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F.EMA GRAS assessment of natural flavor complexes: Sage oil, Orris Root Extract and Tagetes Oil and related flavoring ingredients

Nigel J. Gooderham [a], Samuel M. Cohen [b], Gerhard Eisenbrand [c], Shoji Fukushima [d], F. Peter Guengerich [e], Stephen S. Hecht [f], Ivonne M.C.M. Rietjens [g], Thomas J. Rosol [h], Jeanne M. Davidsen [i], Christie L. Harman [i], Shannen E. Kelly [i], Sean V. Taylor [j,*]

[a] Dept. of Metabolism, Digestion, Reproduction, Imperial College London, Sir Alexander Fleming Building, London, SW7 2AZ, United Kingdom
[b] Havlik–Wall Professor of Oncology, Dept. of Pathology and Microbiology, University f Nebraska Medical Center, 983135 Nebraska Medical Center, Omaha, NE, 68198-3135, USA
[c] University of Kaiserslautern, Kühler Grund 48/1, 69126, Heidelberg, Germany
[d] Japan Bioassay Research Center, 2445 Hirasawa, Hadano, Kanagawa, 257-0015, Japan
[e] Tadashi Inagami Professor of Biochemistry, Dept. of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN, 37232-0146, USA
[f] Wallin Professor of Cancer Prevention, Masonic Cancer Center and Dept. of Laboratory Medicine and Pathology, Cancer and Cardiovascular Research Building, 2231 6th St., S.E., Minneapolis, MN, 55455, USA
[g] Division of Toxicology, Wageningen University, Stippeneng 4, 6708 WE, Wageningen, the Netherlands
[h] Department of Biomedical Sciences, Heritage College of Osteopathic Medicine, Ohio University, 1 Ohio University, Athens, OH, 45701, USA
[i] Flavor and Extract Manufacturers Association, 1101 17th Street, N.W., Suite 700, Washington, D.C., 20036, USA
[j] Scientific Secretary to the FEMA Expert Panel, 1101 17th Street, N.W., Suite 700, Washington, D.C., 20036, USA

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ABSTRACT

In recent years, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) has conducted a program to re-evaluate the safety of natural flavor complexes (NFCs) used as flavor ingredients. This publication, twelfth in the series, details the re-evaluation of NFCs whose constituent profiles are characterized by alicyclic or linear ketones. In its re-evaluation, the Expert Panel applies a scientific constituent-based procedure for the safety evaluation of NFCs in commerce using a congeneric group approach. Estimated intakes of each congeneric group of the NFC are evaluated using the well-established and conservative Threshold of Toxicological Concern (TTC) approach. In addition, studies on the toxicity and genotoxicity of members of the congeneric groups and the NFCs under evaluation are reviewed. The scope of the safety evaluation of the NFCs contained herein does not include added use in dietary supplements or any products other than food. Thirteen (13) NFCs derived from the *Boronia, Cinnamomum, Thuja, Ruta, Salvia, Tagetes, Hyssopus, Iris, Perilla* and *Artemisia* genera are affirmed as generally recognized as safe (GRAS) under conditions of their intended use as flavor ingredients based on an evaluation of each NFC and the constituents and congeneric groups therein.

1. Introduction

For six decades, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) has been the primary, independent body evaluating the safety of flavoring ingredients for use in human foods in the United States. Flavor ingredients are evaluated for consideration as "generally recognized as safe" (GRAS) for intended use consistent with the 1958 Food Additive Amendment to the Federal Food Drug and Cosmetic Act (Hallagan and Hall, 1995, 2009; Hallagan et al., 2020). To date, the FEMA Expert Panel has concluded that more than 2700 flavoring ingredients have met the GRAS criteria for their intended uses.

Flavoring ingredients can be broadly classified into one of two categories: chemically defined substances or complex mixtures. In past years, the FEMA Expert Panel has conducted two re-evaluation cycles of chemically defined FEMA GRAS flavoring materials. In 2015, a project was initiated to extend the re-evaluation program to include FEMA GRAS natural flavor complexes (NFCs). At the beginning of this project, the Panel reviewed its procedure for the safety evaluation of NFCs published in 2005 (Smith et al., 2005) and subsequently published an

* Corresponding author. Scientific Secretary to the FEMA Expert Panel, Flavor and Extract Manufacturers Association, 1101 17th Street, N.W., Suite 700, Washington, D.C., 20036, USA.
E-mail address: staylor@vertosolutions.net (S.V. Taylor).

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Abbreviations

$BMDL_{10}$	Lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence
CAC	Codex Alimentarius Commission
CF	Correction factor
CFR	Code of Federal Regulations
CG	Congeneric group
CHO	Chinese hamster ovary (cells)
DTC	Decision tree class
ECHA	European Chemicals Agency
EFFA	European Flavour Association
EFSA	European Food Safety Authority
EMA	European Medicines Agency
FAS GATS	Foreign Agricultural Service Global Agricultural Trade System
FCC	Food Chemicals 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
GD	Gestation day
GLP	Good laboratory practices
GRAS	Generally recognized as safe
HMPC	Committee on Herbal Medicinal Products
HPBL	Human peripheral blood lymphocytes
IFEAT	International Federation of Essential Oils and Aroma Trades
IOFI	International Organization of the Flavor Industry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JFFMA	Japan Fragrance and Flavor Materials Association
LOD	Limit of detection
MF	Mutation frequency
MOE	Margin of Exposure
MS	Mass spectrometry
NCE	Normocytic erythrocytes
NFC	Natural flavoring complex
NTP	National Toxicology Program
NOAEL	No-observed-adverse-effect-level
OECD	Organization for Economic Co-Operation and Development
PCI	Per capita intake
SD	Sprague-Dawley (rat)
TD_{50}	Dose giving a 50% tumor incidence
TGR	Transgenic rodent gene mutation (assay)
TTC	Threshold of toxicological concern
UDS	Unscheduled DNA synthesis
WHO	World Health Organization

update in 2018 (Cohen et al., 2018a). The procedure is a scientifically based approach in which data are collected on the annual usage and constituent composition of the NFC under evaluation. The constituents of most of the NFCs are products of well-characterized plant biochemical pathways (Schwab et al., 2008) and as a result, the constituents can usually be arranged into a limited number of well-defined groups of compounds with similar chemical and biological characteristics, referred to as congeneric groups. For each constituent congeneric group, the metabolism, toxicity and genotoxicity of members of the group are evaluated and the estimated intake is calculated and evaluated using the Threshold of Toxicologic Concern (TTC) approach (EFSA Scientific Committee, 2019; Kroes et al., 2000). The updated procedure was first applied to *Citrus*-derived NFCs (Cohen et al., 2019). Subsequently, the FEMA Expert Panel has applied the procedure for the safety evaluation of additional NFCs that have been grouped together based on similar chemical compositions or botanical taxonomy, including the group of *Mentha*-derived NFCs, dill, caraway and buchu NFCs (Cohen et al., 2020); *Cassia*, *Cinnamomum* and *Myroxylon*-derived NFCs (Rietjens et al., 2020); clove, cinnamon leaf and West Indian bay leaf-derived NFCs (Gooderham et al., 2020a); lavender, guaiac and coriander-derived and related NFCs (Fukushima et al., 2020); the eucalyptol-containing NFCs (Eisenbrand et al., 2021); simple phenol-containing NFCs including origanum oil, thyme oil, and related NFCs (Cohen et al., 2021); allspice, anise fennel-derived and related NFCs (Rietjens et al., 2023); lemongrass, chamomile, citronella-derived and related NFCs (Rosol et al., 2023) and asafetida, onion and garlic oil NFCs (Davidsen et al., 2023a). In the tenth manuscript in this series, the Panel reviewed NFCs containing allylalkoxybenzene constituents with suspected genotoxic potential, such as estragole, methyl eugenol, elemicin, safrole, myristicin and parsley apiole, deriving updated $BMDL_{10}$ values from benchmark dose analyses using Bayesian model averaging for safrole, estragole and methyl eugenol and estimated $BMDL_{10}$ values for myristicin, elemicin and parsley apiole by read-across using relative potency factors (Davidsen et al., 2023b). These updated $BMDL_{10}$ values were applied in the safety evaluation, using the margin of exposure (MOE) approach when the estimated intake of an allylalkoxybenzene constituent exceeded the TTC for compounds with structural alerts for genotoxicity (EFSA Scientific Committee, 2019; Kroes et al., 2004), as for NFCs derived from basil, estragon (tarragon), mace, nutmeg, parsley and Canadian snakeroot.

From 2015 through 2021, the FEMA Expert Panel has issued numerous calls for data to gather detailed chemical analyses for the thirteen (13) NFCs listed in Table 1. Members of the International Organization of the Flavor Industry (IOFI), including FEMA, the Japan Fragrance and Flavor Materials Association (JFFMA), the European Flavour Association (EFFA), and the International Federation of Essential Oils and Aroma Trades (IFEAT), provided data on these NFCs derived from the *Boronia, Cinnamonum, Thuja*, *Ruta*, *Salvia*, *Tagetes, Iris, Hyssopus, Perilla* and *Artemisia* genera currently used for flavoring food.

2. History of food use

The botanicals listed in Table 1 have, in general, been used to flavor beverages and foods and in traditional medicine. *Thuja occidentalis* L., also known as cedar leaf, is prevalent in the northeastern United States and eastern Canada. Historically, Ojibwa Indians, as well as other indigenous eastern North American peoples, used the leaves of this botanical in teas (Kuhnlein and Turner, 1991). It later was named arborvitae ("tree of life") by the French explorer Jacques Cartier after local Indians treated him and his crew for scurvy using a tea prepared from the leaves and bark of the tree (Kuhnlein and Turner, 1991). Subsequent production of the oil derived from this botanical began in the mid-nineteenth century by farmers in northern New York and Vermont for use in fine fragrances and room fresheners (Guenther, 1952; Lawrence, 1979).

The camphor tree, *Cinnamomum camphora*, like other members of the *Cinnamomum* genus, is a historical and culturally prominent species. While the cinnamon and cassia producing species of the *Cinnamomum* genus are better known, camphor is produced in the bark of the *C. camphora* species. During the Middle Ages, camphor was extensively used in Arabia as flavoring and perfume and was burned as incense in Hindu religious ceremonies (Rabiu et al., 2011). Prior to World War I, much of the global supply of natural camphor came from Japan and Taiwan's *Cinnamomum camphora* forests (Guenther, 1950; Rabiu et al.,

Table 1
NFCs evaluated by the Expert Panel.

Name	FEMA No.	Estimated daily intake (μg/person/day)[a]	Most recent annual volume (kg)[b]
Boronia Absolute (*Boronia megastigma* Nees)	2167	0.8	8
Camphor Japanese White Oil (*Cinnamomum camphora* (L.) Nees et Eberm.)[c]	2231	23	230
Cedar Leaf Oil (*Thuja occidentalis* L.)[d,e]	2267	7	74
Hyssop Oil (*Hyssopus officinalis* L.)	2591	0.09	0.9
Orris Concrete Liquid Oil (*Iris florentina* L., *I. pallida, I. germanica)*	2829	59	570
Orris Root Extract (*Iris florentina* L., *I. pallida, I. germanica)* – White flag extract	2830	170	1660
Rue Oil (*Ruta graveolens* L.)[f]	2995	0.9	9
Sage Oil (*Salvia officinalis* L.)	3001	590	5700
Sage Oleoresin (*Salvia officinalis* L.)	3002	110	1130
Tagetes Oil (*Tagetea erecta* L.; *T. patula* L.; *T. glandulifera Schrank*), Marigold oil[4]	3040	23	230
Wormwood Oil (*Artemisia absinthium* L.), Absinthium oil	3116	0.2	2
Osmanthus Absolute (*Osmanthus fragrans* Lour.)	3750	9	91
Perilla Leaf Oil (*Perilla frutescens* L.), Shiso oil	4013	0.1	1

[a] Estimated intake using PCI × 10 ("eaters only") calculation is shown.
[b] Harman, C.L. and Linman, M.J. 2023. Flavor and Extract Manufacturers Association of the United States (FEMA) 2020 Poundage and Technical Effects Survey, Washington DC, USA.
[c] Federal Code 21 CFR §172.510, natural flavoring substances from *Cinnamomum camphora* (L.) Nees et Eberm. when added to food under conditions of intended use, must be safrole-free.
[d] GRAS 3 footnote: Provided it is used at levels such that no thujone is detectable in the finished food, using the standard AOAC method.
[e] Code of Federal Regulation 21 CFR §172.510 thujone free in finished food.
[f] Code of Federal Regulation 21 CFR §184.1699 – Oil of rue (*Ruta graveolens* L., *Ruta montana* L., *Ruta bracteosa* L., *Ruta calepensis* L.): Refers to FCC specifications; Use Levels Restriction: Baked goods and baking mixes (10 ppm), Frozen dairy desserts and mixes (10 ppm), Soft candy (10 ppm), All other food categories (4 ppm).

2011). Currently, this tree is grown in China, Japan and adjacent regions in Southeast Asia (Ravindran et al., 2003). White camphor oil is a by-product of the process used for the isolation of natural camphor from crude camphor oil distilled from the chipped tree bark. Following the crystallization and separation of camphor crystals, the resulting oil is fractionated and the lightest fraction, white camphor oil, is used in flavorings and perfumes (Guenther, 1950; Ravindran et al., 2003).

Rue (*Ruta graveolens* L.) is a biennial or perennial herb common in the dry regions of southern Europe and North Africa (Fenaroli et al., 1975). In Ancient Greece, the herb was used as seasoning and in condiments. Later, the Ancient Romans used fresh or dried herb in small quantities due to their strong odor and flavor to spice wine, brines, seafood, meats, sauces and salads. Similarly, the bitter leaves of rue have been used to impart flavor in Mediterranean food and beverages (Arctander, 1960).

Sage (*Salvia officinalis* L.) is a commonly used culinary herb that originated in southern Europe. Historically, sage was used to flavor foods and beverages, and was also used for food preservation (Altindal and Altindal, 2016). Currently, leaves of this botanical are used in Mediterranean and European teas, pastries, cheeses, breads, meats, butter, vegetarian dishes, cakes, sauces, soups and pastas (Altindal and Altindal, 2016). Furthermore, fresh sage leaves are considered an important ingredient in wine, bitters and aperitifs (Arctander, 1960; Fenaroli et al., 1975). In the United States, sage is commonly used to season chowders, pork, poultry stuffing and baked fish (Raghavan, 2007; Guenther, 1949).

Tagetes, commonly known as marigold, is thought to be native to Central America. It is abundant in Argentina, southwestern United States, southern Mexico, South Africa, Australia, Africa and the Mediterranean (Arctander, 1960; Fenaroli et al., 1975; Neher, 1968). Plants of the *Tagetes* genus are used as ornamental plants as well as in traditional medicine (Neher, 1968; Vasudevan et al., 1997). Additionally, the florets of *Tagetes* have been used to add coloring to food. In Mexico, the botanical was ground with water or wine to drink as a tea (Hernández, 1942).

Wormwood (*Artemisia absinthium* L.) is native to central and southern Europe, though it is cultivated in Europe, North Africa, Brazil and the midwestern United States. This botanical has been used in traditional medicinal formulations to treat rheumatic pains (Guenther, 1952) as well as in flavor and fragrance formulations (Arctander, 1960). Wormwood is perhaps best known for its use in absinthe, a distilled alcoholic beverage popular in 19th century Europe, and for absinthism, a chronic illness that was associated with heavy absinthe consumption. The belief that thujone, a major constituent of wormwood oil, was the cause of absinthism led to the prohibition of absinthe and wormwood extracts for food purposes in numerous countries beginning in 1910. While distilled spirits labeled "absinthe" are again available in European and US markets, the presence of α- and β-thujone in food and beverages is controlled in both markets by regulation 1334/2008 in the Europe (European Commission, 2008) and 21 C.F.R. § 172.510 in the USA.

Endemic to Western Australia, *Boronia megastigma* Nees is a small shrub related to the citrus tree that can grow up to six feet in height. The shrub is also referred to as brown boronia and is characterized by reddish brown flowers and a fresh, fruity odor (Lim, 2014). Due to its appealing fragrance, boronia has historically been used in perfumery (Arctander, 1960). The absolute of the flowers of the botanical, boronia absolute, is also used as a flavoring to impart richness to peach, plum, raspberry and other fruity flavors (Arctander, 1960).

Osmanthus (*Osmanthus fragrans* Lour.), also known as sweet olive or fragrant olive, is a shrub species indigenous to southeastern Asia, including southern China and Japan. Historically, medicinal preparations derived from the flowers were used to treat coughs, stomach pains, and rheumatism (Wang et al., 2017). The flowers of the botanical are characterized by a light, jasmine-like scent that has made it ideal for perfumery and flavoring purposes. The flowers are sometimes mixed with black or green tea leaves to make osmanthus tea in China or used to flavor rice wine. Additionally, Osmanthus flowers are used as flavorings in bakery and confectionary products (Arctander, 1960; Kaiser and Lamparsky, 1981) and are commonly used to produce jams and traditional Chinese desserts such as osmanthus tong yuan.

Hyssop (*Hyssopus officinalis* L.) is a shrub in the *Lamiaceae* family indigenous to Europe and the Middle East. The shrub has subsequently been naturalized in North America, where it is commonly found in gardens and along roadsides and has a minty, sage-like aroma and flavor profile. There are several biblical references to the use of hyssop and in ancient Egypt, hyssop was used for religious purification by priests who would consume it as a condiment with bread (Tucker and DeBaggio, 2000). However, it is likely that the hyssop plant referred to in the Bible is the carvacrol-rich Syrian hyssop, *Majorana syriaca*, not *H. officinalis* (Fleisher and Fleisher, 1988). Hyssop has historically been brewed into teas to treat mild medical afflictions of the nose and throat. Hyssop oil is used in the production of bitters, tonics, and French-type liqueurs such as chartreuse (Arctander, 1960; Kokkini et al., 2003; Ravindran, 2017).

Orris (*Iris pallida, I. germanica, I. florentina*) is a perennial flower native to the Mediterranean region and has a long history of use in traditional medicines. The rhizomes, or roots, of the orris plant were used medicinally as early as 1500 BCE by the Egyptians and 200 AD in Asia (Lust, 2009; Wang et al., 2010). Upon its introduction to North

America in the 1600s, Native American communities also utilized orris root to brew medicinal teas and to prepare topical salves or poultices (Crisan and Cantor, 2016). Due to its pleasant aroma, orris was also cultivated in the Greek and Roman empires for use in perfumes (Cumo, 2013). More recently, orris extracts have been used in the cosmetic and perfumery industries. Furthermore, orris root is used to flavor a variety of foods including gin, wine, and other alcoholic beverages, ice cream, confectionaries, and baked goods (Crisan and Cantor, 2016).

Perilla (*Perilla frutescens*) is native to southeast Asia and the Indian highlands, where it has traditionally been grown as a crop (Nitta and Ohnishi, 1999). Perilla is also known as purple mint (McKeon, 2016) with a flavor and aroma profile reminiscent of mint and basil (Laureati et al., 2014). The leaves and stems of the plant are often treated as a vegetable and used as a garnish for raw fish or to color pickles (Nitta and Ohnishi, 1999). Perilla leaf is prominent in Chinese, Korean, Japanese, Indian and Thai cuisine where it is used as a spice, cooked as potherb and combined with fish, rice, vegetables and soups. Furthermore, perilla is used in stir-fries, tempuras, and salads (Ravindran and Shylaja, 2006).

3. Current use

The NFCs listed in Table 1 are used to flavor numerous foods including hard and soft candies, alcoholic and non-alcoholic beverages, meats and sauces, baked goods, chewing gum and condiments. Table 1 lists the annual usage in the USA (Harman and Linman, 2023) and the estimated intake for each NFC under consideration. All the NFCs have reported usage of less than 22,500 kg, thus the PCI × 10 'eaters only' method is used to calculate the estimated *per capita* intake, which assumes that the annual volume is consumed by 10% of the population. For the NFCs listed in Table 1, only three, Sage Oil (FEMA 3001), Sage Oleoresin (FEMA 3002) and Orris Root Extract (FEMA 2830), have an annual usage greater than 1000 kg. Three NFCs, Orris Concrete Liquid Oil (FEMA 2829), Camp and Tagetes Oil (FEMA 3040) have an annual usage between 100 and 1000 kg. The remaining NFCs under consideration have an annual usage of less than 100 kg.

While the botanicals from which these NFC are derived all have a history of use as food, only sage is currently commonly used as a spice or food. The United States Department of Agriculture (USDA) Foreign Agricultural Service (FAS) Global Agricultural Trade System (GATS) online database reports that 4,200,000 kg of sage leaves were imported into the USA in 2020 (USDA, 2020). Dried sage leaves have an average essential oil content ranging from 1.1 to 2.8% (Abu-Darwish et al., 2013; Attokaran, 2017). Assuming a 1.1% volatile oil content for dried sage leaves, 46,000 kg of sage essential oil would be consumed from the consumption of sage leaves as food in the USA in 2020. The yield of oleoresin from dried sage varies depending on the extraction solvent. Extraction of dried sage leaves with hexane yields approximately 5% oleoresin with a volatile oil content of 10% while extraction with a 30:70 hexane:acetone mixture yields approximately 7% oleoresin containing 6–7% volatile oil. (Attokaran, 2017). Based on these yields, an estimated 210,000 to 295,000 kg of sage oleoresin was consumed from the use of dried sage leaves as a food in the USA in 2020. In addition to the consumption as dried leaves, an unknown amount of fresh sage leaves, sourced from kitchen gardens as well as food markets, are also consumed in the USA.

4. Manufacturing methodology

Several of the NFCs listed in Table 1 are essential oils and are produced by distillation techniques. Hyssop Oil (FEMA 2591), Perilla Leaf Oil (FEMA 4013), Rue Oil (2995), Tagetes Oil (FEMA 3040) and Wormwood Oil (FEMA 3116) are produced by steam distillation of the leaves and flowering tops of the plant (Arctander, 1960; Fenaroli et al., 1975). Similarly, Cedar Leaf Oil (FEMA 2267) is produced by steam distillation of the leaves and branch ends of the eastern arborvitae *Thuja occidentalis* L. (Arctander, 1960; Fenaroli et al., 1975). Sage Oil (FEMA 3001) is produced via steam distillation of the partially dried leaves of the plant (Arctander, 1960; Fenaroli et al., 1975).

Spice oleoresins such as Sage Oleoresin (FEMA 3002) are prepared by the extraction of the spice with a volatile solvent such as acetone, isopropanol, methanol, hexane or a chlorinated hydrocarbon followed by removal of the solvent from the extract by distillation. Alternatively, following the collection of the volatile oil of the spice by distillation, the non-volatile spice fraction is extracted with an approved solvent, concentrated by solvent removal then combined with the volatile portion collected earlier in the process. Acceptable solvents for the manufacture of spice oleoresins and allowable levels of residual solvents in the finished oleoresin vary across different countries. In the USA, permissible solvents and allowable levels of residual solvents are listed in 21 C.F.R. § Sec. 173 subpart C and in the FCC monograph on spice oleoresins (Food Chemical Codex, 2023). In addition, the FCC standard on spice oleoresins requires the essential oil of an oleoresin to be similar in its physical and chemical properties, including its infrared spectrum, to that distilled from the spice of the same origin.

Boronia Absolute (FEMA 2167) and Osmanthus Absolute (FEMA 3750) are collected via a two-step process. First, the flowers are extracted with a non-polar solvent such as hexane, toluene or petroleum ether, following which the solvent is removed, resulting in an extract called a concrete (Arctander, 1960; Surburg and Panten, 2006). Absolutes are extracts prepared from the concrete by mixing the concrete with ethanol, heating the solution, followed by a cooling step, filtering of the mixture to remove waxes and finally evaporating the ethanol (Fenaroli et al., 1975; Lis-Balchin, 2002). While concretes and absolutes prepared from fragrant flowers are more commonly used as perfumery ingredients, a few, such as boronia absolute, lavender absolute, orange blossoms absolute and others also have been historically used as flavor ingredients.

Similarly, Camphor Japanese White Oil (FEMA 2231) is collected via a two-step process. When ready to process, the *Cinnamomum camphora* tree is harvested and all parts of the tree, including roots and stump, are mechanically chipped. The chipped wood is steam distilled yielding a crude semi-crystalline camphor oil. This crude camphor oil contains visible white crystals of solid camphor. Following removal of the crystals, camphor oil is fractionally distilled into three separate oils. The lightest fraction, or white camphor oil, contains the least amount of camphor. This fraction is commonly used as a flavoring in food. The medium and heavy fractions are typically called brown and blue camphor oil, respectively (Guenther, 1950; Ravindran et al., 2003).

Orris Root Extract (FEMA 2829) and Orris Root Concrete Liquid (FEMA 2830) are derived from the rhizomes of the botanical. For Orris Root Extract (FEMA 2830), the rhizomes are harvested and undergo extraction with a solvent. Although the term 'concrete' typically refers to a non-polar extract of a flower following the removal of the extraction solvent, Orris Concrete Liquid Oil (FEMA 2830) is produced via steam distillation of the orris rhizomes and use of the term 'concrete' is atypical in this case.

5. Chemical composition

The constituent profiles of the NFCs listed in Table 1 were characterized by analysis of their volatile constituents by gas chromatography-mass spectrometry (GC-MS) to identify constituents by comparison against a standardized library. A flame ionization detector (FID) was used for detection and quantitation of each analyte. Identified and unidentified GC peaks were reported as the area percent of the chromatogram. The constituent data for each NFC were compiled and the mean % for constituents present at levels greater than or equal to 1.0% are listed in Appendix A. For each identified constituent, its Cramer decision tree class (DCT) was determined, and a congeneric group was assigned based on its structure (Cohen et al., 2018a; Cramer et al., 1978). The procedure for the assignment of congeneric groups is outlined in the NFC safety evaluation procedure (Cohen et al., 2018a), and the congeneric groups used are consistent with the chemical groups used

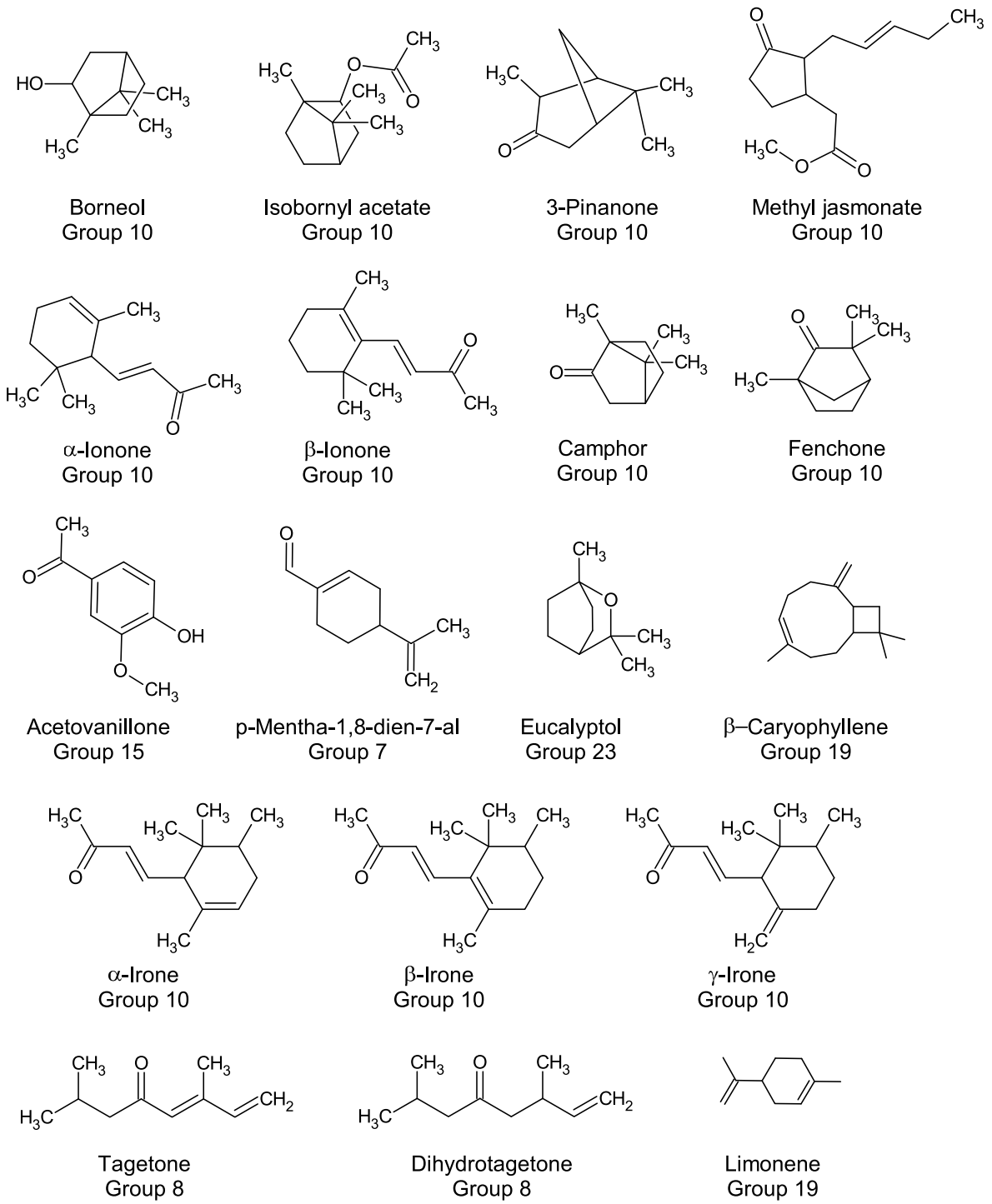


Fig. 1. Structures of commonly found constituents in the NFCs under consideration and their respective congeneric groups.

by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its evaluation of chemically defined flavor materials. The Cramer DCT assigned to each congeneric group is determined by assignment to the most conservative class for the constituents within each group.

Structures of the constituents commonly reported in the NFCs under consideration are shown in Fig. 1. Pie charts depicting the constituent congeneric group profiles for each NFC are shown in Fig. 2. Boronia Absolute (FEMA 2167), Cedar Leaf Oil (FEMA 2267), Hyssop Oil (FEMA 2591), Sage Oil (FEMA 3001) and Wormwood Oil (FEMA 3116) show high percentages of Group 10 (Alicyclic ketones, secondary alcohols and related esters) constituents, while all of the NFCs except Orris Concrete Liquid Oil (FEMA 2829), Orris Root Extract (FEMA 2830), Osmanthus Absolute (FEMA 3750) and Rue Oil (FEMA 2995) contain Group 19 (Aliphatic and aromatic hydrocarbons) constituents. Rue Oil (FEMA 2995) and Tagetes Oil (FEMA 3040) are characterized by a high percentage of Group 8 (Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters) constituents. Orris Concrete Liquid Oil (FEMA 2829) and Orris Root Extract (FEMA 2830) are characterized by significant percentages of Group 1 (Saturated aliphatic, acyclic, linear primary alcohols, aldehydes, carboxylic acids and related esters) constituents and Osmanthus Absolute (FEMA 3750) is characterized by Group 1 and Group 5 (Unsaturated linear and branched-chain

aliphatic, non-conjugated aldehydes, related primary alcohols, carboxylic acids and esters) constituents. Perilla Leaf Oil (FEMA 4013) contains approximately 50% *p*-metha-1,8-dien-7-al or perilla aldehyde, a Group 7 (Saturated alicyclic primary alcohols, aldehydes, acids and related esters) constituent. Lastly, Camphor Japanese White Oil is characterized by the presence of eucalyptol, a Group 23 (Aliphatic and aromatic ethers) constituent, and Group 19 constituents.

The constituent profile of Sage Oleoresin (FEMA 3002) is variable and will depend on the volatile oil content of the source material, the approved solvent used in its preparation, and how it is standardized for use in food. Because of the concentrated nature of spice oleoresins, they are often standardized using a food grade ingredient that also provides an associated solubility profile for the standardized oleoresin. For example, for oil-based applications, an oleoresin may be standardized with an edible vegetable oil. Alternatively, an oleoresin may be standardized with a polysorbate ester that results in a water-soluble standardized oleoresin. Oleoresins may be spray-dried with a modified starch or dispersed on a food grade carrier such as salt or dextrose (Reineccius, 1994). For example, although sage oleoresin may contain approximately 30% essential oil with 70% resinous material, after standardization with a food-grade diluent, it will contain a lower percentage of essential oil and resin (Fig. 3). While a spice oleoresin is always composed of essential oil, resinous material and the standardization agent, the customization of spice oleoresins for specific applications does not allow the determination of a single chemical composition. Nevertheless, since the added constituents are food grade, the safety evaluation can be based on the estimated percentage of essential oil which for Sage Oleoresin (FEMA 3002) is estimated to be 4–10% of its content.

6. Safety Evaluation

The procedure for the safety evaluation for NFCs (Fig. 4) is guided by a set of criteria as outlined in two publications (Smith et al., 2004, 2005) with an update in 2018 (Cohen et al., 2018a). 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, including genotoxicity, of the identified constituents by application of the 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; in Step 13 the overall safety is evaluated along with considerations of safety for use by children, given their lower body weights; lastly in Step 14, the final determination of GRAS status is made. The safety evaluation is presented below in which each step of the procedure (Cohen et al., 2018a) (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 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) 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:*

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

where:

*The annual volume of use of NFCs currently used as flavorings for food is reported in flavor industry surveys (*Gavin et al., 2008; Harman and Linman, 2023; *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:*

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

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).[1] *At this Step, if the conditions of use*[2] *for the NFC result in levels that differ from intake of the same constituents in the food source, it should be reported.*

Sage Oil (FEMA 3001) is derived from a popular culinary herb that is available in both fresh and dried forms in Western food markets and is often grown in home gardens. The USDA's FAS GATS online database reports that 4,200,000 kg of sage was imported into the USA in 2020 (USDA, 2020). Using the USA import volume for sage leaves in 2020 and conservative estimates of the essential oil and oleoresin content of sage leaves, the annual volumes of essential oil and oleoresin consumed from the consumption of sage leaves are 46,000 kg and 210,000 kg, respectively. The oleoresin of sage leaves contains the essential oil and resinous components of the spice from which it is derived. The volume of the resin fraction of sage oleoresin, the total volume minus the essential oil fraction, is conservatively estimated to be 164,000 kg. Based on these volumes, the estimated *per capita* intakes for the consumption of sage oil and the resin fraction of sage leaves are 380 μg/person/day and 1300 μg/person/day, respectively.

In Table 2, the ratio for the consumption of Sage Oil (FEMA 3001) from sage leaves as food compared to the consumption from added flavoring is reported to be 0.6, indicating that the consumption as added flavoring is significantly higher than that from food. In Table 3, the ranges of the estimated intakes from the consumption of both the

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

[2] 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.

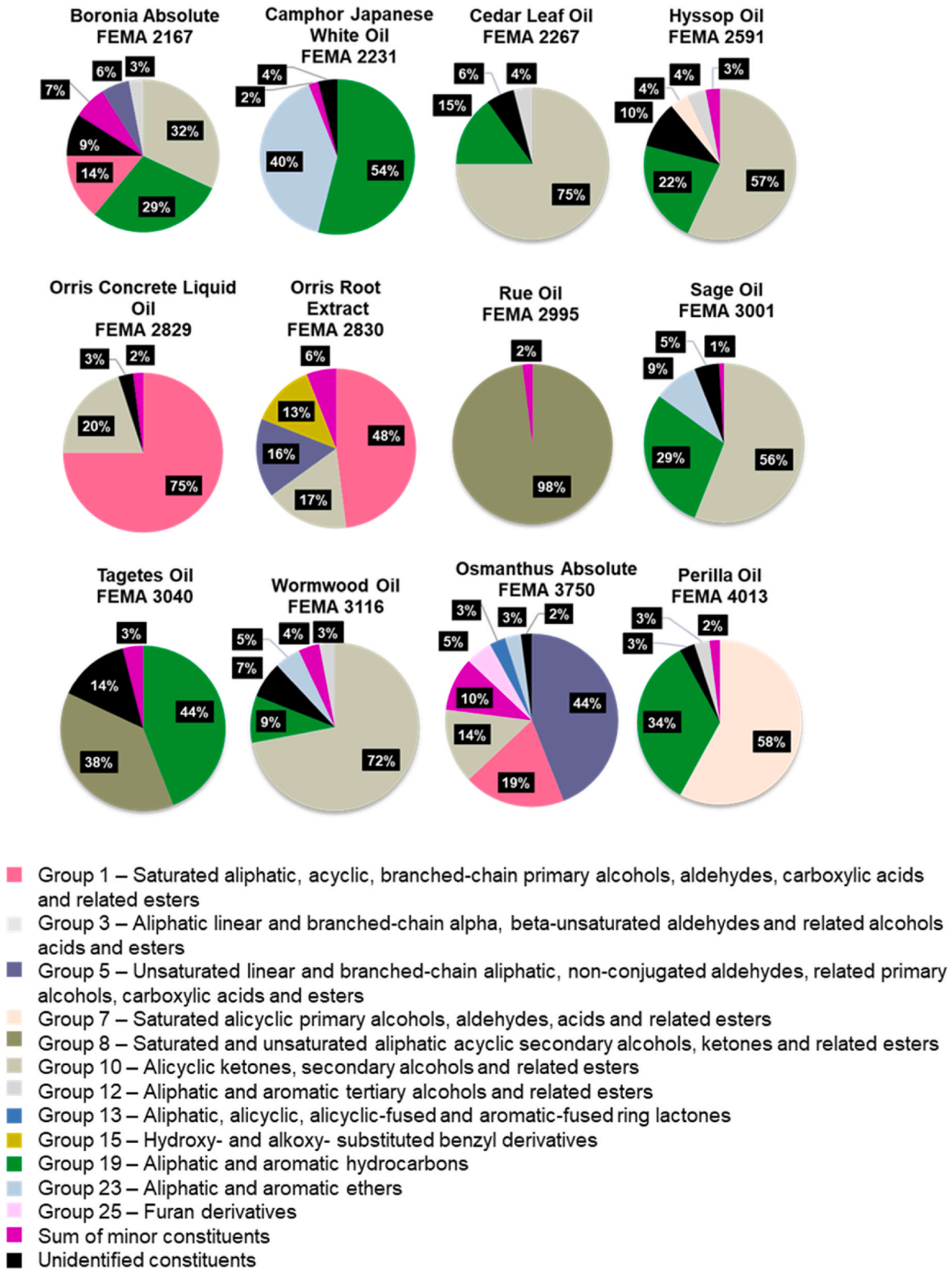


Fig. 2. Constituent congeneric group profiles for the NFCs under consideration.

essential oil and resinous fractions of Sage Oleoresin (FEMA 3002) are reported and consumption ratios of the estimated intake from food versus from added flavoring are calculated. Here, the estimated intakes from the consumption of both the essential oil and resinous fractions of Sage Oleoresin (FEMA 3002) as added flavoring are significantly lower than the estimated intakes from the consumption of food. These calculations, based on import volume data, do not account for the consumption of sage leaves sourced domestically in the USA.

The botanicals of the other NFCs listed in Table 1 are not widely used in foods. As a result, the estimated intake of the remaining NFCs from the consumption from food is assumed to be minimal and that consumption of these NFCs is primarily as added flavoring.

Fig. 3. Sage Oleoresin (FEMA 3002), containing approximately 30% volatile oil, is standardized by dilution with a food grade standardization agent, such as vegetable oil or salt, resulting in Sage Oleoresin (FEMA 3002) composed of 4% essential oil, approximately 87% standardization agent and 9% non-volatile resins. Sage Oleoresin (FEMA 3002) (standardized) containing 4–30% essential oil is used as a flavoring ingredient.

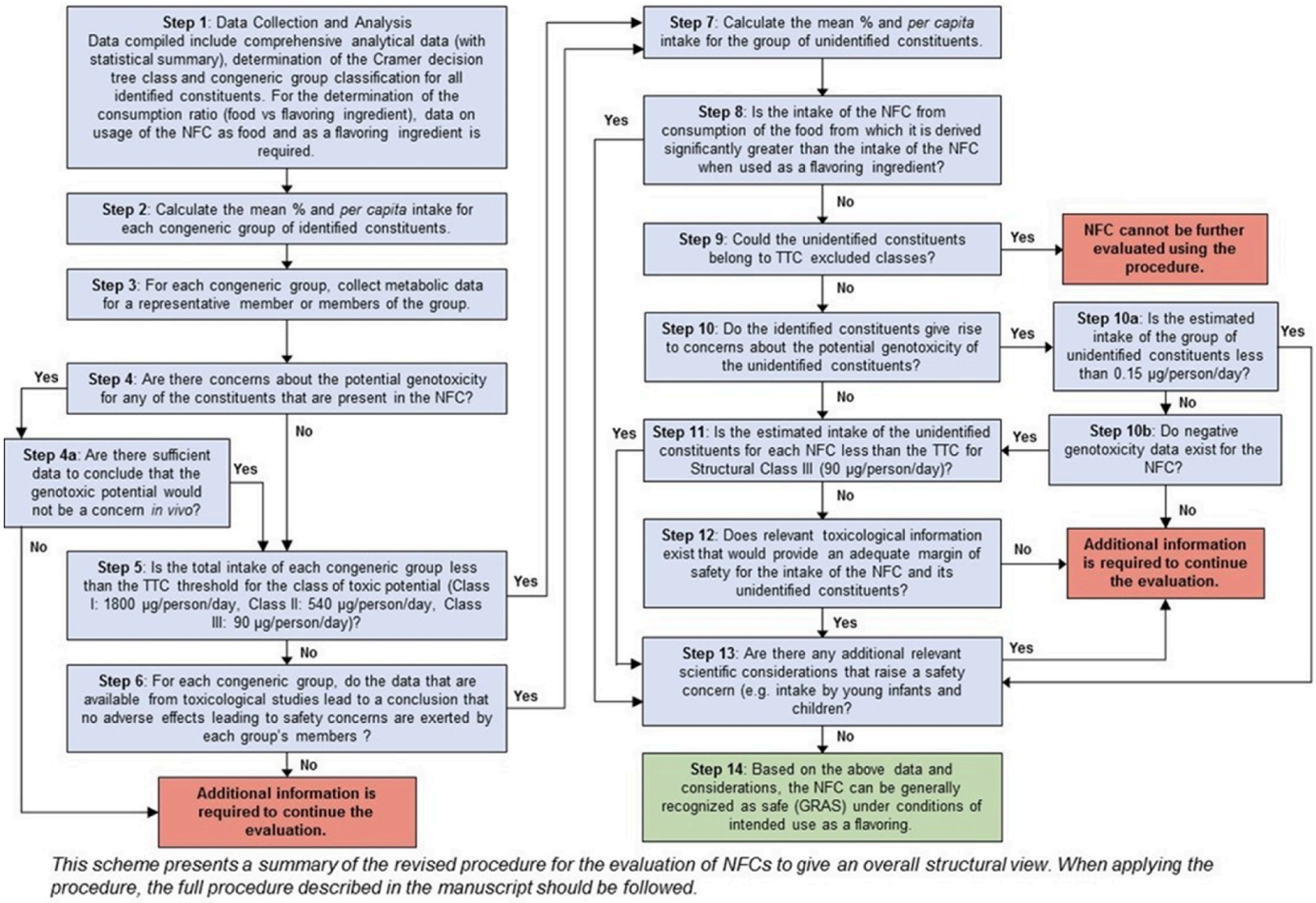


Fig. 4. Procedure for the safety evaluation of NFCs (Cohen et al., 2018a).

Table 2
Consumption ratios for Sage Oil (FEMA 3001).

NFC	Estimated Intake from Food (μg/person/day)	Estimated Intake from Flavoring (μg/person/day)[a]	Consumption Ratio Food:Flavoring
Sage Oil (FEMA 3001)	380	590	0.6

[a] In this analysis, the estimated intake from flavoring is calculated using the PCI x 10 (eaters only) method that assumes consumption by 10% of the population. The estimated intake for each NFC from food (spice) is calculated on a *per capita* basis, assuming consumption by the entire population.

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).

The constituents identified in each NFC are sorted by congeneric group and a summary report for each NFC is provided in Appendix A. Congeneric groups are recorded in order from highest to lowest mean %, with only mean % greater than or equal to 1% of the total NFC reported. Minor constituent percentages (<1% of the total NFC) are summed for the listed congeneric groups and the total mean % of each congeneric group is shown.

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.

For each NFC, the DTC for each identified constituent and congeneric group listed in Appendix A is determined and reported.

Step 2

Determine (a) the mean percentage (%) of each congeneric group in NFCs, and (b) the daily per capita intake[3] of each congeneric group. The value (a) is calculated by summing the mean percentage of each of the constituents within a congeneric group, and the value (b) is calculated from consumption of the NFC and the mean percentage.

Calculation of PCI for each constituent congeneric group of the NFC:

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

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.

The summary report for each NFC, provided in Appendix A, provides the subtotal mean % and estimated intake values (PCI × 10) for each constituent congeneric group.

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 at Step 13 of the procedure.

Proceed to Step 4.

Appendix A lists the identified constituent congeneric groups for each NFC. A recent FEMA Expert Panel publication outlined the use of metabolic data in the safety evaluation of flavoring substances and provided a summary of the expected metabolism for each congeneric group (Smith et al., 2018). Safety assessments for flavoring ingredients of several of the congeneric groups present in the NFCs under consideration have been published by the FEMA Expert Panel. These encompass flavoring ingredients of Group 10 (Alicyclic ketones, secondary alcohols and related esters) including β-ionone and related ionone flavoring ingredients, Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) and Group 19 (Aliphatic and aromatic hydrocarbons) which are predominant in the constituent profiles of the NFCs under consideration (Adams et al., 1996, 2011; Marnett et al., 2014). In addition, the Panel has published evaluations for Group 15 (Hydroxy- and alkoxy-substituted benzyl derivatives), Group 13 (Aliphatic, alicyclic, alicyclic-fused and aromatic-fused ring lactones) and Group 3 (Aliphatic linear and branched-chain alpha, beta-unsaturated aldehydes and related alcohols acids and esters) constituents which are present in some of the NFCs (Adams et al., 1998, 2005, 2007, 2008b). The metabolism of eucalyptol, a constituent of Group 23 (Aliphatic and aromatic ethers), was reviewed by the Panel in the safety evaluation of eucalyptus oil and related NFCs (Eisenbrand et al., 2021). Additional information on the metabolism of Group 1 (Saturated aliphatic, acyclic, linear primary alcohols, aldehydes, carboxylic acids and related esters), Group 3 (Aliphatic linear and branched-chain α,β-unsaturated aldehydes and related alcohols, acids and esters), Group 5 (Unsaturated linear and branched-chain aliphatic, non-conjugated aldehydes, related primary alcohols, carboxylic acids and esters), Group 7 (Saturated alicyclic primary alcohols, aldehydes, acids and related esters), Group 8 (Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters) and Group 25 (Furan derivatives) flavoring ingredients has been published by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 1997, 1998, 1999, 2003, 2004, 2006, 2007, 2011, 2012). For the congeneric groups present in these NFCs, data on the constituents of the group or related compounds allow the conclusion that the members of these respective congeneric groups are metabolized to innocuous products.

Step 4

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

If Yes, proceed to Step 4a.

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

Table 3
Consumption ratios for Sage Oleoresin (FEMA 3002).

NFC	Fraction of Essential Oil	Essential oil Estimated Intake PCI x 10 (μg/person/day)[a]	Essential oil Consumption Ratio Food:Flavoring	Non-Essential oil Fraction of Oleoresin Estimated Intake PCI x 10 (μg/person/day)[a,b]	Non-Essential oil Fraction of Oleoresin Consumption Ratio Food: Flavoring
Sage Oleoresin (FEMA 3002)	4–30%	4–35	>10	10–80	>16

[a] In this analysis, the estimated intake from flavoring is calculated using the PCI x 10 (eaters only) method that assumes consumption by 10% of the population. The estimated intake for each NFC from food (spice) is calculated in a *per capita* basis, assuming consumption by the entire population.

[b] For example, an unstandardized Sage Oleoresin (FEMA 3002) containing 30% essential oil, the resinous fraction will be 70% of the oleoresin. If this oleoresin is standardized, i.e. diluted, to contain 4% essential oil, this standardized oleoresin will have a resinous fraction of 9%, and contain 90% of a food grade diluent. The estimated intake for the resinous fraction of Sage Oleoresin (FEMA 3002) is based on the compositional range of 9–70%.

If No, proceed to Step 5.

For the NFCs under consideration, there are several major constituent congeneric groups represented (see Appendix A and Fig. 2). In their review of *in vitro* and *in vivo* genotoxicity studies for Group 10 (Alicyclic ketones, secondary alcohols and related esters), which includes β-ionone and related ionone flavoring ingredients, Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) and Group 19 (Aliphatic and aromatic hydrocarbons) constituents, the FEMA Expert Panel determined a lack of genotoxic potential for constituents of these groups (Adams et al., 1996, 2011; Marnett et al., 2014). In addition, the Panel determined a lack of genotoxic potential for Group 1 (Saturated aliphatic, acyclic, linear primary alcohols, aldehydes, carboxylic acids and related esters), Group 3 (Aliphatic linear and branched-chain α,β-unsaturated aldehydes and related alcohols, acids and esters), Group 5 (Unsaturated linear and branched-chain aliphatic, non-conjugated aldehydes, related primary alcohols, carboxylic acids and esters), Group 7 (Saturated alicyclic primary alcohols, aldehydes, acids and related esters), Group 8 (Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters) flavoring ingredients, as well as Group 25 (Furan derivatives) constituents, including linalool oxide, which have also been reviewed by JECFA (JECFA, 1997, 1998, 1999, 2003, 2004, 2006, 2007, 2011, 2012). The Panel has concluded that *p*-mentha-1,8-diene-7-al, also known as perillaldehyde (FEMA No. 3557), a constituent of Group 7 (Saturated alicyclic primary alcohols, aldehydes, acids and related esters) and a major constituent of Perilla Leaf Oil (FEMA 4013), is not genotoxic (Cohen et al., 2016). In addition, the Panel found that eucalyptol, a Group 23 (Aliphatic and aromatic ethers) constituent, is not genotoxic (Eisenbrand et al., 2021). A review of the minor constituent congeneric groups in Boronia Absolute (FEMA 2167), Camphor Japanese White Oil (FEMA 2231), Cedar Leaf Oil (FEMA 2267), Orris Concrete Liquid Oil (FEMA 2829), Orris Root Extract (FEMA 2830), Rue Oil (FEMA 2995), Sage Oil (FEMA 3001), Wormwood Oil (FEMA 3116), Osmanthus Absolute and Perilla Leaf Oil (FEMA 4013) also indicates no genotoxic concern for the congeneric groups presented. These NFCs proceed to Step 5.

The constituent profiles of Hyssop Oil (FEMA 2591) and Tagetes Oil (FEMA 3040) contain Group 21B (Selected Allylalkoxybenzenes) constituents, including methyl eugenol and estragole which have an allylalkoxybenzene structural motif (see Fig. 5), which raises a genotoxicity concern (Rietjens et al., 2014a). The occurrence and estimated intake of methyl eugenol and estragole in Hyssop Oil (FEMA 2591) and estragole in Tagetes Oil (FEMA 3040) are shown in Table 4. These NFCs proceed to Step 4a.

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.

The structures of estragole and methyl eugenol share a common motif of a benzene ring substituted with a 2′-propenyl substituent located *para* to an alkoxy group. These allylalkoxybenzenes have been shown to be capable of forming DNA adducts upon bioactivation, in which cytochrome P450s catalyze the formation of a 1′-hydroxy metabolite followed by sulfation at this site by a sulfotransferase. Elimination of sulfate from the 1′-sulfoxy metabolites formed creates a DNA reactive species (Daimon et al., 1997; Herrmann et al., 2012, 2014; Jeurissen et al., 2004, 2007; Phillips et al., 1984; Randerath et al., 1984; Rietjens et al., 2005, 2014a; Ueng et al., 2004; Wiseman et al., 1987). Rodent studies have indicated that methyl eugenol and estragole are hepatocarcinogens at high dose levels (Miller et al., 1983; National Toxicology Program, 2000).

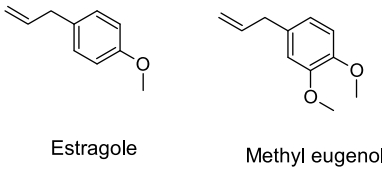


Fig. 5. Structures of estragole and methyl eugenol.

Table 4
NaturalA occurrence and estimated intake of Group 21B (Selected allylalkoxybenzenes) constituents estragole and methyl eugenol for the NFCs under consideration.

Name (FEMA No.)	Constituent of Concern	Mean %	Estimated Intake (μg/person/day)
Hyssop Oil (FEMA 2591)	Methyl eugenol	0.2	0.0002
Hyssop Oil (FEMA 2591)	Estragole	0.1	0.0001
Tagetes Oil (FEMA 3040)	Estragole	0.25	0.06

The direct addition of estragole and methyl eugenol, as well as the related allylalkoxybenzene safrole, as such to food is prohibited in the European Union and limits have been set for the presence of each in finished food categories (European Commission, 2008). In 2016, the FEMA Expert Panel removed methyl eugenol from the FEMA GRAS list, citing the need for additional data to clarify the relevance of DNA adducts formed by methyl eugenol in humans (Cohen et al., 2018b). Later, in October 2018, the United States Food and Drug Administration (FDA) food additive regulations were amended to no longer authorize the use of methyl eugenol as a synthetic flavoring substance and adjuvant for use in food (83 Fed. Reg. 50490. 9 October 2018) in response to a food additive petition. The FDA explained that the basis for the decision was "as a matter of law" on the "extraordinarily rigid" Delaney Clause of the Federal Food, Drug, and Cosmetic Act and further noted that based on the data evaluated, that "it is unlikely that consumption of methyl eugenol presents a risk to the public health from use as a flavoring substance" (83 Fed. Reg. 50490. 9 October 2018).

Estragole and methyl eugenol, as well as other allylalkoxybenzenes

such as safrole, myristicin and elemicin, are naturally occurring constituents in common culinary herbs and spices such as basil, tarragon, allspice, cinnamon, anise, nutmeg and mace. Regarding the natural occurrence of methyl eugenol in herbs, spices and their essential oils and extracts, the FEMA Expert Panel stated, "that these flavorings continue to meet the criteria for FEMA GRAS under their conditions of intended use as flavorings" (Cohen et al., 2018b). In their decision to amend the food additive regulations permitting the addition of synthetic methyl eugenol to food, the FDA states "… there is nothing in the data FDA has reviewed in responding to the pending food additive petition that causes FDA concern about the safety of foods that contain natural counterparts or extracts from such foods" (83 Fed. Reg. 50490. 9 October 2018). Similarly, the European Union established maximum levels for estragole, methyl eugenol and safrole in finished foods that have been flavored with flavorings and/or food ingredients in which these constituents occur naturally (European Commission, 2008).

As presented in Table 4, the estimated intakes of methyl eugenol and estragole from the consumption of Hyssop Oil (FEMA 2591) and estragole from the consumption of Tagetes Oil (FEMA 3040) range from 0.0002 to 0.06 μg/person/day. These values are less than the TTC for compounds with structural alerts for genotoxicity of 0.15 μg/person/day. The TTC of 0.15 μg/person/day is derived based on an analysis of the dose-response data for carcinogenic compounds (Kroes et al., 2004), provided by the Gold database of carcinogens[4] presenting the dose causing a 50% tumor incidence (TD_{50}) (Gold et al., 1984). By linear extrapolation of these TD_{50} data to a 1 in 1 million (1 in 10^6) tumor incidence, an exposure level of 0.15 μg/person/day, associated with a lifetime risk of cancer of 1 in 10^6 was determined as the TTC for compounds with structural alerts for genotoxicity (Kroes et al., 2004). In an EFSA/WHO review of the TTC approach, the 0.15 μg/person/day threshold was considered sufficiently protective for compounds with structural alerts for genotoxicity with the exclusion of high potency carcinogens (the Cohort of Concern) as specified by Kroes and co-workers (EFSA, 2016; EFSA Scientific Committee, 2019; Kroes et al., 2004; Nohmi, 2018). Because the estimated intake for each of the constituents of concern for Hyssop Oil (FEMA 2591) and Tagetes Oil (FEMA 3040) listed in Table 4 is less than the TTC of 0.15 μg/person/day for compounds with structural alerts for genotoxicity, the presence of these constituents does not raise a safety concern and these NFCs proceed to Step 5.

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.

Yes, for the NFCs under consideration, the total estimated intake for each of the congeneric groups present in each NFC is less than the corresponding TTC when applied to the appropriate class of toxic potential (Cramer et al., 1978). These thresholds do not apply for Group 21B (Selected Allylalkoxybenzenes) constituents with genotoxic potential such as methyl eugenol and estragole, which were evaluated in Steps 4 and 4a. All the NFCs under consideration proceed to Step 7.

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 exposure (MOE) 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.

Not Required at Step 5.

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 daily *per capita* intakes for the group of unidentified constituents reported for each NFC under consideration are listed in Table 5 and in Appendix A.

Table 5
Estimated intake of unidentified constituents.

Name	FEMA No.	Estimated daily intake (μg/person/day)
Boronia Absolute	2167	0.08
Camphor Japanese White Oil	2231	0.9
Cedar Leaf Oil	2267	0.45
Hyssop Oil	2591	0.009
Orris Concrete Liquid Oil	2829	1.7
Orris Root Extract	2830	0.5
Rue Oil	2995	0.0009
Sage Oil	3001	29
Sage Oleoresin	3002	10–80
Tagetes Oil	3040	3
Wormwood Oil	3116	0.1
Osmanthus Absolute	3750	0.2
Perilla Leaf Oil	4013	0.005

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.

For the NFCs under consideration, except for Sage Oil (FEMA 3001) and Sage Oleoresin (FEMA 3002), consumption as food/spice cannot be determined or the NFC is not derived from a botanical commonly used as food. In the case of Sage Oil (FEMA 3001), as reported in Step 1, it is estimated that the annual consumption of sage oil from the consumption of sage leaves as food is significantly less than the annual consumption of

[4] Gold database currently maintained by Llasa Ltd. https://www.lhasalimited.org/products/lhasa-carcinogenicity-database.htm.

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

Sage Oil (FEMA 3001) as flavoring. For Sage Oleoresin (FEMA 3002), the estimated intakes of both the essential oil and resin fractions from use as flavoring are significantly lower that the estimated intakes for the consumption of sage leaves as food. As a result, Sage Oleoresin (FEMA 3002) proceeds to Step 13. The remaining NFCs 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, 2016; Kroes et al., 2004).*

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

If No, proceed to Step 10.

The unidentified constituents are not expected to belong to any TTC excluded classes. As previously discussed, this group of NFCs is collected from various flowers, leaves and woody plant fibers by steam distillation of solvent extraction. The oils are primarily composed of low molecular weight monoterpenoid and sesquiterpenoid ketone, alcohols, esters and hydrocarbons or products of the carotenoid pathway. Based on the identified constituents, production methods and current literature, it is not expected that the unidentified constituents would belong to TTC-excluded classes. 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.

For the NFCs listed in Table 5, with the exception of Hyssop Oil (FEMA 2591) and Tagetes Oil (FEMA 3040), the identified constituent profiles do not contain any constituents with potential genotoxicity and therefore do not give rise to concern about the potential genotoxicity of the unidentified constituents. In Step 4, it was determined that these NFCs are primarily composed of aliphatic ketones and mono- and sesquiterpenoid constituents that do not have genotoxic potential. The unidentified constituents are likely products of the isoprene and lipid oxidation pathways and are not likely to exhibit genotoxic potential.

The constituent profile of Hyssop Oil (FEMA 2591) contains low naturally occurring amounts of estragole and methyl eugenol and the constituent profile of Tagetes oil (FEMA 3040) contains low naturally occurring amounts of estragole which have suspected genotoxic potential, as discussed in Step 4. Allylalkoxybenzene compounds such as estragole, methyl eugenol, safrole, elemicin and myristicin are represented in the current mass spectral libraries and are readily detected and identified by GC-MS. These compounds may be part of the unidentified fraction at concentrations below the respective limit of detection (LOD). Depending on the analytical method employed to collect the data contributed to this safety evaluation, the LOD is estimated to be 0.01–0.1% of the NFC. The estimated intake of an unidentified constituent occurring at the upper end of this range, at a concentration of 0.1%, in the NFCs under consideration range from 0.00009 to 0.024 μg/person/day which is less than the TTC for compounds with structural alerts for genotoxicity of 0.15 μg/person/day. A review of available genotoxicity and toxicological studies on the NFCs under consideration is presented later in the manuscript. These studies reported no evidence of genotoxic potential. Based on these data, it is concluded that the unidentified constituents in the NFCs under consideration do not raise a concern for genotoxicity. These NFCs proceed to Step 11.

Step 10a

*Is the estimated intake of the group of unidentified constituents less than 0.*15 μg*/person/day? 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.

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.

Not Required.

Step 11

*Is the estimated intake of the unidentified constituents (calculated in Step 7) less than the TTC (Kroes et al., 2004; 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, as shown in Table 5, the estimated intake of the fraction of unidentified constituents for each NFC under consideration does not exceed the TTC for Structural Class III, 90 μg/person/day. These NFCs 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 to reduce the fraction of unidentified constituents. Resubmit for further evaluation.

Not required at Step 11.

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

[6] This can be based on (1) Expert judgment, (2) Nature of the identified ingredients, and/or (3) Knowledge on the production/extraction process (see also Koster et al. (2011) and EFSA (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 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).

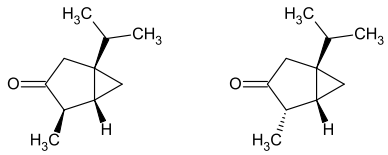
concern before proceeding to Step 14.

If No, proceed to Step 14.

The FEMA Expert Panel concurs with other food ingredient safety evaluation bodies that the TTC is applicable to the entire population (EFSA, 2012, 2016; EFSA Scientific Committee, 2019). An evaluation to consider possible exposure of children, given their lower body weights, and the potential for toxicokinetic and toxicodynamic differences as compared to adults, was conducted for each NFC under consideration. The NFCs under consideration would not be added to foods consumed by infants (CAC, 2007; CAC, 2017; CAC, 2019), indicating that exposure of infants is not expected.

The estimated intakes of naturally occurring potential genotoxic constituents in Group 21B (Selected Allylalkoxybenzenes) from Hyssop Oil (FEMA 2591) and Tagetes Oil (FEMA 3040) were considered. For Hyssop Oil (FEMA 2591), the estimated intakes of methyl eugenol and estragole were more than 700-fold lower than the TTC of 0.15 μg/person/day for compounds with structural alerts for genotoxicity. For Tagetes Oil (FEMA 3040), the estimated intake of estragole of 0.06 μg/person/day is less than 3-fold below the TTC of 0.15 μg/person/day for compounds with structural alerts for genotoxicity, exceeding the TTC for individuals with lower body weights. In cases where the intake of a naturally occurring genotoxic carcinogen from food exceeds the TTC for genotoxic substances, the FEMA Expert Panel applies a Margin of Exposure (MOE) approach (EFSA, 2009). The MOE is calculated based on the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence ($BMDL_{10}$) determined from the mathematical modeling of *in vivo* study data on tumor formation in experimental animals. For this safety evaluation, a $BMDL_{10}$ value for estragole of 10 mg/kg bw/day was calculated with BMD modeling using Bayesian algorithms and model averaging (Davidsen et al., 2023b). The MOE for the estimated intake of estragole from the consumption of Tagetes Oil (FEMA 3040) is 10,000,000, greatly exceeding the threshold of 10,000 that the FEMA Expert Panel, in agreement with EFSA, determined to be of low public health concern and of low priority for risk management actions (EFSA, 2005, 83 Fed. Reg. 50490. 9 October 2018). Because the MOE determination is based on carcinogenicity studies conducted over the lifetime of the test animal, the MOE analysis is considered to apply across the human lifetime. In summary, the estimated intakes of estragole and methyl eugenol in Hyssop Oil (FEMA 2591) and the estimated intake of estragole in Tagetes Oil (FEMA 3040) are either more than 3-fold below the TTC for compounds with structural alerts for genotoxicity or have MOEs substantially greater than 10,000 and therefore these occurrences do not raise a concern for intake of the respective NFCs as flavoring for children.

With the exception of Sage Oil (FEMA 3001), the estimated intake of the constituent congeneric groups for each NFC is substantially less than the corresponding TTC for the group, with none close to the TTC threshold, indicating no concern for intake by children. For Sage Oil (FEMA 3001), the estimated intake of Group 10 (Alicyclic ketones, secondary alcohols and related esters) constituents exceeds the TTC for structural Class II when considering the lower body weights of children. α-Thujone and β-thujone (Fig. 6) are major Group 10 constituents in Sage Oil (FEMA 3001). Based on dose-response modeling of effects in the National Toxicology Program's 2-yr toxicity study, a lower confidence limit for a benchmark response of 10% ($BMDL_{10}$) of 11 mg/kg bw/day was calculated for thujone based on the occurrence of clonic seizures in male rats (Lachenmeier and Uebelacker, 2010; NTP, 2011). This study is summarized below in the section "Biochemical and Toxicological Supporting Information Relevant to the Safety Evaluation". Based on this $BMDL_{10}$, an MOE of greater than 2000 is calculated for the estimated intake of Group 10 constituents in Sage Oil (FEMA 3001), which is also protective for children, considering their lower body weights. Since the known health effect of concern for thujone is potential neurotoxicity, an MOE greater than 100 is likely sufficiently protective. Therefore, the consumption of α- and β-thujones from these NFCs is of low concern for children from a public health point of view and of low priority for risk management actions.



α-Thujone
Group 10

β-Thujone
Group 10

Fig. 6. Stuctures of α- and β-thujone.

In addition, due to concerns for neurotoxicity from intake of α- and β-thujone, these substances are regulated. Historically, absinthism, a condition characterized by neurological problems and mental deterioration, was associated with the consumption of absinthe, an alcoholic beverage prepared with α,β-thujone-containing wormwood extracts (Dolan et al., 2010). In the United States, the Federal Code prohibits direct addition of thujone to food. Finished foods flavored with the essential oils of plants that naturally contain thujone (e.g., wormwood, white cedar, oak moss, and tansy) must be "thujone-free", i.e. contain less than 10 ppm of thujone (Dolan et al., 2010; FDA, 2018; Manfreda, 2007). In 1979, the Codex Alimentarius Commission (CAC) proposed the following maximum thujone limits in food and beverages: 0.5 mg/kg for ready-to-eat foods and beverages in general; 5 mg/kg in alcoholic beverages containing less than 25% vol. ethanol; 10 mg/kg in alcoholic beverages above 25% vol. ethanol; 25 mg/kg in food containing sage; 35 mg/kg in bitters and 250 mg/kg in sage stuffings (CAC, 1979). With the exception of the 250 mg/kg limit for sage stuffings, the CAC proposal was introduced into the European Union law in 1988 (EEC, 1988), which re-legalized the production of absinthe from wormwood as well as the food use of other thujone-containing plants. Later studies on the toxicity of α,β-thujone and on the composition of absinthe liquors raised doubts that α,β-thujone was the causative agent in absinthism and suggested that ethyl alcohol or impurities therein more likely caused the symptoms (Lachenmeier and Uebelacker, 2010). Currently in the European Union, there are limits on the levels of thujone in allowed finished foods containing flavor ingredients: 10 mg/kg in alcoholic beverages, except those produced from *Artemisia* species, 35 mg/kg in alcoholic beverages produced from *Artemisia* species and 0.5 mg/kg in non-alcoholic beverages produced from *Artemisia* species. There are currently no limits for thujones in sage stuffings in the European Union (European Commission, 2008).

Because sage teas and infusions for oral use are used in herbal medicine in Europe, the European Medicines Agency (EMA) has reviewed the metabolism and toxicity of α,β-thujone (EMA, 2012) and has published a monograph on *S. officinalis* (EMA, 2016) recommending a daily exposure to α,β-thujone of less than 6 mg from medicinal herbal products. This limit was derived by the Committee on Herbal Medicinal Products (HMPC) based on an analysis of study data and the consideration of the dietary intake from food. The first study cited is a study in rats in which thujone was administered by oral gavage at doses of 0, 5, 10 or 20 mg/kg bw/day 6 times per week for 14 weeks and a NOEL for convulsions of 10 mg/kg for male rats and 5 mg/kg for female rats was reported (Margaria, 1963). HMPC applied an uncertainty factor of 100 to this NOEL of 5 mg/kg for female rats and, assuming a body weight of 70 kg (HMPC criteria) and calculated a safe limit for thujone intake of 3.5 mg/person/day. The second study cited is a benchmark dose analysis that determined a $BMDL_{10}$ of 11 mg/kg bw/day for the occurrence of clonic seizures in male rats administered thujone in a chronic study (Lachenmeier and Uebelacker, 2010; NTP, 2011). An uncertainty factor of 100 was used to calculate an acceptable daily intake of 0.11 mg/kg bw, yielding a limit dose of 6.6 mg/day for a 60 kg person (Lachenmeier and Uebelacker, 2010). Based on the safe limits calculated from these studies, 3.5 mg/day – 6.6 mg/day, HMPC stated that an intake in the

range between 3 and 7 mg/day does not pose special concerns. However, for the upper limit of the additional intake from medicinal products, the highest safe amount should be reduced by the possible intake via food (estimated to be approximately 1 mg/day), which resulted in the recommended limit of 6 mg/person/day (EMA, 2012). The expected intake of α,β-thujone from the consumption of Sage Oil (FEMA 3001) is 180 μg/person/day, significantly less than the limit proposed by the EMA indicating no safety concern. Given this margin and the self-limiting nature of sage oil, there is no safety concern for consumption by children, considering their lower body weights.

The FEMA Expert Panel conducted an additional analysis, identifying sources of uncertainty in Appendix B. Within the safety evaluation and the discussions below, the limitations in data and methodology applied are discussed. As discussed throughout the safety evaluation procedure, conservatism is incorporated into the evaluation with the use of the TTC approach (Kroes et al., 2000; Munro et al., 1996), safety factors to account for inter-species and intra-individual variability in the calculation of MOE and in the use of the TTC and MOE approaches to evaluate potential genotoxic constituents.

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.

The FEMA Expert Panel has determined that the following NFCs are affirmed as GRAS, under conditions of intended use as flavoring ingredients: Boronia Absolute (FEMA 2167), Camphor Japanese White Oil (FEMA 2231), Cedar Leaf Oil (FEMA 2267), Hyssop Oil (FEMA 2591), Orris Concrete Liquid Oil (FEMA 2829), Orris Root Extract (FEMA 2830), Rue Oil (FEMA 2995), Sage Oil (FEMA 3001), Sage Oleoresin (FEMA 3002) Tagetes Oil (FEMA 3040), Wormwood Oil (FEMA 3116), Osmanthus Absolute (FEMA 3750) and Perilla Leaf Oil (FEMA 4013).

7. Biochemical and Toxicological Supporting Information Relevant to the safety evaluation

The NFCs considered in this manuscript are characterized by the presence of Group 10 (Alicyclic ketones, secondary alcohols and related esters), Group 8 (Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters) and/or Group 7 (Saturated alicyclic primary alcohols, aldehydes, acids and related esters) constituents. The FEMA Expert Panel has published safety evaluations for Group 10 flavoring ingredients as well as an evaluation of the genotoxicity of *p*-mentha-1,8-dien-7-al, a Group 7 constituent and major constituent of Perilla Oil (FEMA 4013) (Adams et al., 1996; Cohen et al., 2016, 2020). The Panel has also published safety assessments for Group 19 (Aliphatic and aromatic hydrocarbons), Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) and Group 3 (Aliphatic linear and branched-chain alpha, beta-unsaturated aldehydes and related alcohols acids and esters) flavoring ingredients, as well as an evaluation of the toxicity and genotoxicity of eucalyptol, a Group 23 (Aliphatic and aromatic ethers) constituent, and Group 21B (Selected Allylalkoxybenzenes) constituents which are also represented in the NFCs under consideration (Adams et al., 2008a, 2011; Cohen et al., 2019; Davidsen et al., 2023b; Eisenbrand et al., 2021; Fukushima et al., 2020; Marnett et al., 2014; Rietjens et al., 2014a).

Furthermore, the Panel has published evaluations of several congeneric groups of flavoring ingredients that are present in some of the NFCs under consideration: Group 15 (Hydroxy- and alkoxy-substituted benzyl derivatives) and Group 13 (Aliphatic, alicyclic, alicyclic-fused and aromatic-fused ring lactones) (Adams et al., 1998, 2005). Toxicity studies for Group 1 (Saturated aliphatic, acyclic, linear primary alcohols, aldehydes, carboxylic acids and related esters), Group 3 (Aliphatic linear and branched-chain α,β-unsaturated aldehydes and related alcohols, acids and esters), Group 5 (Unsaturated linear and branched-chain aliphatic, non-conjugated aldehydes, related primary alcohols, carboxylic acids and esters), Group 7 (Saturated alicyclic primary alcohols, aldehydes, acids and related esters), Group 8 (Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters) and Group 25 (Furan derivatives) flavoring ingredients have been published by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 1997, 1998, 1999, 2003, 2004, 2006, 2007, 2011, 2012).

A discussion of studies on Group 7, Group 8 and Group 10 constituents of the NFCs under consideration that have become available since the publication of the Panel's last evaluation of flavoring ingredients in these groups is presented below. Studies on the NFCs are also discussed. A summary of genotoxicity studies reviewed is provided in Table 6.

7.1. Group 7 constituents: genotoxicity

7.1.1. p-Mentha-1,8-dien-7-al (FEMA 3557)

7.1.1.1. Genotoxicity. The FEMA Expert Panel conducted a review of *in vitro* and *in vivo* genotoxicity studies for *p*-mentha-1,8-dien-7-al (also known as perillaldehyde) and concluded that *p*-mentha-1,8-dien-7-al does not raise a genotoxic concern (Cohen et al., 2016). In this evaluation, a Good Laboratory Practice (GLP) and OECD guideline-compliant combination study consisting of an *in vivo* comet assay in male rats with analysis in the liver and duodenum and an *in vivo* micronucleus assay with analysis of micronucleus induction in polychromatic erythrocytes of the bone marrow was reviewed (Beevers, 2014). The study authors concluded that there was no increase in micronucleated polychromatic erythrocytes of the bone marrow of male rats following oral gavage administration of doses up to 700 mg/kg/day. In the *in vivo* comet assay, a small but statistically significant increase in DNA damage in the liver was detected in the comet assay at the highest dose (700 mg/kg bw/day). Considering that DNA migration was not observed at the lower doses tested (175 and 350 mg/kg/day) and that the increase at the high dose was concomitant with changes in the liver enzymes and evidence of perturbation of hepatocyte function, the DNA damage may be due to a mechanism other than genotoxicity. The FEMA Panel concurred with the conclusion of the study authors that *p*-mentha-1,8-dien-7-al was not genotoxic under the conditions of these assays (Beevers, 2014; Cohen et al., 2016). Following the Panel's publication, a pathology peer-review was conducted that included a blinded review of the slides of liver tissues collected during the study. This review verified that precursor tissue changes indicative of hepatocellular toxicity were observed in the livers of rats in the 700 mg/kg/day dose group and noted that the comet assay % tail intensity results fell within the laboratories historical control range. Based on these observations, the peer-review group concurred that the results in the high dose group in the *in vivo* comet assay are secondary to toxicity and not a direct effect of treatment with *p*-mentha-1,8-dien-7-al (Maronpot et al., 2018).

Another GLP Ames assay was conducted in accordance with Japan's Industrial Safety and Health Act test guidelines along with an OECD guideline-compliant TGR assay using Muta® mice (Honma et al., 2021). In the Ames test, *p*-mentha-1,8-dien-7-al was tested in *S. typhimurium* strains TA100, TA98, TA1535 and TA1537 and *E. coli* WP2*uvrA* at concentrations of 0, 10, 19.5, 39, 78, 156, 313, 625 and 1250 μg/plate with and without S9 metabolic activation. Treatment with *p*-mentha-1,8-dien-7-al did not result in an increased number of revertant colonies in any strain in the presence or absence of S9 metabolic activation. Cytotoxicity was observed at $\geq$ 313 μg/plate for all treatments. In the *in vivo* transgenic rodent gene mutation (TGR) assay, male Muta® Mice (CD2-LacZ80/HazfBR) (6/group) were administered *p*-mentha-1,8-dien-7-al at 0 (corn oil), 125, 250, 500 or 1000 mg/kg/day by oral gavage for 28 days. The liver and glandular stomach were collected 3 days following the final treatment and genomic DNA was extracted from each. The mutant frequency (MF) was estimated using *lacZ* positive selection. Five mice each from the control and the 250, 500, and 1000

Table 6
Summary of genotoxicity studies.

Name of Substance Tested	Test Type (System)	Concentrations/Doses Tested	Results	Reference
a. Group 7 Constituents				
Perillaldehyde	Bacterial reverse mutation in *S. typhimurum* TA100, TA98, TA1535, TA1537, and *E. coli* strain WP2*uvr*A[a]	Up to 1250 μg/plate	Negative[a]	Honma et al., 2021
b. Group 8 Constituents				
2-Nonanone	*In vitro* comet assay human lung carcinoma epithelial A549 cells	6 and 14 mM	Negative	Kreja and Seidel, 2002
2-Nonanone	*In vitro* comet assay Chinese hamster V79 cells	6 and 14 mM	Negative	Kreja and Seidel, 2002
2-Nonanone	*In vitro* micronucleus assay human lung carcinoma epithelial A549 cells	6 and 14 mM	Negative[b]	Kreja and Seidel, 2002
2-Nonanone	*In vitro* micronucleus assay Chinese hamster V79 cells	6 and 14 mM	Negative[b]	Kreja and Seidel, 2002
2-Undecanone	Bacterial reverse mutation in *S. typhimurum* TA100, TA98, TA1535, TA1537, and *E. coli* strain WP2*uvr*A[a]	Up to 5000 μg/plate	Negative[a]	ECHA, 2017b
2- Undecanone	*In vitro* chromosomal aberration in CHO cells[a]	Up to 25 μg/mL	Negative[a]	ECHA, 2006c
2- Undecanone	Mouse lymphoma L5178Y TK ± assay[a]	Up to 35 μg/mL[b] Up to 107 μg/mL[c]	Negative[a]	ECHA, 1995
Group 10 Constituents				
Borneol	Bacterial reverse mutation - *S. typhimurium* TA97, TA98, TA100[a]	1 mg/mL	Negative[a]	Azizan and Blevins, 1995
Borneol	Bacterial reverse mutation - *S. typhimurium* TA98 and TA100, TA1535, TA1537 and TA1538[a]	Up to 5000 μg/plate	Negative[a]	Simmon et al., 1978
Borneol	Bacterial reverse mutation *E. coli* WP2 *uvrA* (trp-)[b]	0.4–3.2 mg/plate	Negative[b]	Yoo, 1986
l-Borneol	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA1535 and TA1537 and *E. coli* WP2*uvr*A[a]	5, 15, 50, 150, 500, 1500 μg/plate	Negative[a]	Bowles, 2013
Borneol	Rec assay – *B. subtilis* M45- and H17+[b]	Up to 10 mg/disc	Positive[b]	Yoo, 1986
l-Borneol	*In vitro* mammalian cell micronucleus assay in HPBL[a]	25-475 μg/mL[b,d] 55-600 μg/mL[c,d] 5-100 μg/mL[b,e]	Negative[a]	Roy, 2013
Borneol	*In vitro* comet assay - primary rat hepatocytes	0, 0.5, and 3 mM	Negative at concentrations < IC_{50}	Horváthová et al., 2009
Isobornyl acetate	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537[a]	Up to 316 μg/plate	Negative[a]	ECHA, 2019a
Isobornyl acetate	Mammalian *hrpt* gene mutation assay in Chinese hamster lung fibroblasts (V79)[a]	Up to 200 μg/mL	Negative[a]	ECHA, 2019b
Camphor	Bacterial reverse mutation - *S. typhimurium* TA97a, TA98, TA100 and TA102	1–2000 μg/plate	Negative[a]	Gomes-Carneiro et al., 1998
d-Camphor	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA1535 and TA1538[c]	4, 20, 100, 500 and 2500 μg/plate	Negative[c]	Anderson and Styles, 1978
d-Camphor	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537[a]	Up to 50 μg/plate[b] Up to 150 μg/plate[c]	Negative[a]	Marzin, 1998
d-Camphor	Bacterial reverse mutation - *S. typhimurium* TA97, TA98, TA100 and TA1535[a]	Up to 667 μg/plate	Negative[a]	NTP, 1992a
Camphor	Mouse lymphoma L5178Y TK ± assay[a]	0.1–4.5 mM	Negative[a]	ECHA, 2013
d-Camphor	*In vitro* chromosomal aberration in CHO cells[a]	250–1500 μg/mL[b] 500–600 μg/mL[c]	Negative[a]	NTP, 1992a
Dihydrotagetone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537[a]	Up to 5000 μg/plate	Negative[a]	Bowles, 2008
Dihydrotagetone	*In vitro* chromosomal aberration assay in HPBL[a]	24–385 μg/mL[a,d] 24–385 μg/mL[b,e]	Negative[a]	Morris, 2009
Fenchone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100 TA1535 and TA1537 and *E. coli* WP2*uvr*A[a]	Up to 5000 μg/plate	Negative[a]	Thompson, 2014
Fenchone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537[a]	Up to 10,000 μg/plate	Negative[a]	ECHA, 2006b; Seifried et al., 2006
Fenchone	Mouse lymphoma L5178Y TK ± assay[a]	250, 500, 600 and 650 μg/mL	Negative[b] Positive[c]	ECHA, 2006a; Seifried et al., 2006
Fenchone	*In vitro* micronucleus– HPBL[a]	190–1520 μg/mL[a,d] 190–1520 μg/mL[b,e]	Negative[a]	Morris, 2014
α-Ionone	Bacterial reverse mutation - *S. typhimurium* TA98 and TA100[a]	0.01–50 μg/plate	Negative[a]	Kasamaki et al., 1982
α-Ionone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537[a]	0.3–5000 μg/plate (test 1); 156–5000 μg/plate or 78–2500 μg/plate (test 2); 20–1250 μg/plate (test 3)	Negative[a]	Bowen, 2011
α-Ionone	*In vitro* chromosomal aberration assay in Chinese hamster B241 cells[a]	25 μg/mL	Positive[a]	Kasamaki et al., 1982
α-Ionone	*In vitro* micronucleus – HPBL[a]	160, 170, 180 μg/mL[c,d] 40, 50 and 65 μg/mL[b,e];	Negative[a]	Lloyd, 2013

(*continued on next page*)

Table 6 (*continued*)

Name of Substance Tested	Test Type (System)	Concentrations/Doses Tested	Results	Reference
β-Ionone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100 TA1535 and TA1537[a]	570 μg/plate	Negative[a]	Florin et al., 1980
β-Ionone	Bacterial reverse mutation preincubation - *S. typhimurium* TA98 TA100 TA1535 and TA98 or TA1537[a]	1–180 μg/plate	Negative[a]	Mortelmans et al., 1986
β-Ionone	Bacterial reverse mutation - *S. typhimurium* TA98 TA100 TA1535 TA1537 and TA102	Up to 1000 μg/plate	Negative[a]	Ballantyne, 2011
β-Ionone	*In vitro* micronucleus – HPBL[a]	30, 50, 60 μg/mL[b,d] 80, 100,120 μg/mL[c,d] 5, 15, 17.5 μg/mL[b,e]	Negative[a]	Stone, 2011
Ionone – mixed isomers	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537[a]	10–5000 μg/plate	Negative[a]	Sokolowski, 2004
Ionone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538[a]	33–5000 μg/plate	Negative[a]	Richold and Jones, 1980
Ionone	*Umu* test – *S. typhimurium* TA1535 (*umuC'-'lacZ*)	490 μg/ml	Positive[b]	Ono et al., 1991
α-Irone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537[a]	33–5000 μg/plate	Negative[a]	Sokolowski, 2000
β-Irone	Bacterial reverse mutation - *S. typhimurium* TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2*uvr*A	0.15–5000 μg/plate	Negative[a]	Thompson, 2012
α,β-Thujone	Bacterial reverse mutation - *S. typhimurium* TA97, TA100, TA1535 and *E. coli* strain WP2*uvr*A/pKM101	1–1000 μg/plate	Negative[a]	NTP, 2011
α -Thujone	Bacterial reverse mutation - *S. typhimurium* TA97, TA100, TA1535 and *E. coli* strain WP2*uvr*A/pKM101	10–10,000 μg/plate	Negative[a]	NTP, 2011
in vivo				
Perillaldehyde	*In vivo* transgenic rodent gene Mutation (TGR) assay	125–1000 mg/kg/day	Negative	Honma et al., 2021
α-Ionone	*In vivo* micronucleus test – ICR mice – femoral bone marrow (i.p.)	Up to 1200 mg/kg bw	Negative	Krsmanovic and Huston, 2006; McGinty et al., 2007
β-Ionone	*In vivo* micronucleus assay – male NMRI mice – femoral bone marrow (i.p.)	250, 500, 750 mg/kg bw (24 h) 750 mg/kg bw (48 h)	Negative	Engelhardt and Leibold, 2003
α,β-Thujone	*In vivo* micronucleus assay – male and female B6C3F1 mice	6.25–50 mg/kg bw/day	Negative -male Positive - female	NTP, 2011
b. Natural Flavor Complexes				
in vitro				
Camphor Japanese White Oil	Bacterial reverse mutation – *S. typhimurium* TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2*uvrA*	Up to 5000 μg/plate	Negative[a]	ECHA, 2014
Cedar leaf oil	Bacterial reverse mutation – *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2[a]	50–5000 μg/plate	Negative[a]	ECHA, 2017a
Hyssop oil	Bacterial reverse mutation – *S. typhimurium* TA98 and TA100[a]	93, 185, 463 μg/plate	Negative[a]	De Martino et al., 2009
Orris root extract (tincture)	Bacterial reverse mutation – *S. typhimurium* TA 98, TA100, TA1535, TA1537, TA1538[a]	0.1–150 μL/plate	Negative[a]	Heck et al., 1989b; Jagannath, 1984
Orris root extract (resinoid)	Bacterial reverse mutation – *S. typhimurium* TA 98, TA100, TA1535, TA1537, TA1538[a]	Up to 5000 μg/plate	Negative[a]	Mulky, 1985
Rue tincture	Bacterial reverse mutation – *S. typhimurium* TA98 and TA100[a]	10–120 μg/plate	Positive[f]	Paulini et al., 1987; Schimmer et al., 1994
Sage oil	Bacterial reverse mutation – *S. typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	23–9100 μg/plate	Negative[a]	Brusick, 1982; Heck et al., 1989a
Sage oil	Bacterial reverse mutation – *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvrA*[a]	50–5000 μg/plate	Negative[a]	ECHA, 2018
Sage oil	Bacterial reverse mutation – *S. typhimurium* TA 98, TA100, TA1535, TA1537[a]	230, 460, 910 μg/plate	Negative[a]	Zani et al., 1991
Sage oil	Bacterial reverse mutation – *S. typhimurium* TA102[a]	Up to 4570 μg/plate	Negative[a]	Vukovic-Gacic et al., 2006
Sage oil	Bacterial reverse mutation – *S. typhimurium* TA98 and TA100[a]	90, 180, 460 μg/plate	Negative[a]	De Martino et al., 2009
Sage extract	Bacterial reverse mutation – *S. typhimurium* TA98 and TA100	10,000 μg/plate	Negative[b]	Mahmoud et al., 1992
Sage oil	Rec assay *Bacillus subtilis*	10, 30 μL/mL	Negative	Zani et al., 1991
Sage oil	Rec assay *Bacillus subtilis*[a]	10 mg/disk	Equivocal[b] Positive[c]	Ueno et al., 1984
Sage oil	Mouse lymphoma assay – TK ± L5178Y[a]	3.6–90 μg/mL[b] 2–46 μg/mL[c]	Negative[b]; Positive[c]	Cifone, 1982; Heck et al., 1989a
Sage oil	Mouse lymphoma assay – TK ± L5178Y[a]	9–73 μg/mL[b] 0.5–55 μg/mL[c]	Negative[b]; Positive[c]	Cifone, 1984; Heck et al., 1989a
Sage oil	Unscheduled DNA synthesis test – rat primary hepatocytes	0.9–730 μg/mL	Negative	Curren, 1986

(*continued on next page*)

Table 6 (*continued*)

Name of Substance Tested	Test Type (System)	Concentrations/Doses Tested	Results	Reference
Wormwood oil	Bacterial reverse mutation assay *S. typhimurium* TA98, TA100, TA1535, TA1537, *E. coli WP2uvrA*/pKM101[a]	Up to 1600 μg/plate	Negative[a]	Mee, 2017b
Wormwood oil	*In vitro* micronucleus induction assay– HPBL[a]	220–290 μg/mL[b,d] 80–370 μg/mL[c,d] 135–180 μg/mL[b,e]	Negative	Mee, 2017a
Osmanthus absolute	Bacterial reverse mutation assay *S. typhimurium* TA98, TA100, TA1535, TA1537, *E. coli WP2uvrA*/pKM101[a]	Up to 5000 μg/plate	Negative[a]	Mee, 2020
Perilla extract-methanolic	Bacterial reverse mutation assay – *S. typhimurium* TA98 and TA100[a]	Up to 100 mg/mL	Negative[g]	Morimoto et al., 1982
Perilla extract-aqueous	Bacterial reverse mutation assay – *S. typhimurium* TA98 and TA100[a]	Up to 100 mg/mL	Negative[a]	Morimoto et al., 1982
Perilla extract-methanolic	Rec assay – *B. subtilis*	Up to 100 mg/mL	Positive	Morimoto et al., 1982
Perilla extract-aqueous	Rec assay – *B. subtilis*	Up to 100 mg/mL	Negative	Morimoto et al., 1982
Perilla extract-aqueous	Bacterial reverse mutation assay – *S. typhimurium* TA97, TA98, TA100, TA102[a]	Up to 1000 μg/plate	Positive[h]	Fujita et al., 1990
Perilla extract-ether	Bacterial reverse mutation assay – *S. typhimurium* TA97, TA98, TA100, TA102[a]	Up to 1000 μg/plate	Negative[a]	Fujita et al., 1990
Perilla extract-ethanolic	Bacterial reverse mutation assay – *S. typhimurium* TA97, TA98, TA100, TA102[a]	Up to 1000 μg/plate	Negative[a]	Fujita et al., 1990
Perilla extract – Terpenoid constituents	Bacterial reverse mutation assay *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	Up to 200 μg/plate	Negative[a]	Pharmaceutical Affairs and Food Sanitation Council of Japan, 2004
Perilla extract – Terpenoid constituents	*In vitro* chromosomal aberration in Chinese hamster fibroblast cells	Up to 5 mg/mL	Negative[a]	Pharmaceutical Affairs and Food Sanitation Council of Japan, 2004
Perilla oil	*In vitro* chromosomal aberration in Chinese hamster fibroblast cells[b]	Up to 0.04 mg/mL	Equivocal[b]	Ishidate et al., 1984
in vivo				
Perilla extract – Terpenoid constituents	*In vivo* micronucleus assay ICR mice (oral.)	0, 500, 1000, 2000 mg/kg bw	Negative	Pharmaceutical Affairs and Food Sanitation Council of Japan, 2004
Sage oil	*In vivo* micronucleus assay – male ddY mice (i.p.)	0, 50, 100, 200 mg/kg bw	Negative	Hachiya, 1987

[a] With and without S9 metabolic activation system.
[b] Without S9 metabolic activation system.
[c] With S9 metabolic activation system.
[d] Short (3 or 4h) treatment time.
[e] Long or continuous (24 h) treatment time.
[f] Positive in TA98 with S9 metabolic activation. Negative in TA98 without S9 metabolic activation and negative in TA100 with and without S9.
[g] Negative in TA98 with S9 metabolic activation. Cytotoxic in TA98 without S9 and in TA100 with and without S9.
[h] Positive in all strains with and without S9 metabolic activation, except negative in TA98 with S9.

mg/kg bw/day dose groups were used for the mutation assays (note that in the 1000-mg/kg-day perillaldehyde treatment group, one death occurred on day 5 before the treatment). The MFs of *lacZ* genes in the glandular stomach and liver tissues from treated mice were not significantly greater than MFs of the transgenes in the corresponding tissues from the control mice. The authors concluded that the data showed no evidence of *in vivo* mutagenic potential of *p*-mentha-1,8-dien-7-al at doses up to 1000 mg/kg/day in mice.

7.2. Group 8 constituents: genotoxicity and developmental toxicity

7.2.1. 2-Nonanone (FEMA 2785)

7.2.1.1. Genotoxicity. The *in vitro* genotoxic, clastogenic and mutagenic potential of 2-nonanone were evaluated in assays using human lung carcinoma epithelial A549 cells and Chinese hamster V79 cells (Kreja and Seidel, 2002). The IC50 value (concentration resulting in 50% reduction of colony formation) for 2-nonanone was 2 mM. 2-Nonanone was negative for the induction of micronuclei in both cell lines at 6 and 14 mM in the absence of metabolic activation in a micronucleus assay. The comet assay was performed using 2-nonanone concentrations of 6 and 14 mM in both human lung carcinoma epithelial A549 cells and Chinese hamster V79 cells. Tail moments were not significantly different from the control at the concentrations tested in both cell lines (Kreja and Seidel, 2002). Due to the lack of a standardized procedure for the performance and evaluation of results of the *in vitro* comet assay, the relevance of the results cannot be assessed (Gooderham et al., 2020b).

7.2.2. 2-Undecanone (FEMA 3093)

7.2.2.1. Genotoxicity. In an OECD guideline-compliant bacterial reverse mutation assay, *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2*uvrA* were treated with 2-undecanone at concentrations up to 5000 μg/plate in both the presence and absence of an S9 metabolic activation system. There were no increases in the mean number of revertant colonies at any dose either with or without S9 (ECHA, 2017b). In an OECD guideline-compliant *in vitro* chromosome aberration study, 2-undecanone was tested in Chinese hamster ovary cells at concentrations up to 25 μg/mL both with and without S9 metabolic activation. There were no statistically significant increases in chromosome aberrations under the conditions tested (ECHA, 2006c). In a forward mutation assay in mouse lymphoma (TK locus) L5178Y cells, 2-undecanone was assayed at concentrations up to 35 μg/mL in the absence of an S9 metabolic activation system and up to 107 of 0.1–4.5 μg/mL in the presence of S9. 2-Undecanone was negative for mutagenicity under the conditions tested (ECHA, 1995).

7.3. Group 10 constituents: genotoxicity and developmental toxicity

7.3.1. Borneol (FEMA 2157)

7.3.1.1. Genotoxicity. Several Ames assays have been conducted to determine the mutagenicity of borneol. Borneol was negative for mutagenicity when concentrations of 1 mg/mL were incubated with *S. typhimurium* strains TA97, TA98 and TA100 both in the presence and absence of an S9 metabolic activation system derived from the livers of Aroclor 1254-induced rats (Azizan and Blevins, 1995). Mutagenicity was not observed when borneol was tested at concentrations up to 5000 μg/plate in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 both in the presence and absence of an S9 metabolic activation system (Simmon et al., 1978) or when borneol was tested at concentrations of 0.4–3.2 mg/plate in *Escherichia coli* strain WP2*uvrA* in the absence of S9 (Yoo, 1986). Mutagenicity was not observed when *l*-borneol was tested at concentrations ranging from 5 to 1500 μg/plate in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2*uvrA* with and without the addition of S9 metabolic activation derived from the livers of phenobarbitone/β-naphthoflavone induced rats (Bowles, 2013).

In a rec assay using *B. subtilis* strains M45-and H17+, borneol was positive for mutagenicity when tested at concentrations up to 10 mg/disc in the absence of metabolic activation (Yoo, 1986). The rec assay has not been standardized in an OECD guideline for genotoxicity testing, and OECD has noted that indicator tests such as the rec assay should be correlated to the results of other assays that measure DNA damage or mutagenicity that can be passed on to subsequent generations (OECD, 2015).

In a GLP and OECD guideline-compliant *in vitro* mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL), *l*-borneol was tested at concentrations of 25–475 μg/mL for 4 h in the absence of S9, 55–600 μg/mL for 4 h in the presence of S9 and 5–100 μg/mL for the non-S9 activated 24 h exposure. The S9 metabolic activation system was derived from the livers of Aroclor 1254-induced rats. *l*-Borneol was negative for the induction of micronuclei in both non-activated and S9-activated test systems in HPBL under the conditions tested (Roy, 2013).

In an *in vitro* comet assay, borneol (>99% pure) was incubated with primary rat hepatocytes at concentrations ranging from 0.5 to 3 mM for 2 h. At concentrations less than 2.5 mM borneol, no DNA damage was observed in primary rat hepatocytes (Horváthová et al., 2009). Significant ($p < 0.001$) increases in mean tail DNA (%), indicative of DNA damage, were observed at concentrations of 2.5 and 3 mM borneol, but a decrease in cell viability was observed at these higher concentrations, indicating that the response may be related to cytotoxicity. Due to the lack of a standardized procedure for the performance and evaluation of results of the *in vitro* comet assay, the relevance of the results cannot be assessed (Gooderham et al., 2020b).

7.3.2. Isobornyl acetate (FEMA 2160)

7.3.2.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, isobornyl acetate was tested in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 in the presence and absence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats. In both plate incorporation and pre-incubation assays, isobornyl acetate was not mutagenic at concentrations up to 316 μg/plate in both the presence and absence of S9 (ECHA, 2019a). Isobornyl acetate was also negative for mutagenicity in an OECD and GLP guideline-compliant *hrpt* gene mutation assay in Chinese hamster V79 cells in both the presence and absence of S9 metabolic activation. No mutagenicity was observed when V79 cells were treated with isobornyl acetate at concentrations up to the limit of cytotoxicity, 200 μg/mL in the presence and absence of S9 derived from the liver of Aroclor 1254-induced rats (ECHA, 2019b).

7.3.2.2. Reproductive and developmental toxicity. A GLP and OECD guideline compliant reproductive toxicity study was conducted to provide information regarding the effects of isobornyl acetate on gonadal function, estrous cycles, mating behavior, conception, parturition, lactation and the growth and development of offspring up to 60 days postpartum (Lewis, 2017; Politano et al., 2017). The study also provided preliminary data on potential developmental toxicity. The design of the study was an oral gavage, one-generation reproduction protocol in rats, with an evaluation through sexual maturity in the F1 generation. One hundred male and 100 female rats were assigned to four dosage groups consisting of 25 males and 25 females (0, 30, 100, and 300 mg/kg bw/day). The test substance or corn oil control was administered to male rats once a day starting 84 days before the cohabitation period, through the cohabitation period of up to 14 days and continuing until the day prior to euthanasia. Female rats were dosed once a day beginning 14 days before the cohabitation period, through the cohabitation period of up to 14 days, and continuing through the day of euthanasia (through day 25 of gestation for rats that did not deliver or day 22 of lactation for rats that delivered a litter). For P(F0) generation males, viability, clinical observations, body weights, feed consumption, mating/fertility, organ weights, gross/microscopic observations, and sperm assessments were evaluated. For P(F0) generation females, viability, clinical observations, body weights, feed consumption, estrous cycling, mating/fertility, natural delivery/litter observations, organ weights, gross/microscopic observations, and ovarian follicle counts were evaluated. For the F1 generation, viability, body weights, anogenital distances, sexual maturation, nipple eruption, and gross observations were evaluated. There were no deaths in either the P(F0) or F1 generation rats. P(F0) generation rats in the 300 mg/kg bw/day male and female groups showed some excess salivation that the authors attributed to the test substance. Body weight, body weight gain and feed consumption values were not affected by treatment with isobornyl acetate in any P(F0) or F1 generation rats. Isobornyl acetate did not cause effects on mating and fertility, reproductive or non-reproductive organ weights, or sperm motility or concentration in male P(F0) generation rats. Isobornyl acetate did not affect reproductive or non-reproductive organs, estrous cycles, mating or fertility parameters, or natural delivery in female P(F0) rats. The F1 generation showed no compound-related lesions or changes in body weight, body weight gain, feed consumption, or organ weights, effects on nipple eruption or effects on sexual maturation. A reproductive NOAEL in the P(F0) generation rats and a NOAEL for viability and growth of the F1 generation of greater than 300 mg/kg bw/day was determined (Lewis, 2011; Politano et al., 2017).

7.3.3. d-Camphor (FEMA 2230) and d,l-Camphor (FEMA 4513)

7.3.3.1. Genotoxicity. No evidence of mutagenicity was observed in an Ames assay when *S. typhimurium* strains TA97a, TA98, TA100, and TA102 were incubated for 72 h with (+) camphor at concentrations of 1–2000 μg/plate in the presence or absence of an S9 metabolic activation system derived from the livers of Aroclor 1254-induced rats (Gomes-Carneiro et al., 1998). Mutagenicity was not observed when camphor was tested at concentrations ranging from 4 to 2500 μg/plate in *S. typhimurium* strains TA98, TA100, TA1535 and TA1538 in the presence of an S9 metabolic activation system prepared from the livers of Aroclor 1254-induced rats (Anderson and Styles, 1978). In another Ames assay, mutagenicity was not observed when camphor was tested at concentrations ranging from 4 to 2500 μg/plate in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 at concentrations up to 50 μg/plate in the absence of S9 and up to 150 μg/plate in the presence of S9 prepared from the livers of Aroclor 1254-induced rats (Marzin, 1998). Camphor was also non-mutagenic in an Ames assay conducted in *S. typhimurium* strains TA97, TA98, TA100 and TA1535 at

concentrations up to 667 μg/plate in the presence and absence of S9 prepared from the livers of Aroclor 1254-induced rats (NTP, 1992a).

In an *in vitro* chromosomal aberration study in Chinese hamster ovary (CHO) cells, *d*-camphor was negative for the induction of aberrations at concentrations up to 1500 μg/mL in the absence of S9 metabolic activation and up to 600 μg/mL in the presence of S9 (NTP, 1992a). A GLP and OECD guideline-compliant forward mutation assay in mouse lymphoma (TK locus) L5178Y cells was also conducted to assess the mutagenicity of camphor. Camphor was assayed at concentrations of 0.1–4.5 mM in the presence and absence of metabolic activation and was negative for mutagenicity under the conditions tested (ECHA, 2013).

7.3.3.2. Developmental toxicity. Developmental toxicity of *d*-camphor was evaluated in Sprague-Dawley (SD) rats and Himalayan rabbits (Leuschner, 1997). In a reproductive and developmental toxicity study, sexually mature and mated female SD rats (20/dose) were administered *d*-camphor via oral gavage at doses of 0 (propylene glycol), 216, 464 and 1000 mg/kg bw/day during gestation days (GD) 6 through 17. Signs of toxicity, body weight and food intake were observed daily, and on GD 20, dams were euthanized and macroscopically examined, and their fetuses were examined for external, skeletal, and visceral abnormalities. There were no substance-related effects in the low dose dams. In the 464 and 1000 mg/kg bw/day groups, salivation and reduced food intake were observed. Necropsy revealed that 2 mid-dose dams and 5 high-dose dams developed ulcers in the cardiac region of the stomach. In the high-dose group, pronounced signs of toxicity including clonic convulsion, pilo-erection, reduced motility, and body weight gain were evident. Necropsy revealed one dam in the high-dose group that developed a thickened, rough gastric cardia. In fetuses, there were no substance-related variations, malformations, retardation or effects on prenatal fetal development. Based on these observations, the FEMA Expert Panel determined a NOAEL of 216 mg/kg bw/day for maternal toxicity and 1000 mg/kg bw/day for fetal developmental toxicity in SD rats.

In rabbits, there was no evidence of developmental toxicity when *d*-camphor was orally administered to pregnant Himalayan rabbits at doses of 0 (propylene glycol), 147, 316 and 681 mg/kg bw/day during gestation days (GD) 6 through 18. On GD 29, dams were euthanized. Macroscopic examinations of the dams and examination of the fetuses for external and skeletal anomalies were performed. Reduced body weight gain and reduced food consumption was observed in the 681 mg/kg bw/day dose group. No substance related effects were observed in the 147 or 316 mg/kg bw/day dose groups. There were no substance-related variations, retardations or malformations in the fetuses at any dose level. It was concluded that when orally administered during the fetal period of organogenesis, *d*-camphor elicits no teratogenic properties on prenatal fetal development in rats and rabbits (Leuschner, 1997). Based on these observations, the FEMA Expert Panel determined a NOAEL of 316 mg/kg bw/day for maternal toxicity and 681 mg/kg bw/day for fetal developmental toxicity in Himalayan rabbits.

In a developmental toxicity study, female SD rats were administered *d*-camphor on days 6–15 of gestation by oral gavage at doses of 0 (corn oil), 100, 400 or 800 mg/kg bw/day. Maternal signs of toxicity included increases in relative and absolute liver weights, decreases in food consumption, increased water consumption, and a decrease in weight gain at the highest dose level (800 mg/kg bw/day). There were no effects on fetal growth, viability or morphological development (NTP, 1992b). For this study in SD rats, the FEMA Expert Panel determined a NOAEL of 400 mg/kg bw/day for maternal toxicity and 800 mg/kg bw/day for fetal developmental toxicity. In a developmental study in rabbits, *d*-camphor was administered by oral gavage to pregnant rabbits at doses of 0 (corn oil), 50, 200 and 400 mg/kg bw/day on GD 6–19 (NTP, 1992c). There was a decreasing trend in maternal weight gain with increasing dose. There was no effect on fetal growth, viability or morphological development. The FEMA Expert Panel concurs with the NOAEL of 400 mg/kg bw per day (the highest dose tested) determined by the study authors for developmental and maternal toxicity.

An *in vitro* study on the effect of camphor reported a decrease in sperm motility and sperm viability at concentrations of 1–10% camphor, using a stock solution of 1:1 w/v in chloroform (Jadhav et al., 2010).

For an assessment of reproductive and developmental toxicity, 20-day old male BALB/c mice (6/group) were administered 30 mg/kg bw/day of *d,l*-camphor dissolved in olive oil via oral gavage for either 10 (group 1) or 20 (group 2) days. Control groups received daily equal amounts of olive oil as the experimental groups for the same periods of time, and one sham group received no intervention. At the end of the testing period all mice were anesthetized, and their testes were removed to obtain serial sections for histological staining. Serial sections from testes of each group were studied by light microscopy. Results indicated that there was less vascularization in testis tissue in the experimental groups than in the control group, and only a small portion of seminiferous tubules began canalization in the treatment groups. The non-canalized tubes showed a high density of cells and microscopic assessment showed that the interstitial tissue of the tubules was less developed than in the controls. Internal diameters of seminiferous tubules in experimental groups were significantly smaller in the treatment groups compared to the control. The number of released spermatocytes was significantly lower in experimental groups, and it appeared that maturation and release of spermatocytes was delayed and compressed to layers in the tubules near the central canal. The authors concluded that low doses of *d,l*-camphor can affect the development of testicular tissue and reduce proliferation of the seminiferous epithelium, therefore reducing reproductive function of the testes in mice; however, reproductive function was not tested in this study (Nikravesh and Jalali, 2004). The Panel indicated that this manuscript had serious limitations and does not support the author's conclusions due to a lack of use of appropriate terminology for germinal epithelial cells, inadequate use of nonbiased stereology and quantitation of tubules, lack of staging of tubules and identification of germinal epithelial cells, inadequate research methods and statistics and no measurements of body and organ weights and reproductive hormones. In addition, there was clear photographic evidence of spermatid maturation in the testes.

7.3.3.3. Summary of reproductive and developmental toxicity. Reproductive and developmental studies in rats and rabbits indicated maternal toxicity without effects observed in the offspring (Leuschner, 1997; NTP, 1992b, c). Most conservative NOAEL derived from these studies was 216 mg/kg bw/day for maternal toxicity reported in SD rats (Leuschner, 1997). Camphor was a reported constituent for several NFCs under consideration. Based on this NOAEL, an MOE of greater than 109,000 was calculated for the estimated intake of camphor from the consumption of Sage Oil (FEMA 3001) and an MOE of greater than 1.9 million was calculated for the estimated intake of camphor from the consumption of Sage Oleoresin (FEMA 3002) containing 30% volatile oil. For Cedar Leaf Oil (FEMA 2267) and Camphor Japanese White Oil (FEMA 2231), which contain only 2 and 0.5% camphor, respectively, the MOE values are greater than 92 million.

7.3.4. Dihydrotagetone

7.3.4.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, dihydrotagetone was non-mutagenic when tested at concentrations up to 5000 μg/plate in *S. typhimurium* strains TA98, TA100 TA1535 and TA1537, and *E. coli* strain WP2*uvrA* both in the presence and absence of an S9 metabolic activation system derived from the livers of rats induced with phenobarbitone/β-naphthoflavone (Bowles, 2008). In a GLP and OECD guideline-compliant *in vitro* chromosomal aberration assay in HPBL, dihydrotagetone was tested at concentrations of 24–385 μg/mL for 4 h in the absence and presence of an S9 metabolic activation system derived from the livers of rats induced with

phenobarbitone/β-naphthoflavone and for 24 h in the absence of S9 (Morris, 2009). Dihydrotagetone did not induce an increase in the frequency of cells with chromosomal aberrations and was non-clastogenic under the conditions tested.

7.3.5. Fenchone

7.3.5.1. Genotoxicity. No evidence of mutagenicity was observed in a GLP and OECD guideline-compliant reverse mutation assay in which *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and *E. coli* WP*uvrA* were treated with fenchone using both the Ames plate incorporation and pre-incubation methods with doses ranging from 50 to 5000 μg/plate both with and without the addition of an S9 metabolic activation system derived from the livers of phenobarbitone/β-naphthoflavone-treated male rats (Thompson, 2014). Fenchone was also negative in another OECD guideline-compliant reverse mutation assay in which *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were treated with fenchone in the presence and absence of an S9 metabolic activation system derived from the livers of Aroclor 1254-treated rats and hamsters. Fenchone was negative for mutagenicity when tested at concentrations up to 10,000 μg/plate in both the absence and presence of S9 metabolic activation (ECHA, 2006b; Seifried et al., 2006).

In an OECD guideline-compliant mouse lymphoma assay, fenchone was tested in a forward mutation assay in mouse lymphoma L5178Y TK ± cells at concentrations of 250, 500, 600 and 650 μg/mL in the presence and absence of an S9 metabolic activation system derived from the livers of Aroclor 1254-induced male SD rats. In the absence of S9, a greater than two-fold increase in the mutant frequency was observed at 650 μg/mL fenchone, a concentration in which the relative total growth of the cells was less than 10%, indicating significant cytotoxicity (OECD, 2016). In the presence of S9, a greater than two-fold increase in the mutant frequency was observed at concentrations of 500 μg/mL and higher. At concentrations of 500–600 μg/mL fenchone, the relative total growth of the cells was 15–38%. In summary, the study authors concluded that fenchone was negative for mutagenicity in mouse lymphoma L5178Y TK$^{\pm}$ cells in the absence of S9 and positive in the presence of S9 up to the limits of cytotoxicity (ECHA, 2006a; Seifried et al., 2006).

In a GLP and OECD guideline-compliant *in vitro* micronucleus study, fenchone was incubated with HPBL in 4h incubation experiments at concentrations ranging from 190 to 1520 μg/mL in the presence and absence of an S9 metabolic activation system derived from the livers of phenobarbitone/β-naphthoflavone-treated male rats. No increase in micronuclei formation was observed in the 4 h experiments at the concentrations tested. In a second experiment, fenchone was incubated for 24 h with HPBL in the absence of S9. A small but statistically significant increase in the frequency of micronuclei was observed in the 24 h experiment at 760 μg/mL fenchone. However, this dose level exceeded OECD guidelines for toxicity and the frequency of micronuclei induction was only marginally above the upper range of the vehicle historical control range and therefore considered to be not toxicologically significant. The authors concluded that fenchone was negative for induction of micronuclei under the conditions tested (Morris, 2014).

Based on a review of the results of genotoxicity studies of fenchone in which GLP and OECD guideline-compliant Ames and an *in vitro* micronucleus assay were negative and with due regard for the positive result in in a non-guideline mouse lymphoma L5178Y TK$^{\pm}$ cells in the presence of S9 metabolic activation at toxic concentrations, the FEMA Expert Panel determined, using the weight of evidence approach (Gooderham et al., 2020b), that there is no concern over the genotoxic potential of fenchone.

7.3.6. α-Ionone (FEMA 2594)

7.3.6.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, α-ionone was not mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the presence and absence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats. Assays included three plate incorporation experiments at concentrations ranging from 0.3 to 5000 μg/plate, 78–5000 μg/plate, and 20–2500 μg/plate in the presence and absence of S9. The authors concluded that α-ionone was negative for mutagenicity under the conditions tested (Bowen, 2011). α-Ionone was non-mutagenic in another Ames assay conducted in *S. typhimurium* strains TA98 and TA100 in the presence and absence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats at concentrations up to 50 μg/plate (Kasamaki et al., 1982). In an *in vitro* chromosomal aberration assay in Chinese hamster B241 cells, *α*-ionone increased the frequency of chromosomal aberrations at a concentration of 25 μg/mL in the presence and absence of S9 metabolic activation (Kasamaki et al., 1982).

In a GLP guideline-compliant *in vitro* micronucleus assay, α-ionone was tested for clastogenic and aneugenic potential in HPBL. α-Ionone was tested at concentrations of 40–65 μg/mL in 24 h treatments in the absence of S9 and 160 to 180 μg/mL in 3 h treatments in the presence of S9 metabolic activation. The highest concentrations analyzed for micronuclei induced 51% and 56% reductions in the replication index in the absence and presence of S9, respectively. α-Ionone did not induce micronuclei in HPBPL under the conditions tested (Lloyd, 2013).

In an OECD and GLP guideline-compliant *in vivo* micronucleus assay, ICR mice (5/sex/dose) were administered α-ionone at doses of 0, 300, 600 or 1200 mg/kg bw by intraperitoneal injection (i.p) and euthanized 24 h following treatment for the collection of femoral bone marrow. In a second experiment, ICR mice (5/sex/dose) were administered α-ionone at doses of 0 and 1200 mg/kg bw and euthanized 48 h following treatment. During the treatment period, lethargy and piloerection were observed in all mice in the 600 and 1200 mg/kg bw dose groups and hunched position was observed in all mice in the 1200 mg/kg bw dose group. A reduction in the ratio of the polychromatic erythrocytes to total erythrocytes in treated groups compared to controls was given as evidence of bioavailability of α-ionone to the bone marrow target tissue. Bone marrow cells were collected after either another 24 or 48 h and examined for micronucleated polychromatic erythrocytes. α-Ionone did not induce any statistically significant increases in the frequency of micronucleated PCEs under the conditions tested (Krsmanovic and Huston, 2006; McGinty et al., 2007).

7.3.7. β-Ionone (FEMA 2595)

7.3.7.1. Genotoxicity. β-Ionone was tested in an Ames assay with and without S9 activation using *S. typhimurium* strains TA98, TA100, TA1513, and TA1537 at a concentration of 570 μg/per plate. The results were negative (Florin et al., 1980). β-Ionone was negative for mutagenicity in an Ames test preincubation assay with and without S9 activation, in *S. typhimurium* strains TA1535, TA98, TA100, and TA97 or TA1537 at concentrations ranging from 1 to 180 μg/plate (Mortelmans et al., 1986). In an OECD and GLP guideline-compliant Ames assay, β-ionone was negative for mutagenicity in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the presence and absence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats at concentrations up to 1000 μg/plate (Ballantyne, 2011).

In a GLP guideline-compliant *in vitro* micronucleus assay, β-ionone was tested for clastogenic and aneugenic potential in HPBL. β-Ionone was tested at concentrations up to 60 μg/mL in 3 h treatments in the absence of S9 and concentrations up to 120 μg/mL in 3 h treatments in the presence of S9 metabolic activation. In a 24 h experiment, β-ionone was tested at concentrations up to 17.5 μg/mL in the absence of S9. The S9 metabolic activation system was derived from Aroclor 1254-induced

male SD rat livers. β-Ionone did not increase the frequency of micronuclei in HPBL when tested up to the limit of cytotoxicity in the presence and absence of S9 (Stone, 2011).

In an OECD and GLP guideline-compliant *in vivo* micronucleus assay, male NMRI mice (5/dose) were administered β-ionone at doses of 0 (olive oil), 250, 500 or 750 mg/kg bw by i.p injection and euthanized 24 h following treatment. In a second experiment, male NMRI mice (5/dose) were administered β-ionone at doses of 0 and 750 mg/kg bw by i.p injection and euthanized 48 h following treatment for the collection of femoral bone marrow. The study authors reported that administration of the test substance led to evident signs of toxicity. Bone marrow cells were examined for micronucleated PCEs. β-Ionone did not demonstrate any statistically significant increases in the frequency of micronucleated PCEs under the conditions tested and was therefore considered to be non-clastogenic and non-aneugenic to mouse erythrocytes *in vivo* (Engelhardt and Leibold, 2003).

7.3.7.2. Developmental toxicity. In a developmental toxicity study, β-ionone was evaluated at doses of 0, 125, 250, 500 or 1000 mg/kg bw/day given in corn oil by oral gavage to female Wistar rats (17–20 rats per group) on GD 6–15 (Pinto et al., 2018). The rats were terminated on gestation Day 21, the gravid uterus was removed and implantations, living and dead fetuses and resorptions were recorded. The living fetuses were weighed, and examined for external, skeletal and visceral abnormalities. Heart, thymus, liver, spleen, kidneys and lungs of sectioned fetuses were also weighed.

There were no unscheduled deaths during the study. At necropsy, no significant alterations in maternal organ weights and no gross abnormalities were observed in the dams in any of the treatment groups or controls. There were no significant differences in pregnancy weight gain or gravid uterus weights between the 125, 250 and 500 mg/kg bw/day treatment groups and controls. In the high dose group, a reduced net maternal weight gain and clinical signs including chromodacryorrhea (secretion of pigmented tears), piloerection and increased vocalization were observed. In addition, the percentage of rats that successfully copulated and had implantation sites detectable on GD 21 was only 60% in the 1000 mg/kg bw/day dose group compared to 94% in control animals. However, a low pregnancy rate, 73%, was also observed in the control group of the second experiment and thus may not have been treatment related. It is also possible that treatment at the high dose level of β-ionone impaired the ongoing implantation and/or induced very early post-implantation losses that were undetected at termination. The study authors concluded that the 1000 mg/kg bw/day dose was toxic to pregnant rats and exceeded the maximum tolerated dose. Under the conditions of the study, a NOAEL of 500 mg/kg bw/day was determined for maternal toxicity.

Examination of the gravid uterus showed no significant differences in the number of *corpora lutea graviditatis* per dam or number of implantation sites per pregnant female in the treatment groups compared to controls. *β*-Ionone did not significantly increase the frequency of malformations nor did it retard fetal growth, reduce the litter size, increase the average number of resorptions per implantations per litter or affect fetal body weights in any of the treatment groups compared to the control group. There were no significant differences in fetal organ weights between the treatment and control groups. There were incidental but significant increases in the incidence of extra liver lobes and skeletal abnormalities in the treatment groups, but these cases showed no dose-related effect and a high incidence of these abnormalities was also noted in the control or historical control groups. The sex ratio on GD21 slightly favored male over female fetuses in the high dose group. The authors concluded that except for higher embryo lethality at a toxic dose of 1000 mg/kg bw/day β-ionone did not cause an embryotoxic effect over the dose range up to 500 mg/kg bw/day. The authors determined a NOAEL for developmental toxicity of 500 mg/kg bw/day for β-ionone under the conditions of this study (Pinto et al., 2018).

In an additional experiment, the embryotoxicity of a single oral dose of β-ionone at 1000 mg/kg bw, given on gestation Day 11, was also evaluated. In this study, there was a reduction in overall and net (after subtracting gravid uterus weight) maternal weight gain and a significant decrease in the weight of the gravid uterus in treated females versus controls. Additionally, the percentage of whole litter losses and the percentage of resorptions per implantation rate were increased in treated rats compared to controls. Examination of fetuses showed no significant differences in the body weight of fetuses or incidences of fetal abnormalities between the treatment and control groups. The study authors concluded that the single 1,000 mg/kg bw/day dose at GD 11 resulted in material toxicity but did not disrupt key events of rat embryogenesis occurring at this point in the pregnancy (Pinto et al., 2018).

7.3.7.3. Subchronic toxicity. In a GLP and OECD guideline-compliant 90-day toxicity study, Wistar rats (10/sex/dose) were administered β-ionone mixed in the feed at dietary concentrations of 0 (control), 100, 1,000 or 10,000 ppm for 3 months. The target doses corresponded to 0, 7.1, 72 and 720 mg/kg bw/day in males and 0, 8.2, 83 and 800 mg/kg bw/day in females, respectively. At study termination, all surviving rats were fasted for 16 h and then euthanized and examined. At the lowest dose, no substance-related findings were obtained. At the middle dose, there were increased urinary casts and urinary transitional epithelial cells in males; a significant increase in the relative liver weight in males and the absolute and relative liver weights in females; centrilobular hypertrophy of hepatocytes in the liver of 3 males; and higher amounts of α_{2u}-globulin in tubular epithelial cells of the kidneys in males. At the highest dose, there was a significant increase in the absolute and relative liver weights in males and females and a significant increase of the absolute and relative kidney weights in males and of the relative kidney weight in females, centrilobular hypertrophy of hepatocytes in the liver of males and females and higher amounts of α_{2u}-globulin in tubular epithelial cells of the kidneys in males. There was an increase in γ-glutamyltransferase, calcium, total protein, albumin, globulins, cholesterol, urinary ketones and urinary transitional epithelial cells in urine of males and females. The significant increase in kidney weight in males was attributed to the accumulation of α_{2u}-globulin, a male rat-specific finding, and is known to be non-relevant to human health (Capen et al., 1999; Flamm and Lehman-McKeeman, 1991; Swenberg, 1993). The testes and epididymal weights of high dose males were significantly increased, but there was no histological correlation to demonstrate a substance-related effect. Female reproductive systems were unaffected and all groups showed no signs of neurotoxicity or decreased motor function in the Functional Observational Battery. Significant substance-related effects in the liver and kidneys in both male and female rats were apparent at the middle and high doses, but no signs of toxicity were observed in the low dose group. There were no mortalities in the treatment groups. Based on these observations, the study authors determined a NOAEL of 100 ppm (7.1 and 8.2 mg/kg bw/day in male and female Wistar rats, respectively), for β-ionone (Kaspers, 2004).

7.3.8. Ionone (mixed isomers)

7.3.8.1. Genotoxicity. A GLP and OECD guideline-compliant Ames assay was conducted with 100% ionone mixed isomers to investigate the potential of this compound to induce gene mutations using the plate incorporation test and the pre-incubation test using *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102 (Sokolowski, 2004). The assay was performed both with and without liver microsomal activation. Concentrations tested were 10, 33, 100, 333, 1000, 2500 and 5000 μg/plate. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment at any dose level in the presence or absence of S9 metabolic activation.

The "*umu*-test" was utilized to analyze the genotoxicity of ionone.

This procedure uses *S. typhimurium* strain TA1535 which has a plasmid carrying the fused gene *umuC'-'1acZ*. In the fused gene, the *umu* operon is induced by DNA-damaging agents and the intensity of the response after DNA damage is measured by β-galactosidase activity which is produced from the fused gene (Oda et al., 1985; Ono et al., 1991). For this assay, 0.2 mL (0.19 g) of ionone (resulting in a dose of 490 μg/ml/OD_{600}, where OD_{600} reflects the cell density before the assay) was incubated in the cell culture for 2 h, in both the presence and absence of S9 metabolic activation. The test was positive in the absence of S9 metabolic activation. In the presence of S9, prepared from livers of male rats induced with phenobarbital and 5,6-benzoflavone, treatment with ionone resulted in cell death. The assay was also run using a 24 h incubation period with a dose of 490 μg/ml/OD_{600} without S9 and 350 μg/ml/OD_{600} with S9. The authors concluded that ionone is genotoxic without S9 activation based on the results of the *umu*-test (Ono et al., 1991).

An Ames assay using *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 was conducted to assess the potential mutagenic effects of ionone. Ionone was tested at concentrations of 0, 9, 90, 900, and 9000 μg/plate.[8] The highest concentration was toxic to the cells, rendering the absence or incomplete formation of a bacterial lawn. There were no substantial increases in the revertant colony numbers of any of the five strains following treatment with ionone at any dose level, either in the presence or absence of liver S9 (Richold and Jones, 1980).

7.3.8.2. Summary on genotoxicity for α-Ionone, β-Ionone and Ionone (mixed isomers). OECD guideline-compliant Ames assays for α-ionone, β-ionone and ionone (mixed isomers) were negative for mutagenicity. α-Ionone was positive in a non-guideline *in vitro* chromosomal aberration assay and ionone (mixed isomers) was positive in the non-guideline "umu-test" in the absence of S9 metabolic activation (Kasamaki et al., 1982; Ono et al., 1991). However, α-ionone and β-ionone were negative for genotoxicity in OECD guideline *in vitro* micronucleus assays (Lloyd, 2013; Stone, 2011). In addition, α-ionone and β-ionone were negative in *in vivo* micronucleus assays when administered by i.p. injection (Engelhardt and Leibold, 2003; Krsmanovic and Huston, 2006; McGinty et al., 2007). In the α-ionone study, the reduction in the ratio of the polychromatic erythrocytes to total erythrocytes in treated groups compared to controls was given as evidence of bioavailability of α-ionone to the bone marrow target tissue. In the β-ionone study, the study authors noted that the administration of the test substance led to evident signs of toxicity. Based on the results of these assays, the FEMA Expert Panel determined, using the weight of evidence approach (Gooderham et al., 2020b), that there is no concern for the genotoxic potential of α-ionone, β-ionone and ionone (mixed isomers).

7.3.9. α-Irone (FEMA 2597)

7.3.9.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, mutagenicity was not observed when α-irone was tested at concentrations up to 5000 μg/plate in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, both in the presence and absence of an S9 metabolic activation system derived from the livers of phenobarbital/β-naphthoflavone induced rats (Sokolowski, 2000).

7.3.9.2. Subchronic toxicity. A 90-day feeding study of α-irone in cottonseed oil was conducted in groups of FDRL rats (15/sex/dose), at dose levels of 0 or 5.2 mg/kg bw/day for males and 0 or 5.9 mg/kg bw/day for females. Rats were euthanized on day 90 and a gross necropsy was conducted. There was no evidence of adverse toxicological effects in males. In females, there was a slight increase in efficiency of food utilization, hematocrit, hemoglobin, and lymphocytes. The researchers determined a NOAEL of 5.2 and 5.9 mg/kg bw/day for males and females, respectively (Oser et al., 1965).

7.3.10. β-Irone

7.3.10.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, mutagenicity was not observed when β-irone was tested at concentrations ranging from 0.15 to 5000 μg/plate in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA both in the presence and absence of an S9 metabolic activation system derived from the livers of rats induced with phenobarbitone/β-naphthoflavone. β-Irone was considered to be non-mutagenic under the conditions of the test (Thompson, 2012).

7.3.11. Thujone

7.3.11.1. Genotoxicity. Genetic toxicology studies with α-thujone and α,β-thujone were conducted in *S. typhimurium* strains and *E. coli* and mouse peripheral blood erythrocytes. α,β-Thujone (1–1,000 μg/plate) and α-thujone (10–10,000 μg/plate) were not mutagenic when tested in *S. typhimurium* strains TA97, TA98, TA100, TA1535 *or E. coli* WP2 *uvrA*/pKM101 both with and without exogenous metabolic activation provided by a rat or hamster liver S9 mix (NTP, 2011). In an *in vivo* micronucleus test, exposure of male mice daily for 3 months via gavage to α,β-thujone at 6.25–25 mg/kg bw/day did not result in an increase in micronucleated erythrocytes in the peripheral blood. NTP reported that female mice administered doses ranging from 6.25 to 50 mg/kg bw/day had a small but statistically significant increase in micronucleated erythrocytes in peripheral blood at the end of the 3-month study at the 50 mg/kg bw/day dose. The highest male dose tested was 25 mg/kg. There did not appear to be a dose-response trend at 6.25, 12.5, and 25 mg/kg for either males or females. Micronucleated normocytic erythrocytes (NCEs)/1000 NCEs were measured to be 1.90 ± 0.19 in male control mice rats and 0.7 ± 0.2 in female control mice that were administered 0.5% methyl cellulose. NTP concluded that the micronucleus test was negative in males and positive in females (NTP, 2011). It is noted that 7 out of 10 female mice administered 50 mg/kg α,β-thujone in the 3 month study died before the end of the study and seizures were reported in female mice dosed with >25 mg/kg, indicating that the maximum tolerated dose was exceeded (OECD, 2002). In addition, the micronucleated NCEs in the female control group were much lower than in the male control group and while the increase in micronucleated NCEs in the high dose female group was significant, it was low and within the range of values observed in the treated male mice. Further testing was not performed to resolve these inconsistencies (NTP, 2011).

NTP reported that there was no evidence of carcinogenic activity of α,β-thujone in male or female $B6C3F_1$ mice administered 3, 6, or 12 mg/kg in the 2-year gavage study. (This study is discussed in further detail below). In summary, NTP reported that there was some evidence of carcinogenicity (neoplasms of the preputial gland and benign pheochromocytomas of the adrenal gland) in male rats administered α,β-thujone for 2 years at the mid-dose, 25 mg/kg bw/day. Both male and female rats in the high dose group, 50 mg/kg bw/day, did not survive to the end of the study. The increase in incidences of preputial gland neoplasms was not dose-related and the incidences of hyperplasia were not increased, hence the NTP rating of "some" evidence rather than "clear" evidence. In addition, the occurrence of benign pheochromocytomas of the adrenal gland, observed only in male rats, is not associated with the induction of corresponding tumors in humans (Greim et al., 2009). There was no evidence of carcinogenic activity of α,β-thujone in female rats administered 12.5 or 25 mg/kg bw/day. NTP stated that the evidence regarding genotoxicity and carcinogenicity appear to be equivocal, however, carcinogenicity in this NTP study was not observed at the lower doses that did not induce seizures (NTP, 2011). Based on a review of the results of *in vitro* mutation assays, the *in vivo* micronucleus

[8] Based on a density of ionone: 0.93 g/mL.

study and carcinogenicity studies reported by the NTP, the FEMA Expert Panel determined, using the weight of evidence approach (Gooderham et al., 2020b), that there is no concern over the genotoxic or carcinogenic potential of thujone.

7.3.11.2. Chronic studies. In 2011, the National Toxicology Program (NTP) published the results of short term and chronic toxicity studies for α,β-thujone in F344/N rats and B6C3F$_1$ mice (NTP, 2011). In two-year carcinogenicity studies, a mixture of α- and β-thujone in 0.5% methyl cellulose was administered by oral gavage to rats at 0, 12.5, 25 or 50 mg/kg bw/day and mice at 0, 3, 6, 12 or 25 mg/kg bw/day for five days per week for 105 weeks. During the study, all animals were observed twice a day. Body weights were measured weekly up to week 13 then every 4 weeks until the end of the study (mice) or day 648/649 (male/female rats) then every 2 weeks. Mean body weights of all dosed rat groups and all dosed mice groups, with the exception of mice administered 25 mg/kg bw/day thujone were generally within 10% of those of the vehicle control groups throughout the study. At termination, complete necropsies and microscopic examinations were performed on all animals.

All male and female rats and female mice in the high dose group died before the end of the study. Survival was 14/50 for the high dose male mice group. A dose-related response in the occurrence of seizures was observed in male and female rats (male rat: control 1/50, low dose 5/50, middle dose 43/50 and high dose 50/50; female rat: control 1/50, low dose 3/50, middle dose 47/50, high dose 50/50). In male mice, seizures were observed only in the high-dose groups (44/50). In female mice, incidental seizures were observed in the control and lowest dose groups and in all animals in the highest dose group. Analysis of the brain tissues found pigment-laden macrophages, consistent with hemosiderin, in and around the choroid plexus in the third ventricle in high dose male and female rats (male rat: control 0/50, low dose 1/50, middle dose 0/50 and high dose 3/50; female rat: control 1/50, low dose 3/50, middle dose 5/50, high dose 19/50), an observation that was considered to be related to the administration of the test substance. Necrosis of the neurons and other types of cells was observed in the brain tissues of high dose male rats (3/50).

In the pituitary gland, statistically significant ($p \leq 0.01$) increased incidences of atrophy of the pars distalis in the high-dose group and dilatation of Rathke's cleft in the middle and high dose groups were observed in female rats. There were a few occurrences of these lesions in treated male rats and a single occurrence of necrosis of the pars distalis in the 50 mg/kg bw/day group. In addition, increased incidences of preputial gland adenoma and carcinoma (control, 3/49; low dose, 1/49; mid dose, 9/50; high dose not reported since did not survive) and benign pheochromocytomas of the adrenal medulla (control, 6/50; low dose, 8/50; mid dose, 12/49; high dose not reported since did not survive) were reported in male rats, but not female rats or mice. The incidences of preputial gland and adrenal medulla tumors in the mid-dose males exceeded the historical control ranges for methyl cellulose gavage studies but these incidences were not statistically significant at $p \leq 0.01$, using the significance value recommended for the evaluation of common tumors (FDA, 2001; Haseman, 1983; OECD, 2014a). Furthermore, the induction of pheochromocytomas in rats and mice is not considered relevant to humans (Greim et al., 2009). In the spleen, pigmentation was observed in middle and high dose male rats and high dose female rats. For male rats, the incidence of mineralization in the kidney was significantly increased in all dose groups compared to the control group. However, renal mineralization in rats is very common, approaching 100% incidences if multiple sections of the kidney are examined and/or if special stains are utilized. It is not considered indicative of an adverse effect (Lord and Newberne, 1990). Incidence of alveolar epithelial hyperplasia in the lung of male rats was increased when compared to the control group in the mid dose group (control 11/50, low dose 17/50, mid dose 19/50, high dose 3/50) but there was not an overall dose response.

For the 105-week study in rats, the doses were selected based on a previous 3-month study in which decreased survival was reported at doses of 75 and 100 mg/kg bw/day and infrequent seizures were observed in a few males and females administered 50 mg/kg bw/day and one female administered 25 mg/kg bw/day. However, based on the poor survival and occurrence of seizures observed for both rats and mice at the high doses in the 105-week studies, the FEMA Expert Panel noted that 50 mg/kg bw/day dose in this study exceeded the maximum tolerated dose. The authors of the study report did not determine a NOAEL for rats or mice in these studies. A lower confidence limit for a benchmark response of 10% (BMDL$_{10}$) of 11 mg/kg bw/day was determined for thujone, using dose-response modeling of chronic seizures in male rats from this NTP 2-year study (Lachenmeier and Uebelacker, 2010; NTP, 2011). Based on this BMDL$_{10}$ and the estimated intakes reported in Table 1, MOE values of 1 x 10^7, 2200, 14,000 and 29,000 were calculated for thujone in Cedar Leaf Oil (FEMA 2267), Sage Oil (FEMA 3001), Sage Oleoresin (FEMA 3002) and Wormwood Oil (FEMA 3116), respectively.

7.4. Natural flavor complexes

7.4.1. Camphor Japanese White Oil

7.4.1.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, camphor white oil (composition not reported) was tested for mutagenicity in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2*uvrA* in the presence and absence of S9 metabolic activation derived from the livers of phenobarbitone/β-naphthoflavone-induced rats. At concentrations up to 5000 μg/plate, camphor white oil was negative in all strains tested in both the presence and absence of S9 metabolic activation (ECHA, 2014).

7.4.2. Cedar leaf oil

7.4.2.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, cedar leaf oil, prepared by the steam distillation of the leaves and twigs of *Thuja occidentalis* (composition not reported), was negative for mutagenicity. The assay was performed in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2*uvrA* pKM101, at concentrations up to 5000 μg/plate in the presence and absence of S9 metabolic activation (ECHA, 2017a).

7.4.3. Hys

7.4.3.1. Genotoxicity. No evidence of mutagenic potential was observed in a reverse mutation assay when *S. typhimurium* strains TA98 and TA100 were incubated for 48 h hyssop oil (18% β-pinene, 29% *iso*-pinocamphone, 11% *trans*-pinocamphone) at concentrations of 93–463 μg/plate in the presence or absence of an S9 metabolic activation system derived from the livers of Aroclor 1254-induced rats, using the plate incorporation method (De Martino et al., 2009).

7.4.4. Orris root extract

7.4.4.1. Genotoxicity. In a GLP guideline-compliant Ames assay, orris root extract (described as a tincture), was negative for mutagenicity when tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 at concentrations of 0.1–150 μL/plate. The assay was performed both with and without S9 metabolic activation derived from the livers of Aroclor 1254-induced rats (Heck et al., 1989b; Jagannath, 1984). In another assay, orris root extract (described as a resinoid), was negative for mutagenicity when tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 at concentrations up to 500 μg/plate, with and without S9 metabolic activation derived from the

livers of Aroclor 1254-induced rats (Mulky, 1985).

7.4.5. Rue oil

7.4.5.1. Genotoxicity. In an Ames assay, commercially available rue tincture (*Ruta graveolens* L.) was tested using the plate incorporation method with *S. typhimurium* strains TA98 and TA100 in the presence and absence of S9 metabolic activation derived from the livers of phenobarbital-induced rats, at concentrations up to 120 μg/plate (Paulini et al., 1987; Schimmer et al., 1994). The composition of the tincture was described as 1:5 in 70% ethanol. A mutagenic response was observed in *S. typhimurium* strain TA98 in the absence of S9 metabolic activation. The rue tincture was negative for mutagenicity in *S. typhimurium* TA98 in the presence of S9 and in TA100 both in the absence and presence of S9. Because this test material is a tincture and not the essential oil of *Ruta graveolens* L., it is not consistent with Rue Oil (FEMA 2995) used as a flavoring ingredient, and therefore this study is not relevant to the safety evaluation of this NFC. It is expected that a tincture would contain some volatile constituents of the botanical, but also an unknown non-volatile fraction.Because of a lack of studies on the essential oil of rue that are similar in composition to Rue Oil (FEMA 2995), an evaluation of the mutagenicity and genotoxicity of the constituents was performed. Rue Oil (FEMA 2995) consists of 99% Group 8 (Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters) constituents, 94% 2-undecanone, 2% 2-nonanone and 2% 2-decanone. As reviewed above, 2-undecanone was negative for mutagenicity in an OECD guideline Ames study and a mouse lymphoma study and was also negative for genotoxicity in an OECD guideline *in vitro* chromosomal aberration assay. Based on these studies on 2-undecanone, which is the major constituent (94%) in Rue Oil (FEMA 2995), the FEMA Expert Panel concluded that there is no concern for genotoxicity for this NFC.

7.4.5.2. Developmental toxicity. In a reproductive/developmental study of rue oil, female nulliparous albino CD-1 outbred mice were mated with young adult males, and GD 0 was established when a vaginal sperm plug was observed. From GD 6 to GD 15, female mice were administered 8, 38, 177 or 820 mg/kg bw/day of rue oil dissolved in corn oil by oral gavage. A control group was sham-treated, and a positive control group received 150 mg/kg bw/day aspirin. Body weights were recorded on GD 0, 6, 11, 15 and 17. Test subjects were observed daily for appearance, food consumption and weight. Dams were subject to Caesarean section on GD 17 under anesthesia to assess the numbers of implantation sites, resorption sites, fetal mortality, body weights of live pups, and urogenital anomalies in dams and congenital abnormalities in fetuses. One third of the fetuses from each litter were subjected to visceral examinations using the Wilson technique. The other two-thirds were cleared in potassium hydroxide, stained and examined for skeletal defects. No significant effects on implantations or resorptions, maternal or fetal survival and soft or skeletal tissue abnormalities were observed in the treated groups compared to the sham-treated control group. The study authors concluded there were no adverse effects observed in nidation (embryo implantation), maternal or fetal survival, or fetal abnormalities with the administration of up to 820 mg/kg bw/day rue oil for 10 days in albino CD-1 mice (Bailey and Morgareidge, 1974).

In a second reproductive/developmental study of rue oil, nulliparous albino rats (Wistar derived stock) were mated with young adult males and GD 0 was established when the vaginal sperm plug was observed. From GD 6–15, females were administered rue oil dissolved in corn oil daily by oral gavage at doses of 10, 45, 209 or 970 mg/kg bw/day. A separate control group was administered the corn oil vehicle alone, and a positive control group was administered aspirin at 250 mg/kg bw/day. Body weights were recorded on GD 0, 6, 11, 15 and 20. Animals were observed daily for appearance, food consumption and weight. Dams were subject to Caesarean section on GD 20 under anesthesia to record the sex, corpora lutea, implantation sites, resorption sites, fetal mortality, body weights of live pups, urogenital abnormalities in the dams and congenital defects in fetuses. One-third of fetuses from each litter underwent visceral examination using the Wilson technique. The remaining two-thirds were cleared in potassium hydroxide and examined for skeletal defects. No significant effects on implantations or resorptions, maternal or fetal survival or skeletal abnormalities were observed in the test groups compared to the vehicle control group. The study authors concluded there were no adverse effects observed in nidation, maternal and fetal survival, or fetal abnormalities with the administration of up to 970 mg/kg bw/day rue oil for 10 days in albino CD-1 mice (Bailey and Morgareidge, 1974).

In a reproductive/developmental study of an aqueous rue extract, nulliparous Swiss albino rats were mated with young adult males and GD 0 was established when the vaginal sperm plug was observed as an indication that copulation had occurred. From GD 0 to GD 4, females were dosed daily *ad libitum* with 10 mL of 5, 10 and 20% aqueous solution of *Ruta graveloens* extract. The extract was prepared by extracting the dried leaves of *Ruta graveolens* in water (60–70 °C), filtration and addition of water to a fixed volume. A separate control group was administered water. Ingested volumes ranged from 8.6 to 8.8 mL/day. Treatment began 18h following an injection of human chorionic gonadotropin. On GD 4, females were terminated by cervical dislocation and embryos were examined. Females administered the rue extract showed no signs of intoxication or differences in weight gain. There was a dose-dependent increase in the percentage of embryos at the morula stage, a decrease in the percentage of normal blastocysts and an increase in abnormal embryos. Increased percentages of abnormal compacted morula and blastocysts were observed in the middle and high dose groups. Additionally, in all groups administered the rue oil, there was an observed delay in embryo transport (Gutiérrez-Pajares et al., 2003). Based on the description of the test substance, an aqueous extract of rue leaves, this test material is not consistent with Rue Oil (FEMA 2995) used as a flavoring ingredient and, therefore, this study is not relevant to the safety evaluation of this NFC.

7.4.6. Sage oil

7.4.6.1. Genotoxicity. In a GLP guideline-compliant Ames assay, sage oil (*Salvia officinalis*) was not mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 or TA1538 in the presence and absence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats, at concentrations up to 9100 μg/plate[9] (Brusick, 1982; Heck et al., 1989a). In an OECD and GLP guideline-compliant Ames assay, sage oil (*S. officinalis*) was non-mutagenic in the presence and absence of S9 metabolic activation in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 *uvrA* at 50–5000 μg/plate using the plate incorporation and preincubation methods. No excessive cytotoxicity or precipitation was observed under any test conditions (ECHA, 2018). In another Ames assay, sage oil (*Salvia officinalis* L.) was tested using both the pre-incubation and plate incorporation methods with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, in the presence and absence of an S9 metabolic activation system derived from the livers of Aroclor-induced rats. Sage oil was found to be non-mutagenic at concentrations of 230, 460 and 910 μg/plate[14] in both the presence and absence of S9 (Zani et al., 1991). In a fourth Ames assay, sage essential oil (37.5% α-thujone, 14.4% 1,8-cineole, 13.8% camphor and minor amounts of other terpenes) was non-mutagenic in *S. typhimurium* strain TA102 at concentrations up to 4570 μg/plate in the presence and absence of an S9 metabolic activation system derived from the liver of phenobarbital/β-naphthoflavone-induced rats (Vukovic-Gacic et al., 2006). A fifth Ames assay reported no mutagenicity for sage oil (37.9%

[9] Based on median density of 0.914 g/mL (Source: Food Chemical Codex 12th Edition, United States Pharmacopeia (USP), Rockville, MD, USA).

β-thujone, 13.9% camphor, 7.6% borneol, 5.9% α-humulene and other minor compounds) in *S. typhimurium* strains TA98 and TA100 at concentrations of 90, 180 and 460 μg/plate in the presence and absence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats. (De Martino et al., 2009). Lastly, an ethanolic sage extract was negative for mutagenicity when tested at 10 mg/plate in *S. typhimurium* strains TA98 and TA102 (Mahmoud et al., 1992), a concentration that exceeds the maximum limit recommended by the OECD guideline (OECD, 1997) of 5 mg/plate.

Positive and equivocal responses were observed in *Bacillus subtilis* M45 Rec^- and H17 Rec^+ incubated with 10 mg/disk of sage oil in the absence and presence of S9 metabolic activation, respectively (Ueno et al., 1984). In another rec assay, sage oil (*Salvia officinalis* L. – 7.73% eucalyptol, 7.3% α-thujone, 5.2% β-thujone, 17.4% camphor other minor constituents) was negative in *B. subtilis* PB1652 and PB1791 at 10 and 30 μL/mL. A response was deemed positive when the ratio between the diameters of the inhibition zones of the rec^- mutant and rec^+ strains exceeded 1.2 (Zani et al., 1991). The rec assay has not been standardized in an OECD guideline for genotoxicity testing, and OECD has noted that indicator tests such as the rec assay should be correlated to the results of other assays that measure DNA damage or mutagenicity that can be passed on to subsequent generations (OECD, 2015).

In a GLP guideline-compliant forward mutation assay, L5178Y mouse lymphoma $TK^{\pm}$ cells were incubated with sage oil at concentrations of 3.6–90 μg/mL in the absence of S9 and concentrations of 2–46 μg/mL in the presence of S9 metabolic activation prepared from the livers of Aroclor 1254-induced male rats. Sage oil was non-mutagenic in the absence of S9 and was positive at all concentrations in the presence of S9 (Cifone, 1982; Heck et al., 1989a). In a follow-up GLP guideline-compliant forward mutation assay, L5178Y mouse lymphoma $TK^{\pm}$ cells were incubated with a stock solution of sage oil prepared in a buffer at pH 7.2 at concentrations of 9–73 μg/mL in the absence of S9 and concentrations of 0.5–55 μg/mL in the presence of S9 metabolic activation prepared from the livers of Aroclor 1254-induced male rats. Sage oil was non-mutagenic in the absence of S9 and was positive at all concentrations in the presence of S9 (Cifone, 1984; Heck et al., 1989a). However, the study authors suggested that these positive responses may have been false positives due to excessive cytotoxicity and/or changes in the osmolality of the medium (Heck et al., 1989a).

In a GLP and OECD guideline-compliant unscheduled DNA synthesis (UDS) assay, sage oil was tested in male SD rat primary hepatocytes at concentrations of 0.9–730 μg/mL and induced no significant increases in the average net nuclear grain counts compared to the negative control (Curren, 1986). It is noted that the UDS assay was removed from the OECD library of standardized assays in April 2014 due to the limitation that it does not detect the mutagenic consequences of unrepaired genetic damage (OECD, 2015).

In an *in vivo* micronucleus assay, sage oil was administered intraperitoneally to male ddY mice at 0 and 50 mg/kg bw four times and at 100 and 200 mg/kg bw once (6/group). The vehicle control group was administered olive oil. The extent of exposure of the bone marrow to the test substance was not reported. No significant increases in the frequency of polychromatic erythrocytes and micronucleated polychromatic erythrocytes in the bone marrow were observed (Hachiya, 1987). Although the current OECD 474 guideline for the *in vivo* micronucleus assay, indicates the criteria for target tissue exposure for a clearly negative test in Paragraph 48, the OECD 474 guideline also describes the practical considerations in the interpretation of the results of the *in vivo* MN assay when all of the criteria for a clearly negative (or clearly positive) result are not fulfilled in Paragraph 50 (OECD, 2014b). In its evaluation of available *in vivo* MN data for flavoring substances, the FEMA Expert Panel relies upon its expert judgment and experience in drawing conclusions for the genotoxic potential. The FEMA Expert Panel has published a more extensive statement regarding its interpretation of *in vivo* genotoxicity study data and the availability of evidence of target tissue exposure (Cohen et al., 2022).

In summary, based on the results of the Ames, rec and forward mutation assays and an *in vivo* micronucleus assay, the weight of evidence indicates that there is no genotoxicity concern for Sage Oil (FEMA 3001).

7.4.7. wormwood oil

7.4.7.1. Genotoxicity. In a GLP and OECD guideline-compliant Ames assay, wormwood oil was non-mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*/pKM101. Wormwood oil was tested at concentrations up to 1600 μg/plate in the absence and presence of S9 prepared from the livers of Aroclor 1254-treated rats. Using the plate incorporation method, there were no significant increases in the numbers of revertant colonies under the conditions tested (Mee, 2017b).

In a GLP and OECD guideline-compliant micronucleus induction assay, HPBL were treated with wormwood oil for 3h at concentrations of 220–290 μg/mL in the absence of S9 metabolic activation and concentrations of 83–370 μg/mL with metabolic activation. A 24h treatment without metabolic activation was performed at concentrations of 135 and 180 μg/mL. The S9 metabolic activation system was prepared from the livers of Aroclor 1254-treated rats. Cytotoxicity (55 ± 5%), determined using the cytokinesis block proliferation index, was observed at the high concentrations of each of the 3h treatments. No significant increases in micronuclei induction were found under all test conditions (Mee, 2017a).

Based on the results of these GLP and OECD guideline compliant *in vitro* Ames and micronucleus induction assays, there is no genotoxic concern for Wormwood Oil (FEMA 3116).

7.4.7.2. Subchronic toxicity. In a 13-week repeated dose toxicity study performed in accordance with the guidelines recommended by the Japanese Ministry of Health, Labor and Welfare, wormwood extract (composition not reported) was administered in the drinking water to Wistar Hannover rats (10/sex/group) at doses of 0, 0.125, 0.5 or 2%. All rats survived until the end of the study and no changes indicating obvious toxicity attributable to the treatment with wormwood extract were observed in the body weights, hematological and serum biochemical examinations, organ weights, and histopathological examinations. A few rats of the 2% group showed slight rejection of the drinking water, but there was no significant difference in the body weight gains of this group compared to the control group. Based on the results of the present study, the NOAEL of wormwood extract in Wistar Hannover rats was estimated to be 2% (equivalent to 1.27 g/kg bw/day in males and 2.06 g/kg bw/day in females), the highest dose tested (Muto et al., 2003). Based on the NOAEL of 1.27 g/kg bw/day for male rats, an MOE of greater than 300 million is calculated for Wormwood Oil based on the estimated intake reported in Table 1.

7.4.8. Osmanthus Absolute

7.4.8.1. Genotoxicity. In a GLP and OECD guideline-compliant Ames assay, osmanthus absolute was non-mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and *E. coli* WP2*uvrA*/pKM101. Osmanthus absolute was tested at concentrations up to 5000 μg/plate in the absence and presence of an S9 metabolic activation system prepared from the livers of Aroclor 1254-treated rats. Using both the plate incorporation and pre-incubation methods, there were no significant increases in the numbers of revertant colonies under the conditions tested (Mee, 2020).

7.4.9. Perilla leaf oil

7.4.9.1. Genotoxicity. In an Ames assay, aqueous and methanolic extracts of leaves of *Perilla frutescens* were tested in *S. typhimurium* strains

TA98 and TA100 at concentrations up to 100 mg/mL with and without S9 metabolic activation prepared from the livers of Kanechlor KC-500-induced male rats using the plate incorporation method. The aqueous extract was negative for mutagenicity in both strains with and without S9. The methanolic extract was negative for mutagenicity in TA98 with S9 metabolic activation. In strain TA100 with and without S9 and in TA98 without S9, the methanolic extract of *Perilla frutescens* was cytotoxic under the test conditions (Morimoto et al., 1982). In another Ames assay, the ether, ethanol, and water extracts of the perilla herb were tested in *S. typhimurium* strains TA97, TA98, TA100 and TA102 using the pre-incubation method with and without S9 metabolic activation at concentrations up to 1000 μg/plate. Positive effects were observed in all four strains of *S. typhimurium* when tested with the water extract in the absence of S9 metabolic activation. In the presence of S9, weak mutagenicity was observed in all strains, except TA98 in which the water extract was negative for mutagenicity. No effects were observed with the ether or ethanol extracts (Fujita et al., 1990).

A methanolic extract of leaves of *Perilla frutescens* was mutagenic in the rec assay at a concentration of 100 mg/mL in the absence of S9 metabolic activation in *Bacillus subtilis* M45 Rec^- and H17 Rec^+. An aqueous extract of leaves of *Perilla frutescens* was negative under the same test conditions (Morimoto et al., 1982). However, as stated previously, the rec assay has not been standardized in an OECD guideline for genotoxicity testing and should not be viewed in isolation but should be considered with results of other mutagenicity assays (Gooderham et al., 2020b; OECD, 2015).

In an *in vitro* chromosomal aberration assay, perilla oil, supplied by the Japan Food Additives Association at the request of the Ministry of Health and Welfare of Japan, was tested at concentrations up to 0.04 mg/mL in Chinese hamster fibroblast cells. Cells were exposed to 3 doses (only high dose specified in the report) for 24 h and no metabolic activation systems were applied. At the maximum dose tested (0.04 mg/mL), the incidence of polyploid cells was 2%. The incidence of cells with structural chromosomal aberrations at 24 h following treatment was 6.0% and was considered an equivocal result by the study authors (Ishidate et al., 1984).

Following the review of these results by the Pharmaceutical Affairs and Food Sanitation Council, Food Sanitation Sub-section, Food Additives Subcommittee with the Japanese government, additional studies were performed (Pharmaceutical Affairs and Food Sanitation Council of Japan, 2004) on an acidic solution or an aqueous ethanol extract of *Perilla frutecens* var. *crispa* Tanaka whose composition was described as mainly terpenoids. In an Ames assay, the perilla extract was negative for mutagenicity in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1537 at concentrations up to 200 μg/plate, in the presence and absence of S9 metabolic activation. In an *in vitro* chromosomal aberration study, perilla extract was negative in CHL cells at concentrations up to 5 mg/mL in the presence and absence of an S9 metabolic activation system. Finally, perilla extract was negative for micronucleus induction in the bone marrow in an *in vivo* micronucleus assay conducted in ICR mice orally administered 500, 1000 or 2000 mg/kg bw/day perilla aldehyde (Pharmaceutical Affairs and Food Sanitation Council of Japan, 2004).[10]

In summary, Ames assays on water, methanolic, ethanolic and ether extracts of perilla reported by Morimoto and Fujita give mixed results and the *in vitro* chromosomal aberration assay on perilla oil reported an equivocal result. However, the composition of the Perilla Leaf extracts used in these studies was undefined and how the composition of these materials relate to Perilla Leaf Oil (FEMA 4013) prepared by steam distillation is not clear. Therefore, the relevance of these mixed results to the safety assessment of Perilla Leaf Oil (FEMA 4031) is ambiguous. Later studies reported by the Japanese government were performed on a perilla extract stated to be composed mainly of terpenoids, although a full compositional analysis was not provided. An Ames study, an *in vitro* chromosomal aberration study and an *in vivo* micronucleus assay of this perilla extract were all negative. Perilla Leaf Oil (FEMA 4013) contains 54% (mean %) *p*-mentha-1,8-dien-7-al (perilla aldehyde) and 34% Group 19 terpene hydrocarbons. The FEMA Expert Panel found no concern for genotoxicity in its review of *p*-mentha-1,8-dien-7-al (Cohen et al., 2016) or Group 19 constituents (Adams et al., 2011; Cohen et al., 2019). Based on lack of genotoxicity potential for the constituents, there is no genotoxicity concern for Perilla Leaf Oil (FEMA 4013).

7.4.9.2. Subchronic toxicity. In a 13-week repeated dose toxicity study, an extract of *Perilla frutecens* var. *crispa* Tanaka whose composition was mainly terpenoids was administered to F344 rats in their drinking water at levels of 0, 2.5, 5 or 10% (10/sex/group) (Yun et al., 1999). There was a significant increase in water consumption in the 5% and 10% treatment groups compared to controls, which was attributed to the presence of sucrose added as a solvent/carrier to the test substance. A trend of suppressed body weight gains was observed in high-dose males at 9 weeks and high-dose females after 7 weeks, although this effect was not statistically significant. No difference in body weight gains was observed in either sex between treatment and control rats at the end of the study.

Hematological tests showed a significant increase in white blood cell (WBC) counts in high-dose females, high and mid-dose males, and a decrease in segmental neutrophils and increase in lymphocytes in all male treatment groups. However, the variation in WBC was not significantly higher than the background data of untreated rats, and there were no histopathological changes related to treatment.

There were increases in blood T-cell count, albumin, total protein in the mid- and high-dose males and an increase in the albumin/globulin ratio in the high-dose males. T-cell counts were increased in all female treatment groups compared to controls and increases in sodium and alkaline phosphatase were reported in the high-dose female group. However, these changes were within the range of the background data of untreated rats, and no related histopathological changes were observed in the liver or hematopoietic system. Therefore, these findings were not considered to be induced by the administration of the perilla extract.

At the end of the study, organ weights were determined for the brain, heart, lung, liver, kidney, adrenal, spleens and testis. Increases in relative liver and heart weights were observed in high-dose males, increases in absolute and relative liver weights were observed in mid- and high-dose females and increases in absolute and total heart weights were observed in females of all treatment groups. However, no histopathological findings accompanying these changes were observed and therefore, these effects were not considered adverse.

In conclusion, up to 10% perilla extract in drinking water was administered to male and female F344 rats for 13 weeks, and no treatment-induced toxic changes were observed. Therefore, the NOAEL of perilla extract was determined to be 10% (13.9 g/kg bw/day) for males and 10% (37.1 g/kg bw/day) for females. Based on the more conservative NOAEL of 13.9 g/kg bw/day, a MOE of greater than 8×10^9 was calculated for the intake resulting from the use of Perilla Leaf Oil (FEMA 4013) as a flavoring ingredient.

8. Recognition of FEMA GRAS status

Most of the NFCs listed in Table 1 were determined to be GRAS under conditions of intended use by the FEMA Expert Panel in 1965 (Hall and Oser, 1965) while the GRAS determinations for Osmanthus Absolute (FEMA 3750) and Perilla Leaf Oil (FEMA 4013) were made later (Oser et al., 1985; Smith et al., 2001). The constituent profiles of the NFCs under evaluation were characterized by the presence of Group 10 (Alicyclic ketones, secondary alcohols and related esters), Group 8 (Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters) and/or Group 7 (Saturated alicyclic primary

[10] A further evaluation of target tissue exposure was not conducted for this study due to the unavailability of a full study report.

Table 7
NFCs affirmed FEMA GRAS.

FEMA No.	Name
2167	Boronia Absolute (*Boronia megastigma* Nees)
2231	Camphor Japanese White Oil (*Cinnamomum camphora* (L.) Nees et Eberm.)
2267	Cedar Leaf Oil (*Thuja occidentalis* L.)
2591	Hyssop Oil (*Hyssopus officinalis* L.)
2829	Orris Concrete Liquid Oil (*Iris florentina* L., *I. pallida, I. germanica)*
2830	Orris Root Extract (*Iris florentina* L., *I. pallida, I. germanica*), White flag extract
2995	Rue Oil (*Ruta graveolens* L.)
3001	Sage Oil (*Salvia officinalis* L.)
3002	Sage Oleoresin (*Salvia officinalis* L.)
3040	Tagetes Oil (*Tagetes erecta* L.*; T. patula* L.*; T. glandulifera Schrank*), Marigold oil
3116	Wormwood Oil (*Artemisia absinthium* L.), Absinthium oil
3750	Osmanthus Absolute (*Osmanthus fragrans* Lour.)
4013	Perilla Leaf Oil (*Perilla frutescens*, Labiatae), Shiso oil

alcohols, aldehydes, acids and related esters) constituents. In the application of the safety procedure, the estimated intakes of the constituent congeneric groups of each NFC were determined to be less than the TTC threshold for their structural class, indicating an adequate margin of safety for these materials. In addition, the weight of evidence indicates a lack of genotoxic potential for these NFCs. Exposure to the allylalkoxybenzenes methyl eugenol and estragole in Hyssop Oil (FEMA 2591) and Tagetes Oil (FEMA 3040) were at estimated intakes less than the TTC of 0.15 μg/person/day for compounds with structural alerts for genotoxicity and did not represent a safety concern. Upon application of the safety evaluation procedure, the FEMA Expert Panel affirms the NFCs listed in Table 7 as GRAS under conditions of intended conditions of use as flavoring ingredients.

CRediT authorship contribution statement

Nigel J. Gooderham: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Samuel M. Cohen:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Gerhard Eisenbrand:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Shoji Fukushima:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **F. Peter Guengerich:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Stephen S. Hecht:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Ivonne M.C.M. Rietjens:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Thomas J. Rosol:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Jeanne M. Davidsen:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing, Supervision. **Christie L. Harman:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Shannen E. Kelly:** Data curation, Writing – review & editing. **Sean V. Taylor:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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, Rietjens and Rosol are members of the Expert Panel of the Flavor and Extract Manufacturers Association. Authors Davidsen, Harman, Kelly and Taylor are employed by Verto Solutions which provides scientific and management support services to FEMA. A full description of the conflict of interest protections and procedures used to ensure that the FEMA Expert Panel decisions are fully objective and based solely on the merits of the available information have been published (Marnett et al., 2013) and are available on the FEMA website at https://www.femaflavor.org/gras#conflict.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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