon administration of deuterated and C-14 labeled pulegone at a dose of 250 mg/kg bw (Thomassen et al., 1991). In a later study in male and female Fischer F344 rats, fourteen urinary metabolites were identified following the administration of a single or multiple 80 mg/kg bw doses of pulegone by gavage. Urinary metabolites, collected at 0–4 h, 4–8 h and 8–12 h following dosing, were isolated by HPLC and analyzed by NMR. Most of the identified metabolites were glucuronic acid and glutathione conjugates. Based on their analyses, three principal metabolic pathways for the metabolism of R-(+) pulegone were outlined, as shown in Fig. 8. In the first pathway, pulegone undergoes direct hydroxylation catalyzed by P450 enzymes to yield a series of ring- and side chain-hydroxylated pulegone metabolites that are either conjugated with glucuronic acid (C1, D1, D2, E1) or further metabolized, conjugated and excreted. In a second major pathway, pulegone undergoes reduction to yield menthone or isomenthone, followed by hydroxylation of ring or side chain positions and then conjugation with glucuronic acid, yielding metabolites F1, F2, G1, and E2. In a third pathway, pulegone undergoes conjugation with glutathione in a Michael-type addition leading to mercapturic acid conjugates (K and L) that are excreted or further hydroxylated and excreted (B1 and B2). Other identified metabolites include an alkyl-substituted phenol and phenylacetic acid derivatives formed by aromatization of the alicyclic ring system (Chen et al., 2001). In a separate analysis, urine collected for 24 h following a single 80 mg/kg bw dose of pulegone was treated with glucuronidase prior to analysis. It was noted that pulegone, menthofuran, 2-(N-acetylcystein-S-yl)menthofuran, piperitone and pulegol were not detected in either hydrolyzed or untreated urine samples. Differences in urine collection practices may explain the observation of pulegone, menthofuran and piperitone metabolites in the urine of rats treated for 6 weeks with 75 or 150 mg/kg bw/day pulegone (Da Rocha et al., 2012). Finally, the glucuronic acid conjugate of 9-hydroxypulegone was not observed but 9-hydroxypulegone was proposed to be an intermediate in the formation of 7a-hydroxy-3,6-dimethyl-5,6,7,7a-tetrahydro-2(4*H*)-benzofuran glucuronide (E3).

A study was conducted with human exposure to pulegone to compare the metabolites between human and animal studies. The goal was to confirm the metabolites previously observed in rats and identify any unique to humans. Groups of 3 male and 3 female healthy human volunteers ingested specific diet entirely free from spices to control for the presence of pulegone at least 24 h in advance of test substance exposure. Groups received single doses of 35 mg of (*R*)-(+)-pulegone or 70 mg of (*S*)-(−)-pulegone in 500 ml of cow's milk at the noontime meal. These dietary levels correspond to estimated exposures of 0.5 mg/kg bw and 1 mg/kg bw of pulegone. Urine samples were collected prior to pulegone exposure at the end of the 24 h adjustment period through the next 48 h for total urine collection of 72 h. The samples were treated with glucuronicidase and sulfatase to convert conjugated metabolites to their respective aglycones prior to GC-MS and high-resolution MS analyses. The major metabolites isolated from human urine were 8,9-dihydromenthofuran, 1-hydroxymenthan-3-one, menthone and 10-hydroxypulegone. Levels of 8,9-dihydromenthofuran formed from 9-hydroxypulegone were generally higher in urine collected from subjects who had ingested (*S*)-(−)-pulegone while 10-hydroxypulegone was more abundant in R-enantiomer samples. The author notes that menthofuran was not detected but found that 10-hydroxypulegone cyclizes to form menthofuran in aqueous solution at room temperature and any pH within hours (Engel, 2003).

7.9.2. Evidence that menthofuran is a metabolite of pulegone

Initial studies investigating the mechanism of hepatotoxicity of pulegone indicated the formation of a toxic metabolite catalyzed by the P450 enzyme class (Gordon et al., 1987) as evidenced by the inhibition or potentiation of hepatoxicity by known inhibitors and activators of this class (Mizutani et al., 1987; Moorthy et al., 1989a). Menthofuran was identified as the major metabolite produced when (R)-(+)-pulegone was incubated with mouse liver microsomes in the presence of an NADPH-generating system (Gordon et al., 1987) and was postulated to form from the cyclization of 9-hydroxypulegone, an intermediate metabolite. An analysis of the pharmacokinetics of pulegone and menthofuran (matched area under the curve and matched time course) after intraperitoneal administration to mice showed that a significant amount of the hepatotoxicity of pulegone could be accounted for by the formation of menthofuran (Thomassen et al., 1988). In a later experiment, a metabolite of (R)-(+)-pulegone, 2-Z-(2′-keto-4′-methylcyclohexylidene) propanal was trapped as a semicarbazide derivative in mice administered a hepatotoxic dose (280 mg/kg i.p.) of (R)-(+)-pulegone and in mice and rats administered menthofuran at dose of 125 or

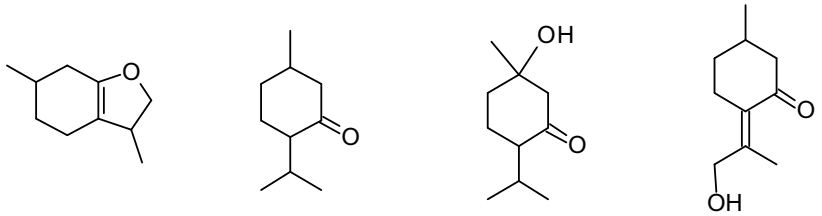


Fig. 9. Metabolites identified from the urine of human volunteers following the ingestion of 0.5 or 1 mg/kg bw pulegone (Engel, 2003).

200 mg/kg. Further experiments indicate that this γ-ketoenal is a reactive intermediate involved in the formation of liver protein adducts observed upon the incubation of (R)-(+)-pulegone and menthofuran with rat and mouse liver microsomal proteins and an intermediate in the formation of mint lactone from menthofuran and shown in the shaded box in Fig. 10. (McClanahan et al., 1989; Thomassen et al., 1992). In a later 2D Western-LC/MS/MS analysis of liver extracts from Sprague-Dawley rats administered 200 mg/kg menthofuran by intraperitoneal injection, four rat liver proteins were identified, serum albumin, mitochondrial aldehyde dehydrogenase, cyctoplasmic malate dehydrogenase and mitochondrial ATP synthase that reacted with an antiserum developed to detect menthofuran – protein adducts (Khojasteh et al., 2012).

In a metabolic study in male and female Fischer F344 rats, conjugated urinary metabolites were identified following the administration of 60 mg/kg, 40 μCi/kg dose of [2–^{14}C] menthofuran by gavage. Urinary metabolites, collected at 0–4 h, 4–8 h and 8–12 h following dosing, were isolated by HPLC and analyzed by NMR. Thirteen metabolites were isolated and identified, including four metabolites, G1, E3, J and G2, that were also isolated and identified in an earlier study on the metabolism of pulegone. The structure of metabolite G1 (see Fig. 10) was reassessed and determined not to be identical with that shown in Fig. 9 but to more likely be one of the octahydro-3,6-dimethyl-7a-hydroxybenzofuran glucuronides. The metabolic scheme proposed by the authors (Fig. 10) is consistent with the pathway proposed by Thomassen and coworkers, in which the γ-ketoenal 2-Z-(2′-keto-4′-methylcyclohexylidene) propanal and mint lactones are intermediates in the formation of 12 of the 13 identified metabolites. In addition, three sulfonic acid metabolites, consistent with the reaction of glutathione and taurine with the proposed γ-ketoenal were identified. Four of the identified metabolites, labeled E3, J, G1 and G2 were also identified as metabolites of (R)-(+)-pulegone in rats providing additional evidence that menthofuran is an intermediate in pulegone metabolism.

7.9.3. Short-term studies of toxicity

Pulegone was administered orally by gavage to groups of 28 female Wistar SPF rats at dosage levels of 0 or 160 mg/kg bw/day for 28 days by oral gavage in a study designed to understand the cyst-like spaces observed in the studies of peppermint oil described above. The clinically treated animals showed slackness, depression, significantly decreased food consumption and body weight ($p < 0.001$). Blood chemical examinations performed on day 27 or 28 of dosing revealed increased plasma glucose, increased alkaline phosphatase, a non-statistically significant ($p < 0.1$) increase in alanine aminotransferase and decreased plasma creatinine in the treated group. Non-statistically significant increased absolute liver weight ($p > 0.1$) and relative liver weight ($p > 0.05$) were also observed, but there were no significant histopathological findings in the liver (Mølck et al., 1998). Cyst-like spaces were not observed.

In a 14-week gavage study (NTP, 2011), a Core Group (10/dose/sex) of male and female F344N Fischer rats were administered 0, 9.375, 18.75, 37.5, 75 or 150 mg/kg bw of pulegone daily, excluding weekends and holidays, by gavage for approximately 14 weeks. Special Study Groups (10/dose/sex) were given three doses of pulegone daily for 3 days and sacrificed on the fourth day or 13 doses over 17 days and sacrificed on Day 18. Body weights and clinical observations were made weekly for the Core Group and on Day 1 and at termination (Days 4, 18

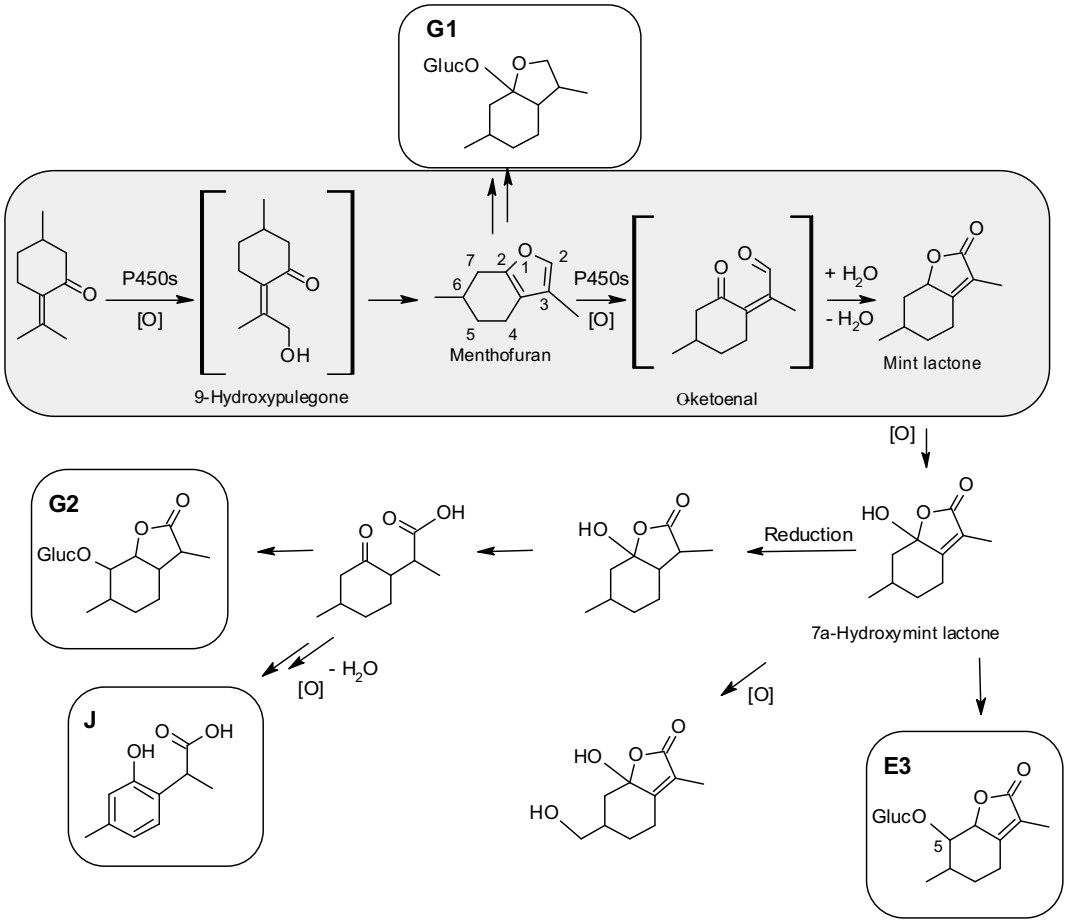


Fig. 10. Proposed pathway for the formation of menthofuran and mint lactone (shaded box) and menthofuran metabolism in rats. Metabolites (boxed) G1, G2, J and E3 were also observed in an analogous study on the metabolism of pulegone in rats.

and 94). At termination of the three study groups, blood was sampled for clinical chemistry determinations and liver samples were prepared for measurement of reduced and oxidized glutathione concentrations. Sperm morphology and vaginal cytology were performed on the male and female groups, respectively, treated with 0, 18.75, 37.5, 75 or 150 mg/kg bw per day. At necropsy, organ weights were measured and histopathological examination was performed on a wide variety of tissues from the Core Group.

One mortality (female) was recorded in the 150 mg/kg bw group prior to the end of the study. Decreases in body weight were reported in the Special Study Group at day 18, in the males at 75 or 150 mg/kg bw per day at Day 94 and in females at 150 mg/kg bw per day at Day 94. Clinical chemistry changes included increased alkaline phosphatase, bile acids/salts, and decreased red blood cell counts at 37.5 mg/kg bw per day in males at Day 94. Increased bile acids/salts and decreased red blood cells and hemoglobin levels were reported at 37.5 mg/kg bw per day in females. At higher dose levels in both males and females, more significant changes were observed in these parameters. Also, other statistically significant changes were reported in hematological and serum liver enzyme measurements. Increased serum levels of reduced glutathione for females and increased levels of oxidized glutathione for males were observed in a dose-dependent manner after Day 18 and Day 94 when compared to control levels.

Measurement of organ weights on Day 94 indicated statistically significant increases in relative liver weight, thymus weight and kidney weight at the 75 and 150 mg/kg bw per day level in males. Relative kidney weight was also increased in the 18.75 and the 37.5 mg/kg bw per day groups. In females, statistically significant increases in absolute and relative liver weights, absolute and relative thymus weights, and absolute and relative kidney weights were reported at the 75 and 150 mg/kg bw per day level. Absolute and relative kidney weights were also increased in the 18.75 and the 37.5 mg/kg bw per day groups of females. Histopathological examination revealed liver and kidney alterations at 75 mg/kg bw per day dose levels. These included hepatocyte hypertrophy and bile duct hyperplasia and nephropathy in males. At 150 mg/kg bw per day, males also showed evidence of chronic hepatic inflammation, hepatocellular necrosis, oval cell hyperplasi and hepatic periportal fibrosis. Females exhibited a similar liver histopathology at the 150 mg/kg bw dose levels. Females in control and treatment groups showed an increasing incidence of mineralization of the glandular stomach and both males and females showed a significant increase in the incidence of bone marrow hyperplasia at 75 and 150 mg/kg bw per day dose levels. No-observed-adverse effects were observed in either males or females at the dose level of 9.375 mg/kg bw per day reported in the study (NTP, 2011). This NOAEL value was used to assess the margin of safety for Group 11 (Pulegone and structurally and metabolically related substances) constituents of Peppermint oil (FEMA 2848) in Table 3 above.

In a report on a 90-day gavage study (NTP, 2011), a Core Group (10/dose/sex) of male and female B6C3F1 mice were administered at 0, 9.375, 18.75, 37.5, 75 or 150 mg/kg bw of pulegone daily, excluding weekends and holidays, by gavage for approximately 14 weeks. A Special Study Group (10/dose/sex) was given a single dose of pulegone daily for 3 days and sacrificed on the fourth day. Body weights and clinical observations were made weekly for the Core Group and on Day 1 and at termination (Day 4 and 95). At termination of both study groups, blood was taken for clinical chemistry determinations and liver samples were prepared for measurement of reduced and oxidized glutathione concentrations. Sperm morphology and vaginal cytology were performed on groups treated with 0, 37.5, 75 or 150 mg/kg bw per day. At necropsy, organ weights were measured and histopathological examinations were performed on a wide variety of tissues for the Core Group. The only significant effects reported included a significant increase in absolute and relative liver weights at 150 mg/kg bw dose level in both sexes. After 95 days, reduced and oxidized glutathione concentrations were increased in the 37.5, 75 and 150 mg/kg bw per day groups of females and in the 75 and 150 mg/kg bw per day groups of males. Oxidized glutathione was significantly increased in the 9.375 and 18.75 mg/kg bw per day groups of females and the 37.5 mg/kg bw per day group of males. Based on the significant increase in the absolute and relative liver weight changes the NOAEL level after 95 days was 75 mg/kg bw per day.

7.9.4. Long term studies of toxicity

In a carcinogenicity bioassay, F344/N rats (50/sex/dose) were administered 0, 18.75 (males only), 37.5, 75 or 150 (females only) mg pulegone/kg bw via corn oil gavage, 5 days per week for up to 104 weeks (NTP, 2011). Due to excessive morbidity and mortality, 75 mg/kg males and 150 mg/kg females were not administered pulegone after week 60 (stop-exposure); these groups were administered the corn oil vehicle until the end of the study. Survival of 37.5 mg/kg males was significantly less than that of the vehicle controls; only two 75 mg/kg stop-exposure males survived and no 150 mg/kg stop-exposure females survived to the end of the study. Compared to those of the vehicle controls, mean body weights were less in 75 mg/kg stop-exposure males after week 13 and in 75 mg/kg and 150 mg/kg stop-exposure females after weeks 21 and 9, respectively. Clinical findings included thinness, lethargy, and ruffled fur in the 75 mg/kg stop-exposure males and 150 mg/kg stop-exposure females. Based on the severe toxicity at 75 mg/kg in males and 150 mg/kg in females, it is likely that these doses exceeded the maximum tolerated dose (MTD) and should not be considered in the overall cancer risk assessment (Foran, 1997; OECD, 2009).

The incidences of urinary bladder papilloma and of papilloma or carcinoma (combined) were significantly increased in 150 mg/kg stop-exposure females but not in males. Given the high morbidity and mortality of the group of female rats, the MTD was clearly exceeded and thus, those tumors should not be considered in an overall risk assessment. In the kidney, incidences of hyaline glomerulopathy were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and all dosed groups of females. The severity of chronic progressive nephropathy (nephropathy) was increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and 75 mg/kg and 150 mg/kg stop-exposure females. The incidence of renal cyst was significantly increased in 75 mg/kg stop-exposure males. In the liver, incidences of diffuse hepatocyte cellular alteration were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and 75 mg/kg and 150 mg/kg stop-exposure females. There were significant increases in the incidences of other liver lesions indicative of hepatocellular cytotoxicity including fatty change, bile duct cyst, hepatocyte necrosis, oval cell hyperplasia, bile duct hyperplasia and portal fibrosis. In the nose, 37.5 mg/kg and 75 mg/kg stop-exposure males and all dosed groups of females had significantly increased incidences of olfactory epithelium degeneration. All dosed groups of females had significantly increased incidences of respiratory metaplasia of the olfactory epithelium and nasal inflammation. These nasal and olfactory changes were likely due to the highly irritating effects of the volatile pulegone. In male rats, incidences of inflammation and ulcer of the forestomach were significantly increased in the 37.5 mg/kg and 75 mg/kg stop-exposure groups, and incidences of forestomach squamous epithelial hyperplasia and proliferation were increased in 75 mg/kg stop-exposure males. This effect is commonly observed in rats administered irritating test materials by corn oil gavage. In the glandular stomach, the incidence of inflammation was significantly increased in 75 mg/kg stop-exposure males. In all dosed groups of females, the incidences of pituitary gland pars distalis adenoma were significantly less than that in the vehicle controls. The incidence of mineralization, a common occurrence in this strain of rats (Frazier et al., 2012; Lord and Newberne, 1990), was significantly increased in 150 mg/kg females, and the incidence of nephropathy in 150 mg/kg females and severity of nephropathy in 150 mg/kg males were increased. Incidences of congestion of the glomerulus were increased in 150 mg/kg males and females. The incidence of osteoma or

osteosarcoma (combined) in all organs of 75 mg/kg females exceeded the historical control ranges. One 150 mg/kg male and one 75 mg/kg female had nasal osteoma; no nasal osteomas have been observed in historical control rats. The incidences of olfactory epithelial degeneration of the nose were significantly increased in all dosed groups of females and in 75 and 150 mg/kg males. Incidences of inflammation, nerve atrophy and olfactory epithelial metaplasia of the nose were significantly greater in 150 mg/kg males and females than in the vehicle control groups and are most likely related to the toxic effects of the volatilized pulegone, which is highly cytotoxic to epithelia. As described later in a discussion on the observed renal effects, the inflammation combined with the secondary hyperparathyroidism due to the renal effects in these rats is likely the mechanism by which these bone tumors occurred. However, the renal effects are not considered relevant to humans and therefore the bone tumors are considered not to pose a cancer risk to humans. It is also noted that the human exposure to pulegone is considerably less than the dose needed to produce nasal inflammation. In the forestomach, incidences of squamous hyperplasia and inflammation were significantly increased in 75 mg/kg males and 150 mg/kg males and females, and the incidences of ulcer were significantly increased in 75 and 150 mg/kg males. Inflammation and ulceration are common observances in rodents administered irritating materials via gavage (Adams et al., 2008; Haseman et al., 1984; NTP, 2011). Furthermore, forestomach changes are considered not relevant to humans (Adams et al., 2008; Proctor et al., 2007).

The National Toxicology Program concluded: *Under the conditions of these 2-year gavage studies, there was no evidence of carcinogenic activity of pulegone in male F344/N rats administered 18.75, 37.5 or 75 (stop-exposure) mg/kg. There was clear evidence of carcinogenic activity of pulegone in female F344/N rats based on increased incidences of urinary bladder neoplasms. A unique renal lesion, hyaline glomerulopathy, was observed in all dosed groups of female rats and in 37.*5 mg/kg *and* 75 mg/kg *stop-exposure male rats. In rats, renal failure secondary to hyaline glomerulopathy and chronic progressive nephropathy contributed to the decreased survival in the* 75 mg/kg *stop-exposure males and* 150 mg/kg *stop-exposure females.*

In a carcinogenicity bioassay, groups of B6C3F$_1$ mice (50/sex/dose) were administered 0, 37.5, 75 or 150 mg pulegone/kg bw in corn oil by gavage, 5 days per week for 104 weeks (NTP, 2011). Survival of all dosed groups was similar to vehicle controls. Mean body weights of 150 mg/kg males and females were less than those of the vehicle controls after weeks 25 and 33, respectively. The incidences of hepatocellular adenomas were 22/50, 31/50, 35/50 and 28/50 in the males and 13/49, 15/50, 13/50 and 27/50 in the females at the 4 doses, respectively. Incidences of hepatoblastoma were 1/50, 3/50, 7/50, and 2/50 in males, but only 1 occurred in females at the high dose. Non-neoplastic changes in the liver were frequently observed in treated mice including foci, focal fatty change, centrilobular hepatocyte hypertrophy, hepatocellular necrosis, pigmentation, bile duct cysts and hyperplasia and oval cell hyperplasia. The statistical significance was $p = 0.058$, 0.008, and 0.150 for pair wise comparisons based on the Poly-3 analysis performed by the NTP for the 37.5 mg/kg, 75 mg/kg and 150 mg/kg doses, respectively, in the males. Using a Fisher exact test for pair wise comparisons, the p values were 0.11, 0.015 and 0.32, respectively. The trend test gave a p value of 0.175 for adenomas. For combined hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma, the trend test gave a p value of 0.038, with pair wise comparisons having p values of 0.064, 0.004 and 0.051, respectively. In females, the p value for the trend test for adenomas was < 0.001, and pair wise comparisons were $p = 0.455$, 0.590 and 0.002, at the 3 doses, respectively. The vehicle control rates were within the historical rates of occurrence for hepatocellular adenoma, hepatocellular carcinoma and hepatoblastoma, either alone, or combined.

In the bone, a benign osteoma was seen in one 75 mg/kg female and an osteosarcoma was seen in one 75 mg/kg female and one 150 mg/kg female. When all organs are combined, the incidences of osteoma and osteosarcoma (combined) in 75 mg/kg females exceeded the historical control ranges for corn oil gavage studies and all study routes (NTP, 2011). However, the incidence of osteosarcoma in female B6C3F$_1$ mice has been reported to be 0–4% based on data from the NTP historical control database and thus the incidences of occurrences reported in the pulegone study were within this historical range and are not considered relevant to human cancer risk (Haseman et al., 1998). The issue of nasal inflammation might also have contributed to these lesions as in the rat, and the presence of hyaline glomerulopathy and renal amyloid may have produced renal consequences affecting parathyroid function and its consequences on bone.

The NTP concluded that: “There was a clear evidence of carcinogenic activity of pulegone in male and female B6C3F$_1$ mice based on increased incidences of liver neoplasms.” However, the occurrence of mouse liver tumors during 2-year studies of various substances (e.g., chloroform) have been determined to be the indirect result of high-dose related chronic toxicity leading to cellular regenerative proliferation. These tumors are considered to be secondary to the toxicity, so it is considered that pulegone is not likely to be carcinogenic to the liver at doses that do not produce hepatotoxicity (Adams et al., 2011; Cohen et al., 2004; Haseman, 1986; Haseman et al., 1985). This interpretation has been applied by the US EPA for several substances such as chloroform (Andersen et al., 2000; EPA, 2001). Furthermore, the incidences in male mice did not show a dose response and were not statistically significant except at the mid-dose. In fact, at the low and high dose, the p value was > 0.05 and the trend test was > 0.05. In females, the incidence of liver tumors was only elevated in the high dose group and was statistically significant at $p < 0.01$ (FDA, 2001; Haseman, 1983; OECD, 2014).

Liver tumors are exceedingly common in B6C3F1 mice, a strain which has been widely used in the NTP for carcinogenicity testing. The historical range for B6C3F1 male mice at NTP for adenomas is 44–54% for gavage studies and 24–72% by all routes. For hepatocellular carcinoma, the range is 16–40% by gavage and 16–52% for all routes. For hepatoblastoma the range is 0.8% by gavage and 0–34% by all routes. In females, the ranges are 6–27% by gavage and 2–62% by all routes for adenomas, 2–10% by gavage and 0–28% for all routes for hepatocellular carcinomas, and 0–2% for hepatoblastomas. This wide range of control values for these tumors led Dr. Joseph Haseman (1983) at the NTP to consider statistical significance for common tumors (defined as tumors with a spontaneous incidence of $> 1\%$) at $p < 0.01$ rather than 0.05 for pair wise comparisons, to avoid over interpretation of results. This statistical consideration is widely used in the evaluation of pharmaceuticals as outlined in the ICH guidelines as used by the US FDA (FDA, 2001), and it is also recommended in OECD guidelines (OECD, 2014).

The overall interpretation of the liver tumors in mice is difficult, not only because of the wide range of historical controls, but the lack of a dose response in the males and the presence of an increased incidence of adenomas but not carcinomas or hepatoblastomas in the females only at the high dose. In the males, the only group that had statistical significance at $p < 0.01$ was the mid-dose groups, but only with the Poly-3 analysis, not the Fisher exact test. The other groups of males were actually $p > 0.05$ as was the trend test. In females, the only group that was significant at $p < 0.01$ was the high dose group. In addition, in the males, the first tumor was observed earlier in the controls and low dose group (479 and 428 days, respectively) compared to the mid and high dose groups (654 and 638 days, respectively). The inconsistent tumor incidences, the delay in first tumor, and the widespread presence of toxic manifestations in the liver support the conclusion that an evaluation of the non-cancer endpoints for the pulegone assay in mice provides adequate protection with respect to cancer risk.

7.9.5. Interpretation of renal pathology results from the 2-year studies of pulegone in male and female rats and mice, and implications for bone pathology

In the 2-year studies of pulegone, the manifestation of hyaline glomerulopathy exhibited a clear dose-response relationship, affecting many dosed male and female rats and mice. The incidence was high in male rats at 75 mg/kg and 150 mg/kg but was also significant in female rats at all doses. Male and female rats were affected with a dose response increase. Hyaline glomerulopathy was irreversible as evidenced by the continued accelerated rate of death in the stop-exposure rats after pulegone administration was halted. In the 3-month rat study, hyaline glomerulopathy consisted of numerous, small, round, eosinophilic globules apparently confined to the glomerular mesangium. In the 2-year mouse and rat studies, hyaline glomerulopathy was characterized by accumulations of an amorphous eosinophilic material within the glomerulus. This lesion has not previously been described in NTP studies and had not been reported in the rat. It was first documented in B6C3F1 mice (Wojcinski et al., 1991) but has been reported in several strains of mice subsequently (Hoane et al., 2016). Its manifestation had not been associated with administration of a specific chemical prior to the study with pulegone, but it has recently been reported in mice in association with subcutaneous administration of antisense oligonucleotides (Frazier et al., 2014).

Rats, particularly male rats, develop spontaneous chronic progressive nephropathy (CPN) that is thought initially to involve progressive damage to the glomerulus, but also tubular toxicity (Hard and Khan, 2004). CPN is considered not to have a counterpart in humans (Hard et al., 2009). The combination of the naturally occurring decline in kidney function due to CPN and damage produced by the pulegone-induced glomerulopathy in rats likely led to a more rapid decline in renal function that precipitated the early deaths observed in the 2-year study. In mice, amyloidosis involving the kidney glomerulus occurs commonly in many strains (Frazier et al., 2012). However, hyaline glomerulopathy is a distinct lesion from chronic progressive nephropathy in rats or amyloidosis in mice (Hoane et al., 2016). Of critical importance in evaluating risk to humans concerning these renal lesions is that this type of hyaline glomerulopathy appears to be a rodent specific lesion with no counterpart in humans. This has been most extensively evaluated in patients treated with antisense oligonucleotides (Frazier et al., 2014), without any evidence that hyaline glomerulopathy observed in mice and the glomerular changes observed in monkeys or other renal toxicity occurs in these patients (Chi et al., 2017; Crooke et al., 2017; Engelhardt, 2016).

As renal disease progresses, hyperphosphatemia develops due to decreased glomerular filtration rates. The increased phosphate levels in the blood coupled with a decreased calcitriol production by the diseased kidney leads to decreased levels of ionized calcium in the blood. The physiologic response to decreased blood calcium is parathyroid gland hyperplasia (Drüeke, 2000) and increased parathyroid hormone (PTH) secretion, which in turn causes increased bone reabsorption and increased calcium absorption from the intestines, which can lead to hypercalcemia. Increased bone reabsorption can also lead to fibrous osteodystrophy (Hruska and Teitelbaum, 1995). As renal failure advances, hypercalcemia can develop, leading to soft tissue mineralization. Furthermore, hypercalcemia and PTH-induced increases in vitamin D3 can have proliferative effects on the adrenal medulla (Rosol et al., 2001). For these reasons, the increased incidences of fibrous osteodystrophy, parathyroid gland hyperplasia, soft tissue mineralization (heart, glandular stomach, blood vessel, and lung) and associated inflammation, and adrenal medulla hyperplasia observed in rats in the 2-year study were considered secondary to the renal failure. Hyperparathyroidism and chronic hypercalcemia have been associated with increased thyroid C-cell hyperplasia and C-cell adenomas (Tomita and Millard, 1992). In the studies described here, a reduced incidence of C-cell hyperplasia was observed in female rats. The mechanisms of this observation are unknown; however, it is likely that the changes in C-cell hyperplasia are related to the renal failure and the related perturbations in calcium homeostasis. These kidney disease effects in rats and mice have no direct correlation to human health.

The classic profile of results includes poor survival, mean body weight changes, chronic nephropathy, and associated renal toxicity that are specific to the rat. Analysis conducted by NTP experts (Haseman et al., 1998) have shown the survival rates of feeding studies in 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; Haseman et al., 2003). This species-specific phenomenon probably reflects the sensitivity of the male rat kidney to chronic progressive nephropathy, focal tubular and lining of the renal papilla 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; Hard et al., 2012, 2013; Lock and Hard, 2004; Travlos et al., 2011).

In the pulegone 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 75 for control males to week 65 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 lining epithelium of the renal papilla and increased renal tubule adenoma in both single section evaluation and step section evaluation.

7.9.6. Short term study investigating bladder pathology observed in female rats in the 2-yr NTP pulegone study

In a short-term oral toxicity study performed to investigate bladder pathology reported for female rats in a long-term bioassay of pulegone (NTP, 2011), Da Rocha et al. (2012) dosed 20 female F344/N rats per study group at 0 (vehicle control), 75 and 150 mg/kg bw/day 5 days per week for 4 or 6 weeks by corn oil gavage. Ten rats from each study group were sacrificed at 4 weeks and the remaining 10 rats in each group at 6 weeks. It was determined that examination of the 4-week tissue samples provided sufficient evidence of effects that they would be used, although the 6-week samples would be processed and preserved in an identical manner. One hour prior to sacrifice all rats were injected i.p with bromodeoxyuridine (BrdU). At necropsy, the urinary bladders were inflated *in situ* with Bouin's fixative and removed with a small part of the duodenum as a positive control for BrdU labeling. Post fixation, the bladders were longitudinally bisected and one half was processed for scanning electron microscopy (SEM). The other half was embedded in paraffin with a portion reserved for immunochemistry analysis with anti-BrdU and the remainder stained for traditional histopathology examination. The livers and kidneys were also weighed and preserved for histopathology analysis.

All test-group rats survived to the termination of the study but one control rat died as the result of a gavage error. It was also noted that on day 4, random animals throughout test and control groups showed bloody nasal discharge and that the facility had a minty aroma. Measures were introduced to minimize the volatilization of pulegone which has a very high vapor pressure of 139 mm Hg. The tops of the cages were fitted with filter tops and gavage preparations were sealed. This resolved the issue and the general condition of the rats improved. It was noted that rats showed alopecia around the mouth and yellow staining of the urogenital area in a dose-related manner. The mean body

weights for the high-dose group were significantly reduced while food consumption remained comparable to both the low-dose and control groups. The low-dose group showed body weights and food consumption comparable to controls. Water consumption was increased for low- and high-dose groups compared to controls. Terminal body weights at week 4 for the high-dose group were reduced and reached significance at week 6. Both doses showed a significant increase in kidney weights. Absolute and relative liver weights were significantly increased for the high-dose group. The livers also showed mild to moderate single cell necrosis in the high-dose group.

There was no evidence of increased or abnormal crystals in the urine. There was an increase in the volume of urine that was accompanied by a decrease in the creatinine concentrations for the high-dose group. The urine showed the presence of four major metabolites of pulegone at 75 and 150 mg/kg bw, respectively: pulegone, piperitone, piperitenone and menthofuran. Control animals showed none of these metabolites in the urine. The concentrations of metabolites are at or above cytotoxic levels as determined *in vitro* in human (1T1 cells) and rat (MYP3 cells) urothelial cell lines (Da Rocha et al., 2012).

At 4 weeks, the bladders showed no histopathology changes when examined by light microscopy. The kidneys showed no histopathological changes. The bladder slices from the high-dose group showed significant increases in BrdU labeling index of the urothelium, with only a slight increase for the low-dose group, indicative of a proliferative response. SEM analysis of the urinary bladder revealed dose-related evidence of superficial urothelial necrosis and a dose related increase in the surface SEM classification (Cohen et al., 1990). The incidence of bladder hyperplasia was most likely due to regeneration in response to the cytotoxicity.

The proposed mode of action/pathway for pulegone pathology in the urinary bladder of female rats is (1) chronic exposure to high concentrations of pulegone; (2) metabolism, excretion, and concentration of pulegone and cytotoxic metabolites, especially piperitenone, in the urine; (3) urothelial cytotoxicity; (4) sustained regenerative urothelial cell proliferation; and (5) development of urothelial tumors (Da Rocha et al., 2012).

7.9.7. Genotoxicity

No evidence of mutagenicity was observed when concentrations of pulegone in the range from 100 to 10,000 μg/plate (NTP, 2011) was incubated with *S. typhimurium* strains TA100 or TA1535 without metabolic activation or with metabolic activation by Aroclor 1254-induced Syrian hamster liver preparations or Sprague-Dawley rat liver preparations (NTP, 2011). In a second phase of the experiment, at concentrations in the range from 3.3 to 2167 or 3333 μg/plate, there was no evidence of mutagenicity when pulegone was incubated with *S. typhimurium* strains TA97, TA98, TA100 or TA1535 in the presence or absence of metabolic activation by induced hamster or rat liver preparations. Incubation of the same strains of *S. typhimurium* with menthofuran at three times the concentration of Aroclor 1254-induced rat or hamster liver preparation failed to show any evidence of mutagenicity.

An additional reverse mutation assay was performed using the same lot of pulegone that was used in the 2-year bioassay at concentrations ranging from 12.5 to 1500 μg/plate in *S. typhimurium* strains TA98 and TA100 and *E. coli* WP2 uvrA/pKM101 in the absence and presence of 10% rat liver S9 (NTP, 2011). Mutagenicity was observed in *S.typhimurium* TA98 and *E. coli* WP2/pKM101 at concentrations above 500 μg/plate in the presence of S-9.

In a separate OECD-compliant study using the plate incorporation method, no evidence of mutagenicity was observed when concentrations of pulegone up to 5000 μg/plate was incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA without metabolic activation or with metabolic activation by S9 liver homogenate prepared from Aroclor 1254 treated male Sprague-Dawley rats (Dakoulas, 2017b). In this same study, Peppermint Oil (FEMA

Table 5
Summary of Ames assay results for pulegone.

Strains	Metabolic Activation	Vehicle Control	Concentration (μg/plate)	Result	Reference
S. typhimurin TA97, TA98, TA100, TA1535	None	Corn oil	0, 3.3, 10, 33, 100, 333, 1000, 2167, 3333 (TA100 and TA98 only)	Negative	NTP 2011 (1st of 3 studies)
S. typhimurin TA97, TA98, TA100, TA1535	10% hamster S9 (Syrian hamster liver)	Corn oil	0, 3.3, 10, 33, 100, 333, 1000, 2167, 3333 (TA100 and TA98 only)	Negative	NTP 2011 (1st of 3 studies)
S. typhimurin TA97, TA98, TA100, TA1535	30% hamster S9 (Syrian hamster liver)	Corn oil	0, 3.3, 10, 33, 100, 333, 1000, 2167, 3333 (TA100 and TA98 only)	Negative	NTP 2011 (1st of 3 studies)
S. typhimurin TA97, TA98, TA100, TA1535	10% rat S9 (Aroclor 1254-induced male Sprague-Dawley rat)	Corn oil	0, 3.3, 10, 33, 100, 333, 1000, 2167, 3333 (TA100 and TA98 only)	Negative	NTP 2011 (1st of 3 studies)
S. typhimurin TA97, TA98, TA100, TA1535	30% rat S9 (Aroclor 1254-induced male Sprague-Dawley rat)	Corn oil	0, 3.3, 10, 33, 100, 333, 1000, 2167, 3333 (TA100 and TA98 only)	Negative	NTP 2011 (1st of 3 studies)
S. typhimurin TA98, TA100 and E. coli WP2uvrA/pKM101	None	Corn oil	0, 100, 150, 200, 250, 400, 500, 1000, 1500 (TA100), 2500 (TA98), 3500 (WP2*uvrA*)	Negative	NTP 2011 (2nd of 3 studies)
S. typhimurin TA98, TA100 and E. coli WP2uvrA/pKM101	10% rat S9 (Aroclor 1254-induced male Sprague-Dawley rat)	Corn oil	0, 100, 150, 200, 250, 400, 500, 1000, 1500 (TA100), 2500 (TA98), 3500 (WP2*uvrA*)	Negative (equivocal response for WP2*uvrA* with 10% rat S9 for one trial (n = 3), max increase of 1.5 fold at 3500 μg/plate	NTP 2011 (2nd of 3 studies)
S. typhimurin TA98, TA100 and E. coli WP2uvrA/pKM101	None	Corn oil	0, 12.5, 50, 75, 125, 250, 500, 750, 1500	TA100: Negative TA98: Negative with one equivocal (n = 3) WP2*uvrA*: Negative	NTP 2011 (3rd of 3 studies)
S. typhimurin TA98, TA100 and E. coli WP2uvrA/pKM101	10% rat S9 (Aroclor 1254-induced male Sprague-Dawley rat)	Corn oil	0, 12.5, 50, 75, 125, 250, 500, 750, 1500	TA100: Negative with one equivocal (n = 4) TA98: Positive (n = 2) WP2*uvrA*: Positive (n = 2)	NTP 2011 (3rd of 3 studies)
S. typhimurin TA98, TA100, TA1535, TA1537, and E. coli WP2uvrA	None	Ethanol	15, 50, 150, 500, 1500, 5000	Negative	Dakoulas (2017b)
S. typhimurin TA98, TA100, TA1535, TA1537, and E. coli WP2uvrA	10% rat S9 (Aroclor 1254-induced male Sprague-Dawley rat)	Ethanol	15, 50, 150, 500, 1500, 5000	Negative	Dakoulas (2017b)

2848), containing 2.1% pulegone, was also found to be negative for mutagenicity at concentrations up to 5000 μg/plate (Dakoulas, 2017a).

No increase in the percentage of micronucleated polychromatic erythrocytes in peripheral blood was observed when B6C3F$_1$ mice were administered, by gavage, 9.375, 18.75, 37.5, 75 or 150 mg/kg bw of pulegone daily for 90 days (NTP, 2011).

No evidence of mutagenicity was observed when concentrations of menthofuran in the range from 100 to 10,000 μg/plate (NTP, 2002) was incubated with *S. typhimurium* strains TA100 or TA1535 without metabolic activation or with metabolic activation by Aroclor 1254-induced Syrian hamsters liver preparations (10%) or Sprague-Dawley rat liver preparations (10%). In a second phase of the experiment, at concentrations up to and including 667 μg/plate, there was no evidence of mutagenicity when menthofuran was incubated with *S.typhimurium* strains TA97, TA98, TA100 or TA1535 in the presence or absence of metabolic activation induced by hamster or rat liver preparations. Incubation of the same strains of *S.typhimurium* with menthofuran at three times the concentration of Aroclor 1254-induced rat or hamster liver preparation failed to show any evidence of mutagenicity.

In reviewing the results of these assays conducted on pulegone, summarized in Table 5 and its potential metabolite menthofuran, the weight of evidence is that pulegone is non-genotoxic based on negative responses in the Ames and *in vitro* micronuclueus assay.

7.9.8. Additional studies relevant to the toxicity of pulegone - peperina (Minthostachys verticillate) oil

7.9.8.1. Short-term toxicity study. In a study relevant to the toxicity of pulegone, a 90-day dietary study was conducted in which the essential oil of *Minthostachys verticillate* (Griseb.), commonly known as peperina was added to the feed Wistar rats (5/sex/dose) at concentrations of 0 (control), 1, 4 or 7 g/feed mash (Escobar et al., 2015). These concentrations correspond to doses of 0, 70, 260 or 460 mg/kg bw/day, respectively, for the control and three test groups. During the study, toxicity signs, body weights and food consumption were monitored daily. At the end of the study, the rats were terminated, blood samples were collected and the liver, kidney and a section of the intestine were removed and preserved for histopathological examinations.

The test article was obtained by steam distillation of the leaves of *M.verticillate* (Griseb.) and analyzed by gas chromatography. Compounds were identified based on retention time by comparison to known standards. The composition of the *M.verticillate* (Griseb.) essential oil was determined to be 64.65% pulegone (FEMA 2963), 23.92% menthone (FEMA 2667), 2% eugenol (FEMA 2467), 1.62% iso-pulegone (FEMA 2964), 1.4% limonene (FEMA 2633) with minor amounts of spathulenol, piperitone and other terpenoid compounds.

For all groups, no significant changes were detected in body weight gains, feed intake or feed conversion efficiency. A non-significant increase in average food intake and body weight gain was noted at the 1 g/kg feed concentration. At the termination of the study, no significant changes in the organ weights of the liver, kidneys and intestine were found. Histopathological analysis of liver, kidney and intestinal tissues found no abnormalities. In conclusion, no adverse effects were observed with the feeding of the essential oil of *M.verticillate* (Griseb.) at concentrations up to 7 g/kg of feed. The NOAEL is determined to be the highest dose tested, 460 mg/kg bw/day (Escobar et al., 2015).

7.9.8.2. Genotoxicity studies. An *in vivo* micronucleus assay was conducted in which BALB/c mice (3/sex/dose) were administered a single dose of *M.verticillate* (Griseb.) essential oil by intra-peritoneal injection at concentrations of 0 (control), 25, 50, 100, 250 and 500 mg/kg (Escobar et al., 2012). The composition of the *M.verticillate* (Griseb.) essential oil was determined to be 60.5% pulegone (FEMA 2963), 18.2% menthone (FEMA 2667), 3.76% limonene (FEMA 2633) with minor amounts of α-pinene (FEMA 2902), β-pinene (FEMA 2903) and eucalyptol (FEMA 2465). The bone marrow from the femur was collected and 1000 polychromatic erythrocytes were analyzed from each mouse. No significant increase in the induction of micronuclei was observed in any of the test groups versus the control group.

An *in vivo* micronucleus assay was conducted at the conclusion of a 90-day dietary study in which Wistar rats (5/sex/dose) were fed the essential oil of *M.verticillate* (Griseb.) at concentrations of 0 (control), 1, 4 and 7 g/feed mash (Escobar et al., 2015). These concentrations correspond to doses of 0, 70, 260 and 460 mg/kg bw/day, respectively, for the control and 3 test groups. The composition of the *M.verticillate* (Griseb.) essential oil was determined to be 64.65% pulegone (FEMA 2963), 23.92% menthone (FEMA 2667), 2% eugenol (FEMA 2467), 1.62% iso-pulegone (FEMA 2964), 1.4% limonene (FEMA 2633) with minor amounts of spathulenol, piperitone and other terpenoid compounds. The bone marrow from the femur was collected and analyzed for all the rats in the study and 1000 polychromatic erythrocytes were analyzed from each rat. No significant increase in the induction of micronuclei was observed in any of the test groups versus the control group. A comet assay performed on blood samples collected at the end of the study revealed no significant tail moment in the treated groups compared to the control group.

7.9.9. Conclusions on the toxicology and genotoxicology of pulegone

The NTP (2011) analysis of its 2-year gavage studies found no evidence of carcinogenic activity of pulegone in male F344/N rats administered 18.75, 37.5 or 75 (stop-exposure) mg/kg but found some evidence of carcinogenic activity of pulegone in female F344/N rats based on increased incidences of benign urinary bladder neoplasms. Later experiments (Da Rocha et al., 2012), however, indicated that the urinary bladder neoplasms observed in female rats were due to urothelial cytotoxicity followed by regenerative cell proliferation. Thus, carcinogenicity would not be expected to occur at exposures below which urothelial cytotoxicity is produced. Furthermore, the dose administered to the female rats that increased the incidence of bladder tumors exceeded the MTD based on the severe toxicity resulting in the discontinuance of chemical administration and due to decreased survival. The weight of evidence from the Ames assay results (see Table 6

Table 6
FEMA GRAS flavor materials affirmed.

FEMA No.	Name
2848	Peppermint Oil (*Mentha piperita* L.), *Mentha* 'MP-11', *Mentha x piperita* 'MP-2', Blue Balsam Mint Oil
3031	Spearmint Extract (*Mentha spicata* L.)
3032	Spearmint Oil (*Mentha spicata* L.), Macho mint oil, Julep mint oil
4219	Cornmint Oil (*Mentha arvensis* L.)
4777	Erospicata Oil (*Mentha spicata* 'Erospicata'), *Mentha spicata* 'Erospicata' oil
4778	Curly Mint Oil (*Mentha spicata* var. *crispa*), *Mentha spicata* L. var. *crispa* oil
2839	Pennyroyal Oil (*Hedeoma pulegioides* (L.) var Pers. (American), *Mentha pulegium* L. var. *eriantha* (European, N. African))
2169	Buchu Leaves Oil (*Barosma betulina* Bartl. et Wendl., *B. crenulata* (L.) Hook, *B. serratifolia* Willd.)
2238	Caraway Oil (*Carum carvi* L.)
2383	Dill Oil (*Anethum graveolens* L.)

Table 7
New FEMA GRAS flavor materials.

FEMA No.	Name
4923	Buchu Leaves Extract (*Barosma betulina* Bartl. et Wendl., *B. crenulata* (L.) Hook, *B. serratifolia* Willd.)
4924	Peppermint Oil Terpeneless (*Mentha piperita* L.)
4925	Spearmint Oil Terpeneless (*Mentha spicata* L.)

for a summary of studies) together with a negative *in vivo* mouse peripheral blood micronucleus study supports the conclusion that pulegone is not a genotoxin. In addition, the genotoxicity assays of peppermint oil performed concurrently with pulegone (OECD-compliant) were negative (Dakoulas, 2017a).

The metabolic study of pulegone in rats by Chen et al. (2001), indicate that pulegone is oxidized by P450s or reduced to menthone forming metabolites that undergo conjugation with glucuronic acid and are excreted. Pulegone and its metabolites also may undergo conjugation with glutathione prior to excretion in the bile or urine. In earlier metabolism studies, menthofuran was isolated from the urine of rats and mice administered hepatotoxic doses (200–300 mg/kg bw/day) of pulegone. Menthofuran forms protein adducts in liver tissue and by this mechanism is considered to contribute significantly to the observed hepatotoxicity of pulegone. Menthofuran was not detected in the Chen et al. (2001) study in which rats were administered single or multiple 80 mg/kg bw doses of pulegone nor was menthofuran detected in a study in humans in which a single dose of up to 70 mg pulegone was administered. These observations suggest that at lower, non-hepatoxic doses, pulegone is metabolized forming glucuronic acid and glutathione conjugates.

For the 14-week NTP study in B6C3F1 mice, the NOAEL was 75 mg/kg bw/day based on significant increase in the absolute and relative liver weight changes at the highest dose group. In the 14-week study in F344N rats, the NOAEL of 9.375 mg/kg bw per day (NTP, 2011) was reported based on increased relative kidney weights at higher doses. This more conservative NOAEL value was used to assess the MoS for Group 11 (Pulegone and structurally and metabolically related substances) constituents of Peppermint oil (FEMA 2848) in Table 3 above.

8. Recognition of GRAS status

The mint and related NFCs discussed here were determined to be generally recognized as safe (GRAS) under conditions of intended use as flavor ingredients by the Flavor and Extract Manufacturers Association (FEMA) in 1965 or in subsequent years. Based on the safety evaluation described in this manuscript, the FEMA Expert Panel has affirmed the GRAS status for the materials listed in Table 6.

In addition, the FEMA Expert Panel determined GRAS status and assigned new FEMA numbers for the three materials listed in Table 7.

Upon evaluation of the scientific data relevant to the safety evaluation of the above listed NFCs used as flavor ingredients, it was concluded that under intended conditions of use that they present no safety issues to humans. The safety of these mint and related oils is also supported by their self-limiting properties as flavor ingredients in food resulting in use levels that do not saturate pathways of metabolism and excretion. The constituents of these mint and related oils have been demonstrated to be rapidly absorbed, distributed, metabolized and excreted. There are adequate margins of safety between conservative estimates of exposure and the no-observed-adverse-effect levels in animal short and long-term toxicity studies in addition to a lack of genotoxic potential and no adverse findings in reproductive studies. The data support no significant risk to humans and the affirmation of GRAS status for Peppermint Oil (FEMA 2848), Spearmint Oil (FEMA 3032), Cornmint Oil (FEMA 4219), Erospicata Oil (FEMA 4777), Curly Mint oil (FEMA 4778), Pennyroyal Oil (FEMA 2839), Buchu Leaves Oil (FEMA 2169), Caraway Oil (FEMA 2238) and Dill Oil (FEMA 2383) and the determination of GRAS status for Buchu Leaves Extract (FEMA 4923), Peppermint Oil, Terpeneless (FEMA 4924) and Spearmint Oil Terpeneless (FEMA 4925) as flavoring ingredients in food.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Drs. Cohen, Eisenbrand, Fukushima, Gooderham, Guengerich, Hecht, and Rietjens, are members of the Expert Panel of the Flavor and Extract Manufacturers Association. The FEMA Expert Panel's Statement on Conflict of Interest Protections and Procedures is available at https://www.femaflavor.org/gras#conflict. Authors Bastaki, Davidsen, Harman, McGowen and Taylor are employed by Verto Solutions which provides scientific and management support services to FEMA.

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Appendix A. Supplementary data

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Transparency document

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