FEMA GRAS assessment of natural flavor complexes: Eucalyptus oil and other cyclic ether-containing flavoring ingredients

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ABSTRACT

In 2015, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) initiated a program for the re-evaluation of the safety of over 250 natural flavor complexes (NFCs) used as flavor ingredients. This publication, the sixth in the series, will summarize the re-evaluation of eight NFCs whose constituent profiles are characterized by significant amounts of eucalyptol and/or other cyclic ethers. This re-evaluation was based on a procedure first published in 2005 and subsequently updated in 2018 that evaluates the safety of naturally occurring mixtures for their intended use as flavoring ingredients. The procedure relies on a complete chemical characterization of the NFC intended for commerce and the organization of its chemical constituents into well-defined congeneric groups. The safety of the NFC is evaluated using the well-established and conservative threshold of toxicological concern (TTC) concept in addition to data on absorption, metabolism and toxicology of the constituents of the congeneric groups and the NFC under evaluation. Eight NFCs derived from the *Eucalyptus, Melaleuca, Origanum, Laurus, Rosmarinus* and *Salvia* genera were affirmed as generally recognized as safe (GRAS) under their conditions of intended use as flavor ingredients based on an evaluation of each NFC and the constituents and congeneric groups therein.

1. Introduction

For sixty years, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) has been the main, independent body evaluating the safety of flavoring ingredients for use in human foods in the United States. Flavor ingredients are evaluated by the Expert Panel to determine if they can be considered "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, over 2700 flavoring ingredients have been determined by the FEMA Expert Panel to meet the criteria for GRAS status under conditions of intended use as flavoring ingredients.

The regular re-evaluation of GRAS flavoring ingredients is a component of the FEMA GRAS program. Flavoring ingredients are generally categorized as either a chemically defined flavoring material or a natural flavor complex (NFC), where a chemically defined flavoring material is commonly a single chemical substance, while NFCs are naturally occurring mixtures, typically derived from plants. The FEMA Expert Panel has published its re-evaluations of many groups of chemically defined FEMA GRAS flavoring ingredients, and in 2015, the Panel

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Abbreviations

ASTA	American Spice Trade Association
BMDL$_{10}$	Lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence
CF	Correction factor
CHO	Chinese hamster ovary (cells)
CFR	Code of Federal Regulations
CG	Congeneric group;
DMAPP	Dimethylallyl diphosphate
DTC	Decision tree class
EFFA	European Flavour Association
EFSA	European Food Safety Authority
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
FID	Flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GLP	Good laboratory practices
GMP	Good manufacturing practices
GPS	Geranyl diphosphase synthase
GRAS	Generally recognized as safe
IFEAT	International Federation of Essential Oils and Aroma Trades
IOFI	International Organization of the Flavor Industry
IPP	Isopentenyl diphosphate
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JFFMA	Japan Fragrance and Flavor Materials Association
LC-MS	Liquid chromatography-mass spectrometry
LOAEL	Lowest-observed-adverse-effect-level
LOD	Limit of detection
MOE	Margin of exposure
MoS	Margin of safety
NFC	Natural flavoring complex
NOAEL	No observed adverse effect level
NTP	National Toxicology Program
OECD	Organization for Economic Co-Operation and Development
PCI	Per capita intake
SCE	Sister chromatid exchange (assay)
SPF	Specific pathogen free (mice)
TD50	Dose giving a 50% tumor incidence
TDI	Tolerable daily intake
TTC	Threshold of toxicological concern
WHO	World Health Organization

Table 1
NFCs evaluated by the Expert Panel.

Name	FEMA No.	Estimated Intake (µg/person/day)[a]	Most recently surveyed annual volume (kg)[b]
Bay Sweet Oil (*Laurus nobilis* L.)	2125	60	580
Cajeput Oil (*Melaleuca leucadendron* L.)	2225	1.3	13[c]
Eucalyptus Oil (*Eucalyptus globulus* Labille)	2466	260	24,600
Laurel Leaves Extract (*Laurus nobilis* L.)	2613	2	22
Marjoram Oleoresin (*Majorana hortensis* Moench (*Origanum majorana* L.))	2659	89	860
Marjoram Oil Sweet (*Majorana hortensis* Moench (*Origanum majorana* L.))	2663	180	1780
Rosemary Oil (*Rosmarinus officinalis* L.), Garden rosemary oil	2992	1990	19,260
Sage Spanish Oil (*Salvia lavandulaefolia* Vahl.)	3003	5.9	56

[a] For high volume materials (greater than 22,500 kg/year), the PCI *per capita* is shown. For materials with a lower surveyed volume (less than 22,500 kg/year, PCI * 10 ("eaters only')) calculation is shown.
[b] Harman, C.L. and Murray, I.J. (2018) Flavor and Extract Manufacturers Association of the United States (FEMA) 2015 Poundage and Technical Effects Survey, Washington DC, USA.
[c] Gavin, C.L., Williams, M.C. and Hallagan, J.B. (2008) Flavor and Extract Manufacturers Association of the United States (FEMA) 2005 Poundage and Technical Effects Update Survey, Washington DC, USA.

expanded its re-evaluation program to include NFCs. As part of this project, the Panel updated its scientifically-based step-wise procedure for the safety evaluation of an NFC that was first published in 2005 (Smith et al., 2005) and updated in 2018 (Cohen et al., 2018a). The safety evaluation procedure requires data on the constituent composition and usage of the NFC. The constituents of most NFCs are products of well-characterized plant biochemical pathways (Schwab et al., 2008)

and, as a consequence, the constituents can usually be arranged into a limited number of well-defined groups of compounds with similar chemical and biological characteristics. These groups are referred to as congeneric groups. Data are collected such that the estimated intake, absorption, metabolism and toxicology can be evaluated for each constituent congeneric group of an NFC. The procedure also assesses the potential toxicity and genotoxicity of the unidentified constituent fraction based on the known constituent profile and available toxicological data on the NFC. For this re-evaluation project, the NFCs have been grouped in accordance with the similarity of their congeneric group profiles in order to facilitate the timely re-evaluation of all the NFCs. The Expert Panel has applied this procedure to the safety evaluation of *Citrus*-derived NFCs (Cohen et al., 2019), NFCs derived from *Mentha,* dill, buchu and caraway botanicals (Cohen et al., 2020), NFCs derived from *Cassia, Cinnamomum* and *Myroxylon* botanicals (Rietjens et al., 2020), NFCs derived from clove, cinnamon leaf and West Indian bay (Gooderham et al., 2020a) as well as lavender, guaiac, coriander-derived and related NFCs (Fukushima et al., 2020). In this manuscript, the NFCs under consideration are those whose constituent profiles are characterized by the presence of Group 23 constituents (Aliphatic and aromatic ethers) that include eucalyptol and other cyclic ethers.

The FEMA Expert Panel has issued a series of calls for data to collect constituent and other characterizing data for FEMA GRAS NFC candidates for re-evaluation. In addition to the International Federation of Essential Oils and Aroma Trades (IFEAT), member companies from the International Organization of the Flavor Industry (IOFI), including FEMA, the Japan Fragrance and Flavor Materials Association (JFFMA) and the European Flavour Association (EFFA), provided data for the NFCs listed in Table 1.

2. History of food use

The culinary herbs oregano, marjoram, rosemary and sage are plants of the *Lamiaceae* family, native to the Mediterranean and adjacent regions, and have a long history of use as flavorings for foods (Tucker and DeBaggio, 2000). These and other culinary herbs are available in dried and/or fresh forms in Western food markets and are popular additions to kitchen gardens. *Origanum majorana*, or sweet marjoram, was a staple in the medieval kitchen for the flavoring of a wide variety of dishes and

was a common substitute for hops in the brewing of ales (Freeman, 1943). Sweet marjoram is a small, shrub-like plant with greenish-white flowers and is similar in appearance to oregano, *Origanum vulgare,* and the generic names and history of use of marjoram and oregano are commonly interchanged (Baranska et al., 2005; Tripathy et al., 2017). Although the plants are similar, sweet marjoram has a distinguishable sweeter taste compared to oregano's stronger phenolic flavor (Baranska et al., 2005). Rosemary (*Rosmarinus officinalis)* is also a shrub-like plant that produces blue-white flowers and whose leaves are popularly used to flavor meats, sauces and other savory cuisines (Andrade et al., 2018; Guenther, 1949; Satyal et al., 2017). Similarly, sage leaves are commonly used as a spice in the preparation of meat and meat products. Spanish sage (*Salvia lavandulaefolia*) is characterized by its pale lavender flowers and a fresh-herbaceous odor but is less familiar than the 'common sage' (*Salvia officinalis*) sold in food markets (Arctander, 1961).

The *Melaleuca leucadendra* tree, commonly known as paperbark, is native to Southeast Asia and Australia (Sakasegawa et al., 2003). The trees are cultivated for their lumber and essential oil, known as cajeput oil. Cajeput, meaning "white wood," draws its name from the tree's characteristic pale bark (Brug, 1947; Motl et al., 1990). Cajeput oil, obtained from steam distillation of the fresh leaves and twigs of the tree, is characterized by its high eucalyptol content and has a history of use as herbal medicine and flavoring in foods and soft drinks (Brophy et al., 2013; Brophy and Lassak, 1988).

The eucalyptus tree is an evergreen indigenous to Australia and adjacent regions in Southeast Asia. However, the cultivation of the tree has expanded globally following the production of the first batches of distilled eucalyptus oil in the 1850s (Guenther, 1950). *Eucalyptus globulus* is cultivated for use in the production of paper and other wood products (Coppen, 1995; Brophy and Southwell, 1992; Rana et al., 2014). The resulting "waste leaf" is collected to produce the essential oil, which when added to food, produces a cooling sensation (Diomede and Salmona, 2017). *E. globulus* has historically been used as a flavoring in baked goods, confectionery, meat and meat products, and alcoholic and non-alcoholic beverages (Harborne and Baxter, 2001). In addition to its use as a flavoring ingredient, eucalyptus oil is used in cosmetics such as lotions and fragrance sprays, aromatherapy and massage oils and in over-the-counter (OTC) medicines such as vapor rub ointments and vaporizing liquids. In the United States, eucalyptus oil may be combined with camphor and menthol in topical OTC antitussive preparations (21 CFR 341.14(b); 341.40(u); 341.74(b),(c),(d)), which are drugs to relieve a cough when inhaled after being applied topically to the skin of the throat or chest in the form of an ointment or from a steam vaporizer or when dissolved in the mouth in the form of a lozenge for a local effect (21 CFR 341.3(c)). These non-food uses are not covered under FEMA GRAS assessment of eucalyptus oil for use as a flavoring ingredient.

Sweet bay leaf (*Laurus nobilis* L.) is a spice that traces back to the ancient Greeks. In Greek mythology, the bay laurel plant was sacred to the god Apollo. According to ancient texts, Apollo returned to Delphi wearing a crown made of laurel after slaying a serpent, and thus laurel became symbolic of victory, as well as distinction and prosperity (Giesecke, 2014). An alternative Greek legend described the nymph daughter of Gaia, Daphne, being turned into a laurel tree, after which the god Apollo fashioned a wreath of laurel leaves to signify his divinity (Brophy et al., 2013). In cooking, its leaves often surrounded anise used

for small Roman wedding cakes and are also commonly found in French spice bouquets (Charles, 2013).

3. Current use

The annual usage and estimated exposure calculations for each NFC are summarized in Table 1 (Gavin et al., 2008; Harman and Murray, 2018). These NFCs are used as flavorings in several food categories, including hard and soft candies, gravies, baked goods, gelatins, meat products and sauces, chewing gum and beverages. Within this group, Eucalyptus Oil (FEMA 2466) has the highest annual usage of 24,600 kg. Since this reported usage is greater than 22,500 kg, the estimated *per capita* intake, 260 µg/person/day, is calculated assuming that consumption is spread among the entire population. For NFCs with reported usage less than 22,500 kg, the PCI × 10 'eaters only' method is used to calculate the estimated *per capita* intake, which assumes that the flavoring ingredient is consumed by 10% of the population. Of these NFCs, Rosemary Oil (FEMA 2992) has the largest estimated intake of 1990 µg/person/day. The estimated intakes for the remaining seven NFCs range from 1.3 to 180 µg/person/day.

Several of these NFCs are derived from botanicals that are commonly used as culinary spices or herbs. For example, marjoram, rosemary and sage leaves are frequently added to food as spices and are widely available in food markets and kitchen gardens. Bay leaves, also called laurel leaves derived from *Laurus nobilis*, are commonly sold in the spice section of US food markets. However, despite the widespread use of marjoram, rosemary, sage and bay in food preparation, quantitative data on their annual usage to accurately estimate their *per capita* consumption are not available. Other NFCs, such as Eucalyptus Oil (FEMA 2466) and Cajeput Oil (FEMA 2225) are derived from botanical sources that are not typically consumed as food.

4. Manufacturing methodology

The essential oil NFCs are produced using distillation techniques. Marjoram Oil Sweet (FEMA 2663), Rosemary Oil (FEMA 2992), Sage Spanish Oil (FEMA 2992) and Bay Sweet Oil (FEMA 2125) are produced by distillation of the leaves of the plant (ASTA, 2008; Lawrence, 1997). Similarly, Cajeput Oil (FEMA 2225) is produced by steam distillation of the plants' leaves and end trimmings (Guenther, 1949; Sakasegawa et al., 2003). Eucalyptus Oil (FEMA 2466) is collected from steam distillation of the leaves of the *Eucalyptus globulus* tree.

Both Laurel Leaves Extract (FEMA 2613) and Marjoram Oleoresin (FEMA 2659) are extracts derived from the leaves of the botanical. Laurel Leaves Extract (FEMA 2613) is produced by the extraction of the dried leaves, followed by a concentration step to remove the extraction solvent and reconstitution. Marjoram Oleoresin (FEMA 2659) is obtained by extraction of the dried herb from the shrub of *Majorana hortensis* Moench [*Origanum majorana* L.] using an approved 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 essential oil by distillation, the non-volatile fraction of the herb is extracted with an approved solvent, concentrated by solvent removal, then combined with the volatile portion collected earlier in the process. Hexane extraction of the dried, powdered marjoram leaves reportedly yields 1.5–2% oleoresin (Attokaran, 2017; Food Chemical Codex, 2020). Following this preparation, oleoresins are often "standardized" with a food-grade ingredient such as a vegetable oil, salt or dextrin to contain a specific concentration of essential oil or other key component. Marjoram Oleoresin (FEMA 2659) in commerce typically contains 8–20% essential oil (Food Chemical Codex, 2020).

5. Chemical composition

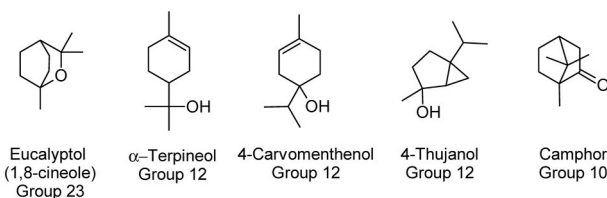The NFCs listed in Table 1 were characterized by the analysis of their

Fig. 1. Structures and congeneric groups of eucalyptol and other commonly reported constituents of the NFCs under consideration.
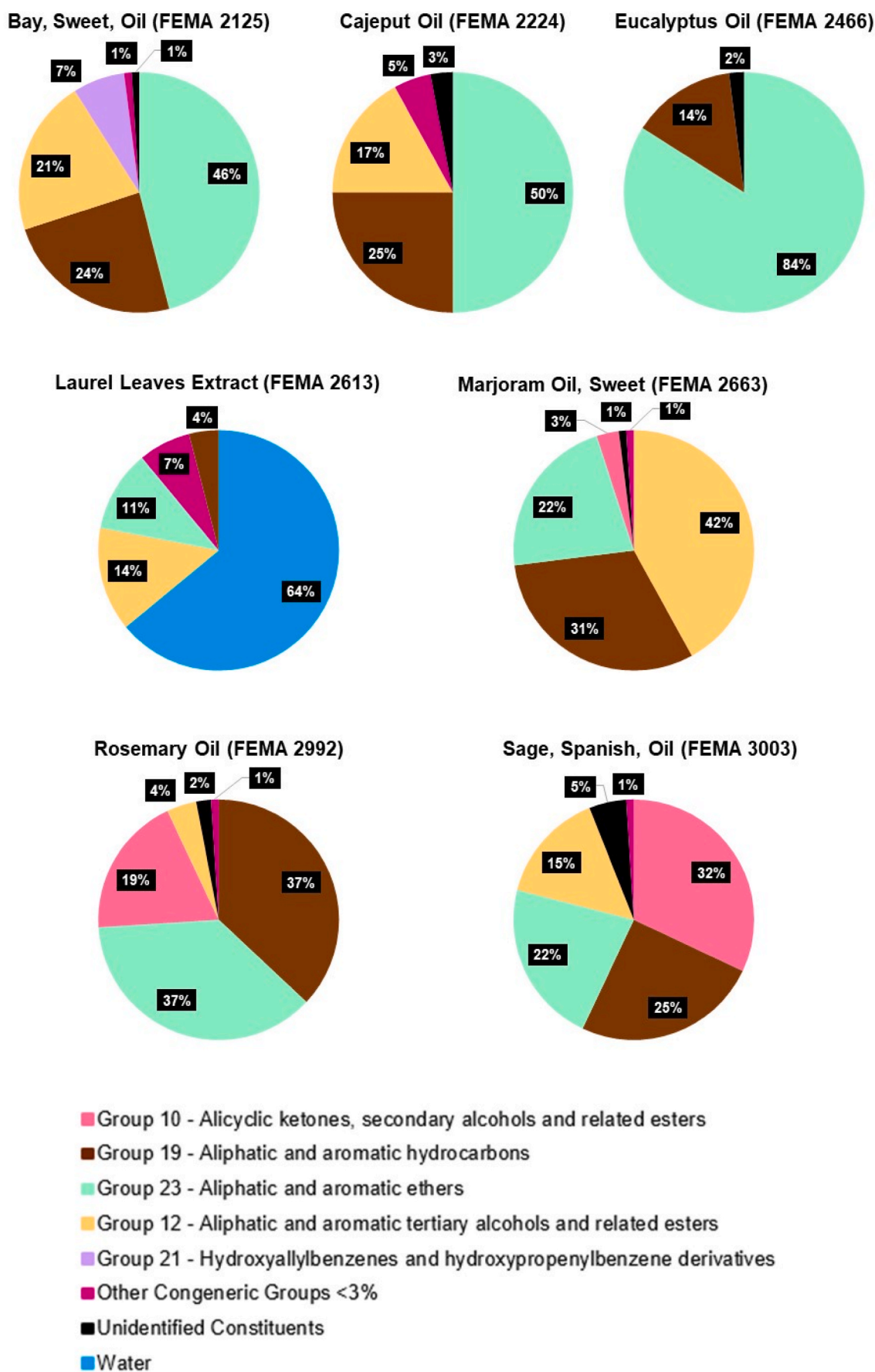
Eucalyptol (1,8-cineole) Group 23 · α–Terpineol Group 12 · 4-Carvomenthenol Group 12 · 4-Thujanol Group 12 · Camphor Group 10

Fig. 2. Constituent congeneric group profiles for the NFCs under consideration.
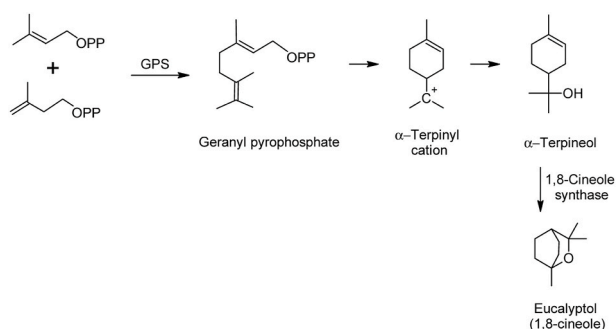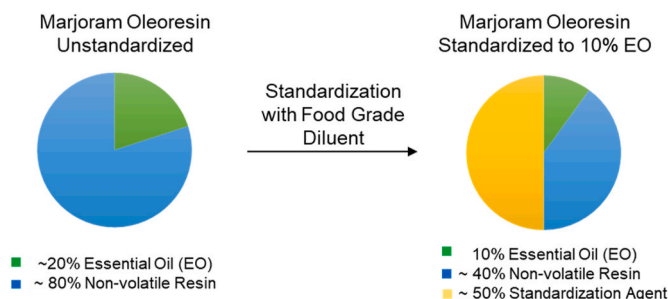
Fig. 3. Biosynthesis of eucalyptol.

Fig. 4. Standardization of raw spice oleoresins, using marjoram oleoresin as an example. Marjoram (raw) oleoresin is standardized by dilution with a food grade standardization agent, such as vegetable oil or salt, resulting in a Marjoram Oleoresin (FEMA 2659) composed of 10% essential oil, approximately 50% standardization agent and 40% non-volatile resins. Marjoram Oleoresin (FEMA 2659) (standardized) containing 8–20% essential oil is used as a flavoring ingredient.

volatile constituents using gas-chromatography mass spectrometry (GC-MS) to identify constituents by comparison against a standardized library, as well as a flame ionization detector (FID) for quantitation of each detected analyte. Identified and unidentified GC peaks are reported as the area percent of the respective chromatogram. The constituent data for each NFC were compiled into statistical summaries (Appendix A). All identifiable constituents were assigned both a Cramer decision tree class and a congeneric group based on the structure of the constituent (Cohen et al., 2018a; Cramer et al., 1978).

The names, congeneric groups and structures of common constituents of the NFCs under consideration are shown in Fig. 1 and the constituent congeneric group profiles for each NFC, with the exception of Marjoram Oleoresin (FEMA 2659), are depicted by pie charts in Fig. 2. The congeneric group profile is dominated by five groups: Group 23 (Alicyclic and aromatic ethers) constituents within which eucalyptol is a primary constituent, Group 19 (Aliphatic and aromatic hydrocarbons), Group 12 (Aliphatic and aromatic tertiary alcohols and related esters), Group 10 (Alicyclic ketones, secondary alcohols and related esters) and Group 21 (Hydroxyallylbenzenes and hydroxypropenylbenzene derivatives). Minor constituent groups include Group 3 (Aliphatic linear and branched-chain alpha, beta-unsaturated aldehydes and related alcohols, acids and esters) and Group 13 (Aliphatic, alicyclic, alicyclic-fused and aromatic-fused ring lactones).

Eucalyptol and other terpenoids are produced in these botanicals via the isoprene biosynthetic pathway. This pathway begins with the condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by geranyl diphosphate synthase (GPS) to give geranyl pyrophosphate. In the biosynthesis of eucalyptol, geranyl pyrophosphate undergoes the elimination of pyrophosphate and cyclization to form an α-terpinyl cation (Fig. 3). From the reactive α-terpinyl cation, α-terpineol is formed. The conversion of α-terpineol to eucalyptol is catalyzed by 1,8-cineole synthase.

Because of the variable nature of the constituent profile of spice oleoresins, they are characterized separately from the essential oil NFCs. Raw spice oleoresins are highly concentrated and consequently, they are
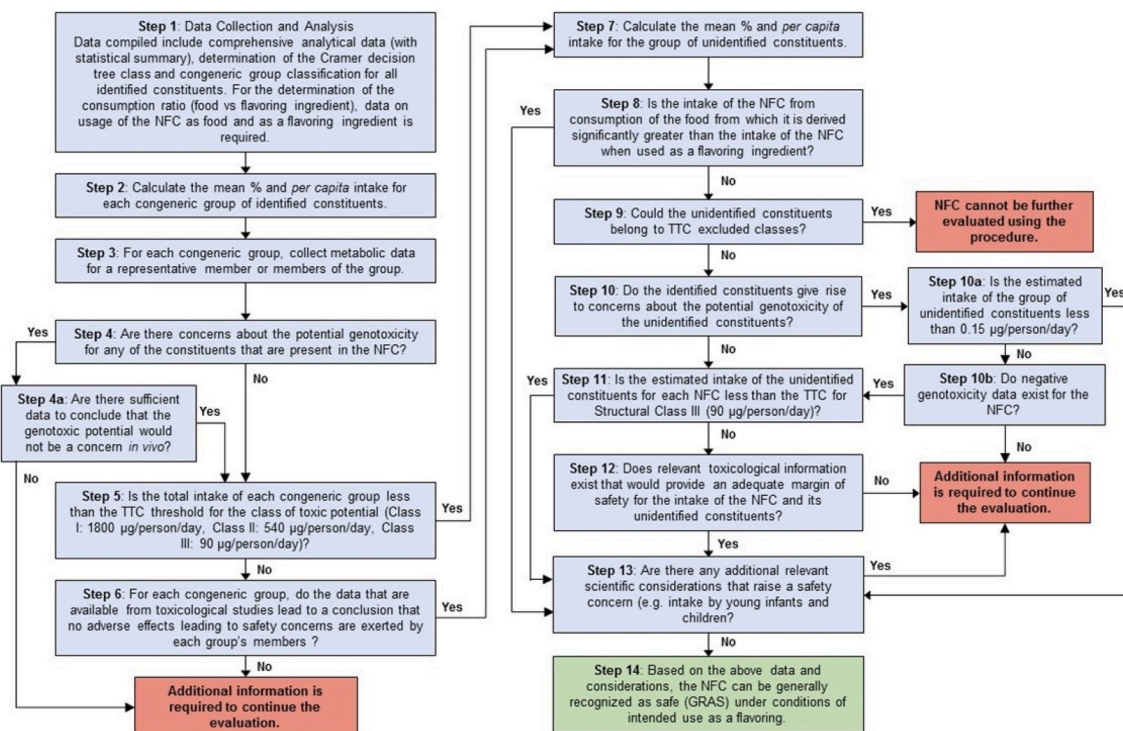
Fig. 5. Procedure for the Safety Evaluation of NFCs (Figure first published in Cohen et al., 2018a).

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, a raw 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 a raw marjoram oleoresin may contain approximately 20% essential oil with 80% resinous material, after standardization with a food-grade diluent, it will contain a much lower percentage of essential oil and resin, as depicted in Fig. 4. 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 Marjoram Oleoresin (FEMA 2659) is estimated to be 8–20% of its content (FCC, 2020).

6. Safety Evaluation

The procedure for the safety evaluation for NFCs was guided by a set of criteria that were initially outlined in two publications (Smith et al., 2004, 2005) and updated in 2018 (Cohen et al., 2018a). Briefly summarized in Fig. 5, 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 ingredient relative to intake from the natural source when consumed as food; Steps 2 through 6 evaluate the exposure and potential toxicity of the identified constituents (organized by congeneric group) based on available data on metabolism and toxicity and on the application of the threshold of toxicological concern (TTC). 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 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.*

Marjoram Oil Sweet (FEMA 2663), Marjoram Oleoresin (FEMA 2659) and Rosemary Oil (FEMA 2992) are derived from the popular culinary herbs marjoram and rosemary which are available in both fresh and dried forms in Western food markets and are often grown in home gardens. Spanish Sage Oil (FEMA 3003) is also derived from a culinary herb but is different from the sage typically sold in food markets. Both Bay Sweet Oil (FEMA 2125) and Laurel Leaves Extract (FEMA 2613) are derived from bay leaves that are also sold as a spice in food markets. However, despite widespread use of these culinary herbs and spices, reliable data on the consumption of these foods, required to calculate a ratio for the consumption of the essential oil from food versus consumption of the essential oil as flavoring, are not available. The other NFCs under consideration, Cajeput Oil (FEMA 2225) and Eucalyptus Oil (FEMA 2466), are derived from botanicals that are not typically consumed as food. For these reasons, in the safety evaluation for all NFCs under consideration, all consumption is assumed to be as flavoring added to food.

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 for each NFC are organized by their respective congeneric groups in Appendix A. The congeneric groups are listed in

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[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.

order of decreasing mean %. Only constituents with a mean % greater or equal to 1% of the total NFC are included. Minor constituent percentages (<1% of the total NFC) are grouped together under each of the listed congeneric groups and the total mean % for each listed congeneric group is reported. Because a detailed constituent profile was not available for Marjoram Oleoresin (FEMA 2659), its constituent profile has been derived based on the volatile oil content of the oleoresin in commerce ranging from 8 to 20%, as described in the FCC FCC (2020).

c. *Assignment of the constituents of 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.

The DTC for each congeneric group, determined by the most conservative constituent in that group, is provided in Appendix A.

Step 2

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

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

where:

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

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

Proceed to Step 3.

The total mean % for the listed congeneric groups in each NFC's summary report is subtotaled and listed in Appendix A with their respective DTC and estimated intake (based on PCI × 10 or PCI, as appropriate).

Step 3

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

Proceed to Step 4

Appendix A lists the constituent congeneric groups for each NFC. For each congeneric group, sufficient data on the metabolism of its constituents or related compounds exist to conclude that members of the respective groups are metabolized to innocuous products. The use of metabolic data in the safety evaluation of flavoring compounds and a
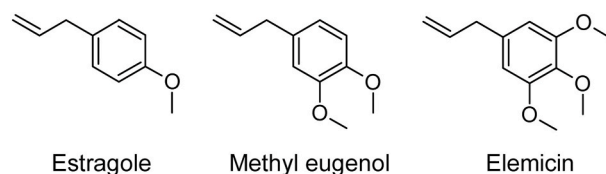
[3] See Smith et al., 2005 for a discussion on the use of PCI × 10 for exposure calculations in the procedure.

Fig. 6. Structures of estragole, methyl eugenol and elemicin.

summary of the expected metabolism of flavoring compounds by congeneric group is described in a recent FEMA Expert Panel publication (Smith et al., 2018). The metabolism of eucalyptol is discussed in more detail in the section *Biochemical and Toxicological* Supporting *Information Relevant to the Safety Evaluation*. Additional metabolic data on Group 19 (Aliphatic and aromatic hydrocarbons) flavoring ingredients can be found in the FEMA Expert Panel safety assessment of this group (Adams et al., 2011) and in the safety evaluation of *Citrus*-derived NFCs (Cohen et al., 2019). The FEMA Expert Panel has also published safety assessments for Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) and Group 10 (Alicyclic ketones, secondary alcohols and related esters) flavoring ingredients and has reviewed the metabolism of Group 21 (Hydroxyallylbenzenes and hydroxypropenylbenzene derivatives) flavoring ingredients (Adams et al., 1996; Marnett et al., 2014; Rietjens et al., 2014). Metabolic data are also provided in the safety assessments for other groups and individual constituents of the NFCs under consideration (Adams et al., 2004; Adams et al., 2005a, b, c; Adams et al., 2002; Adams et al., 1997; Adams et al., 2008; Adams et al., 1998; Adams et al., 1996; Adams et al., 2007).

Step 4

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

If Yes, proceed to Step 4a.

If No, proceed to Step 5.

The FEMA Expert Panel applies a weight of evidence approach in the evaluation of genotoxicity data for flavoring ingredients (Gooderham et al., 2020b). In general, the structural features of the congeneric

Table 2
Natural occurrence and estimated intake of estragole, methyl eugenol and elemicin in the NFCs under consideration.

Name (FEMA No.)	Constituent of Concern	Mean %	Estimated Intake (μg/person/day)
Bay Sweet Oil (FEMA 2125)	Elemicin	0.01	0.004
Bay Sweet Oil (FEMA 2125)	Methyl eugenol	2	1
Bay Sweet Oil (FEMA 2125)	Estragole	0.1	0.07
Laurel Leaves Extract (FEMA 2613)	Methyl eugenol	1	0.03
Laurel Leaves Extract (FEMA 2613)	Estragole	0.3	0.006
Laurel Leaves Extract (FEMA 2613)	Elemicin	0.06	0.001
Marjoram Oleoresin (FEMA 2659)	Methyl eugenol	0.0008–0.002	0.0007–0.002
Marjoram Oleoresin (FEMA 2659)	Estragole	0.007–0.02	0.006–0.02
Marjoram Oil Sweet (FEMA 2663)	Methyl eugenol	0.01	0.02
Marjoram Oil Sweet (FEMA 2663)	Estragole	0.09	0.16
Rosemary Oil (FEMA 2992)	Methyl eugenol	0.01	0.2

Table 3
MOE analysis of estimated intake of estragole in Marjoram Oil Sweet (FEMA 2663) and methyl eugenol in Bay Sweet Oil (FEMA 2125) and Rosemary Oil (FEMA 2992).

Name (FEMA No.)	Constituent of Concern	Estimated Intake (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)	MOE
Bay Sweet Oil (FEMA 2125)	Methyl eugenol	2×10^{-5}	22.2	>890,000
Marjoram Oil, Sweet (FEMA 2663)	Estragole	3×10^{-6}	3.3	>1,200,000
Rosemary Oil (FEMA 2992)	Methyl eugenol	3×10^{-6}	22.2	>6,600,000

groups present in the evaluated NFCs do not raise concerns for genotoxic potential. An evaluation of the available *in vitro* and *in vivo* genotoxicity studies on eucalyptol presented in a later section, *Biochemical and Toxicological* Supporting *Information Relevant to the Safety Evaluation*, determined no concern for potential genotoxicity of eucalyptol. Previous evaluations of other major constituent groups, Group 19 (Aliphatic and aromatic hydrocarbons), Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) and Group 10 (Alicyclic ketones, secondary alcohols and related esters) indicate no genotoxic concern for the constituents of these groups (Adams et al., 1996, 2011; Cohen et al., 2019, 2020; Fukushima et al., 2020; Marnett et al., 2014).

Group 21 (Hydroxyallylbenzenes and hydroxypropenylbenzene derivatives) constituents include eugenol as well as a subset of constituents with an allylalkoxybenzene structural motif. In its recent review of *in vitro* and *in vivo* genotoxicity studies and a two-year bioassay study for carcinogenicity on eugenol, the FEMA Expert Panel concluded that eugenol is genotoxic only at higher concentrations that result in significant cellular toxicity (Gooderham et al., 2020a; Rietjens et al., 2014). However, Group 21 constituents with an allylalkoxybenzene structural motif raise a concern for genotoxic potential (Rietjens et al., 2014). Methyl eugenol, estragole and elemicin, whose structures are shown in Fig. 6, are naturally occurring constituents in many botanicals including common culinary spices and herbs. The occurrence of these constituents and their estimated intakes from Bay Sweet Oil (FEMA 2125), Laurel Leaves Extract (FEMA 2613), Marjoram Oleoresin (FEMA 2659), Marjoram Oil Sweet (FEMA 2663) and Rosemary Oil (FEMA 2992), are shown in Table 2. These five NFCs proceed to Step 4a. The constituent profiles of Cajeput Oil (FEMA 2225), Eucalyptus Oil (FEMA 2466) and Sage Spanish Oil (FEMA 3003) do not indicate a concern for genotoxicity and proceed to Step 5.

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, methyl eugenol and elemicin (Fig. 6) share a common motif of a benzene ring substituted with an alkoxy group located *para* to a 2-propenyl substituent. These allylalkoxybenzene compounds are 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 creates a DNA reactive species (Al-Malahmeh et al., 2017; Hasheminejad and Caldwell, 1994; Herrmann et al., 2012, 2014; Jeurissen et al., 2006, 2007; Phillips et al., 1984; Punt et al., 2008; Randerath et al., 1984; Rietjens et al., 2014; Zhou et al., 2007). Rodent studies have indicated that estragole and methyl eugenol are hepatocarcinogens at high dose levels (Drinkwater et al., 1976; Miller et al., 1983; NTP, 2000).

The direct addition of estragole and methyl eugenol, as well of 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 2018, 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 synthetic flavoring substances and adjuvants for use in food (83 Fed. Reg. 50490. October 9, 2018) in response to a food additive petition. The FDA explained that it had based its decision "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. October 9, 2018).

Estragole, methyl eugenol and safrole, as well as 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 its 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. October 9, 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 shown in Table 2, the estimated intakes of estragole and elemicin from the consumption of Bay Sweet Oil (FEMA 2125), methyl eugenol, estragole and elemicin from Laurel Leaves Extract (FEMA 2613), methyl eugenol and estragole from Marjoram Oleoresin (FEMA 2659) and methyl eugenol from Marjoram Oil Sweet (FEMA 2663) are very low, less than 0.07 µg/person/day. These values are below the TTC of 0.15 µg/person/day for compounds with structural alerts for genotoxicity, as originally derived by Kroes et al. in 2004 (Kroes et al., 2004). This TTC value was determined based on an analysis of the dose-response data for carcinogenic compounds, provided by the Gold database of carcinogens,[4] presenting the dose giving a 50% tumor incidence (TD50) (Gold et al., 1984; Kroes et al., 2004). By linear extrapolation of these TD50 data to a dose resulting in a 1 in 10^6 tumor incidence, an exposure level or TTC at which the lifetime risk of cancer was less than 1 in 10^6 was determined to be 0.15 µg/person/day (Kroes et al., 2004). In a recent EFSA/WHO review of the TTC approach, a 0.15 µg/person/day threshold was proposed and considered sufficiently protective for compounds with structural alerts for genotoxicity with the exclusion of high potency carcinogens (the Cohort of Concern) specified by Kroes and co-workers (EFSA, 2016; Kroes et al., 2004; Nohmi, 2018). Laurel Leaves Extract (FEMA 2613) and Marjoram Oleoresin (FEMA 2659) proceed to Step 5.

In cases where the intake of a naturally occurring carcinogen from food exceeds the TTC for genotoxic substances, a Margin of Exposure (MOE) approach can be applied (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

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

Table 4

Consideration of Groups 19 and 23 in Rosemary Oil (FEMA 2992) where the estimated intake exceeds the TTC value for the congeneric group (CG).

Name (FEMA No.)	DTC	Estimated Intake of CG (µg/person/day)	Estimated Intake of CG (mg/kg bw/day)	NOAEL (mg/kg bw/day)	MoS
Congeneric Group 19 - Aliphatic and alicyclic hydrocarbons					
Rosemary Oil (FEMA 2992)	II	750	0.012	215	>17,900
Congeneric Group 23 - Aliphatic and aromatic ethers					
Rosemary Oil (FEMA 2992)	II	740	0.012	30	>2500

animals. For estragole, a $BMDL_{10}$ of 3.3 mg/kg bw/day was determined based on the most conservative value from the mathematical modeling of *in vivo* carcinogenicity data on tumor formation in female mice (Miller et al., 1983; van den Berg et al., 2011b). For methyl eugenol, a $BMDL_{10}$ of 22.2 mg/kg bw/day was determined based on model averaging of evaluated mathematical models of *in vivo* carcinogenicity data on tumor formation in male and female rats (NTP, 2000; Suparmi et al., 2019). The MOE is calculated as the ratio between the $BMDL_{10}$ and the estimated daily intake of the compound under evaluation. Using this approach, an MOE greater than 10,000 was determined for estragole from the consumption of Marjoram Oil Sweet (FEMA 2663) and for methyl eugenol from the consumption of Rosemary Oil (FEMA 2992) and Bay Sweet Oil (FEMA 2125) (see Table 3). The EFSA has stated and the FEMA Expert Panel concurs with the opinion that MOE values greater than 10,000 that are based on the $BMDL_{10}$ derived from an animal study would be of low public health concern and of low priority for risk management actions (EFSA, 2005). Based on this result, Bay Sweet Oil (FEMA 2125), Marjoram Oil Sweet (FEMA 2663) and Rosemary Oil (FEMA 2992) proceed to Step 5.

Step 5

Is the total intake of each 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 – The estimated intake for the constituent congeneric groups of Bay Sweet Oil (FEMA 2125), Cajeput Oil (FEMA 2225), Eucalyptus Oil (FEMA 2466), Laurel Leaves Extract (FEMA 2613), Marjoram Oleoresin (FEMA 2659), Marjoram Oil Sweet (FEMA 2663) and Sage Spanish Oil (FEMA 3003) are below the TTCs for their respective groups and these NFCs proceed to Step 7. For Rosemary Oil (FEMA 2992), the estimated intakes for Group 23 (Aliphatic and aromatic ethers) and Group 19 (Aliphatic and alicyclic hydrocarbons) exceed the TTC, as indicated in Table 4. The evaluation of Rosemary Oil (FEMA 2992) proceeds to Step 6.

Step 6

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

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

trivial compared to intake of the constituent from consumption of food, then this should be taken into consideration in the safety evaluation (Kroes et al., 2000).

If Yes, proceed to Step 7.

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

The estimated intake of Group 19 constituents exceeded the TTC for Rosemary Oil (FEMA 2992). A review of toxicological studies for Group 19 constituents was conducted for the GRAS re-affirmation of flavoring materials of this group (Adams et al., 2011) and more recently for the GRAS affirmation of *Citrus*-derived NFCs (Cohen et al., 2019). The primary Group 19 constituents of Rosemary Oil (FEMA 2992) are the monoterpene hydrocarbons α-pinene, β-pinene and *d*-limonene. The Margin of Safety (MoS) for Group 19 constituents of Rosemary Oil (FEMA 2992), reported in Table 4, was calculated using a NOAEL of 215 mg/kg bw/day (adjusted daily dose from 300 mg/kg bw/day administered 5 days/week) reported for a two-year toxicity study of the monoterpene hydrocarbon *d*-limonene in female F344N rats (NTP, 1990). A summary of this study is provided later in the manuscript, with a detailed discussion available in the safety evaluation of *Citrus*-derived NFCs (Cohen et al., 2019).

The major Group 23 constituent of Rosemary Oil (FEMA 2992) is eucalyptol. A review of toxicological studies on eucalyptol is summarized in a later section of this manuscript, *Biochemical and Toxicological* Supporting *Information Relevant to the Safety Evaluation*. The MoS was calculated for Group 23 constituents of Rosemary Oil (FEMA 2992) in Table 4, based on the NOAEL of 30 mg/kg bw/day reported in an OECD guideline 28-day toxicity study of eucalyptol in male and female Wistar HanTM rats (Fulcher and Watson, 2013). With the determination of an adequate MoS for both groups exceeding their respective TTC, Rosemary Oil (FEMA 2992) proceeds to Step 7.

Step 7

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

Proceed to Step 8.

For each NFC, the estimated intake and mean % for the group of unidentified constituents is reported in Appendix A and the estimated intakes are summarized in Table 5. For Marjoram Oleoresin (FEMA 2659), the unidentified constituent portion is represented as the range of the estimated intake of the non-volatile portion of the NFC.

Table 5

Estimated intake of unidentified constituents.

Name	FEMA No.	Estimated Intake (µg/person/day)
Bay Sweet Oil	2125	0.6
Cajeput Oil	2225	0.04
Eucalyptus Oil	2466	4
Laurel Leaves Extract	2613	0.0005
Marjoram Oleoresin	2659	8–80
Marjoram Oil, Sweet	2663	1
Rosemary Oil	2992	40
Sage Spanish Oil	3003	0.2

Step 8

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

If Yes, proceed to Step 13.

If No, proceed to Step 9.

No. For the NFCs under consideration, there is a lack of data to calculate the consumption of Bay Sweet Oil (FEMA 2125), Laurel Leaves Extract (FEMA 2613), Marjoram Oleoresin (FEMA 2659), Marjoram Oil Sweet (FEMA 2663), Rosemary Oil (FEMA 2992) and Sage Spanish Oil (FEMA 3003) from the culinary spices and herbs from which they are derived. Cajeput Oil (FEMA 2225) and Eucalyptus Oil (FEMA 2466) are derived from botanicals that are not typically consumed as food. As a result, all consumption of the NFCs under consideration is assumed to be as flavoring added to food. Proceed to Step 9.

Step 9

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

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

If No, proceed to Step 10.

No. The unidentified constituents are not expected to belong to any TTC-excluded class. The identified Group 23, 19, 12 and 10 constituents, including eucalyptol are the products of the isoprene pathway and the identified constituents of Group 21 are products of the shikimate pathway. It is expected that the unknown constituents would also be products of these pathways. Furthermore, all the NFCs under consideration are prepared by steam distillation and extraction processes which would preclude compounds from the TTC excluded classes in the final product. Proceed to Step 10.

Step 10

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

If Yes, proceed to Step 10a.

If No, proceed to Step 11.

No. For the NFCs Cajeput Oil (FEMA 2225), Eucalyptus Oil (FEMA 2466) and Sage Spanish Oil (FEMA 3003), the identified constituent profiles do not contain any constituents with alerts for potential genotoxicity and therefore these NFCs do not give rise to concern about the potential genotoxicity of the unidentified constituents. As mentioned in Step 9, these NFCs' profiles are also highly defined—consisting primarily of eucalyptol, monoterpene hydrocarbons and related terpenoids—and based on the identified constituents, the unidentified constituents are most likely products from the isoprene pathway.

The profiles of Bay Sweet Oil (FEMA 2125), Laurel Leaves Extract (FEMA 2613), Marjoram Oleoresin (FEMA 2659), Marjoram Oil Sweet (FEMA 2663) and Rosemary Oil (FEMA 2992) contain low naturally occurring amounts of estragole, methyl eugenol and/or elemicin 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 0.001 to 2 µg/person/day. Using the most conservative $BMDL_{10}$ for the calculation of MOEs, 1.9 mg/kg bw/day for safrole (van den Berg et al., 2011a), the MOEs for an unidentified allylalkoxybenzene occurring at 0.1% would substantially exceed 10,000 for all the NFCs under consideration. A review of available genotoxicity and toxicological studies on the NFCs under consideration are 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. Proceed to Step 11.

Step 10a

Is the estimated intake of the group of unidentified constituents less than 0.15 µg/person/day (Koster et al., 2011; Rulis, 1989)? A TTC of 0.15 µg/person/day has been proposed for potentially genotoxic substances that are not from the TTC excluded classes materials (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) for each NFC less than the TTC (Kroes et al., 2000; Munro et al., 1996) for Structural Class III (90 µg/person/day)?[6]

If Yes, proceed to Step 13.

If No, proceed to Step 12.

Yes. The estimated intake of the unidentified constituent fraction of each NFC (summarized in Table 5) is less than 90 µg/person/day, the TTC threshold for Structural Class III. 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?

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

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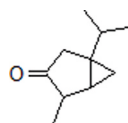
Fig. 7. Structure of thujone.

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.

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 the additional data required to address the concern before proceeding to Step 14.

Table 6
Summary of genotoxicity assays for eucalyptol and NFCs under consideration.

Name	End-point	Test object	Concentration	Results	Reference
a. Eucalyptol FEMA 2465					
Eucalyptol	Reverse mutation in *Salmonella typhimurium*	TA97a, TA98, TA100, and TA102	1000–3000 µg/plate 250–2000 µg/plate	Negative[a]	Gomes-Carneiro et al. (1998)
Eucalyptol	Reverse mutation in *S. typhimurium*	TA98, TA100, TA1535, and TA1537	0–3333 µg/plate	Negative[a]	Haworth et al. (1983)
Eucalyptol	*Rec* assay in *Bacillus subtilis*	M45 and H17	18 µg/disk	Negative	Oda et al. (1978)
Eucalyptol	*Rec* assay in *B. subtilis*	M45 and H17	20 µL/plate	Negative	Yoo (1986)
Eucalyptol	*In vitro* chromosomal aberration	Chinese hamster ovary cells (CHO W B1)	50–800 µg/mL	Negative	Galloway et al. (1987a)
Eucalyptol	Sister chromatid exchange	Chinese hamster ovary cells (CHO W B1)	479–810 µg/mL	Negative	Galloway et al. (1987b)
Eucalyptol	Sister chromatid exchange	Chinese hamster K-1 Cells	0, 3.3, 10, 33.3, 100, 333, 1000 µM	Negative	Sasaki et al. (1989)
b. Natural Flavor Complexes *In vitro*					
Bay Sweet Oil	Reverse mutation in *S. typhimurium* and *E.coli*	TA98, TA100, TA1535, TA1537 and *E.coli* WP2*uvr*A	Up to 150 µg/plate[b] Up to 500 µg/plate[c]	Negative[a]	ECHA (2013a)
Cajeput Oil	Reverse mutation in *S. typhimurium* and *E. coli*	TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	Up to 500 µg/plate	Negative[a]	ECHA (2013b)
Eucalyptus Oil	Reverse mutation in *S. typhimurium* and *E. coli*	TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate	Negative[a]	ECHA (2013e)
Eucalyptus Oil	Chromosomal aberration assay	Human peripheral blood lymphocytes	10, 80, 100 µg/mL[c,d] 200, 325, 350 µg/mL[b,d] 50, 60, 90 µg/mL[c,e]	Negative	ECHA (2013d)
Eucalyptus Oil	Mammalian gene mutation assay	mouse lymphoma L5178Y cells, TK locus	10, 115, 145, 160, 175 µg/mL[c,d] 10, 145, 175, 225, 250 µg/mL[b,d] 10, 50, 100, 150 µg/mL[c,e]	Negative	ECHA (2013f)
Laurel Leaf Extract	Reverse mutation in *S. typhimurium*	TA98 and TA100	10, 30, 50 mg/plate	Negative[c]	Namiki et al. (1984)
Laurel Leaf Extract	Reverse mutation in *S. typhimurium*	TA98 and TA100	10–100 mg/plate	Negative[b]	Rockwell and Raw (1979)
Laurel Leaf Extract	*In vitro* micronucleus assay	Rat hepatocytes	50, 100, 200 mg/L	Negative[c]	Turkez and Geyikoglu (2011)
Marjoram Oil	Reverse mutation in *S. typhimurium*	TA97a, TA98, TA100, TA102 and TA1535	2.2–35 µg/plate (-S9)[c] 4.4–51 µg/plate (+S9)[b]	Negative[a]	Dantas et al. (2016)
Marjoram Oil Sweet	Reverse mutation in *S. typhimurium* and *E. coli*	TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	Up to 5000 µg/plate	Negative[a]	ECHA (2017)
Rosemary Oil	Reverse mutation in *S. typhimurium* and *Escherichia coli*	TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate 10, 33, 100, 333, 1000, 3333 and 5000 µg/plate	Negative[a]	Dakoulas (2014)
Rosemary Oil	*In vitro* mammalian cell micronucleus test	Human peripheral blood lymphocytes	10, 25, 50, 100, 125, 150, 200, 250, 300, 350, 400, 450, 700 µg/mL[c,d] 5, 10, 25, 35, 50, 60, 75, 100, 125 µg/mL[c,e] 25, 50, 100, 150, 175, 200, 225, 250, 275, 300, 350, 400, 450 µg/mL[b,d]	Negative	Roy (2015)
Spanish Sage Oil	Reverse mutation with *S. typhimurium*	TA98, TA100, TA1535 and TA1537	0.6, 1.25, 2.5 µL/plate	Negative[a]	Zani et al. (1991)
Spanish Sage Oil	*Rec* assay in *B. subtilis*	PB 1652 and PB 1791	10 and 30 µL/disk	Negative	Zani et al. (1991)
Sage Spanish Oil	Reverse mutation in *S. typhimurium* and *E. coli*	TA98, TA100, TA1535, TA1537 and *E.coli* WP2*uvr*A	Up to 5000 µg/plate	Negative[a]	ECHA (2018)
In vivo					
Rosemary Oil	*In vivo* chromosomal aberration and micronucleus assay	Male Wistar Rats (3/sex/group)	6.43, 100, 200 mg/kg bw	Negative	Gaiani et al. (2006)
Rosemary Oil	*In vivo* alkaline comet and micronucleus assay	Albino Swiss Mice	300, 1000, 2000 mg/kg	Positive	Maistro et al. (2010)

[a] In the absence and presence of an exogenous metabolic activation system.
[b] In the presence of S9 metabolic activation.
[c] In the absence of S9 metabolic activation.
[d] 3 h or 4 h treatment.
[e] 24 h treatment.

If No, proceed to Step 14.

A low concentration, 0.02%, of naturally occurring β-thujone (Fig. 7) corresponding to an estimated intake of 0.001 μg/person/day was reported in the data collected for Sage Spanish Oil (FEMA 3003). Thujone is historically associated with absinthe, a distilled alcoholic beverage flavored with wormwood oil and popularized in the 19th century in Europe. The chronic consumption of absinthe became associated with "absinthism," a syndrome characterized by convulsions, blindness, mental deterioration and hyper-excitability. The belief that thujone was the toxic agent responsible for the syndrome led to the prohibition of the use of absinthe and wormwood extracts in food 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 regulated by law internationally.

Presently, the direct addition of thujone to food and beverage products is not permitted in the United States and the European Union. However, it may be added through the use of essential oils and derivatives of herbs, barks and fruits. In the European Union, there are clear limits on the levels of thujone in finished consumer foods containing flavoring 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, though there are no limits for thujone in sage stuffings (European Commission, 2008). In the United States, alcoholic beverages containing *Artemisia* species such as wormwood must be "thujone-free" (containing less than 10 ppm) pursuant to 21 C.F.R. § 172.510 and verified by a GC-MS method published by the Alcohol and Tobacco Tax and Trade Bureau (TTB). In consideration of the low levels of thujone reported in Sage Spanish Oil (FEMA 3003), the use of these NFCs in food as flavoring ingredients is unlikely to result in levels in food or beverages that exceed these regulatory limits and is not a safety concern.

Potential exposure to children and infants was also considered because of their lower body weights and the potential for differences in toxicokinetics and toxicodynamics in comparison to adults. For the NFCs under consideration, the estimated intakes for Group 19 (Aliphatic and aromatic hydrocarbons) and Group 23 (Aliphatic and aromatic ethers) in Rosemary Oil (FEMA 2992) constituents exceeded their respective TTC (Table 4). In both cases, the MoS is adequate when calculated for a lower body weight of 20 kg. The estimated intakes for all other constituent congeneric groups were significantly below their respective TTC indicating no safety concern for intake by children by these NFCs.

In addition, the FEMA Expert Panel is aware of accidental poisonings from direct oral consumption of eucalyptus oil in its neat or concentrated liquid form by children and adults and further discusses this issue below under "Additional considerations" for Eucalyptus Oil in the *Biochemical and Toxicological* Supporting *Information Relevant to the Safety Evaluation* section of this manuscript. These accidental poisonings are not from its use as a flavoring ingredient. The *per capita* estimated intake for Eucalyptus Oil (FEMA 2466) is 260 μg/person/day when used as a flavoring ingredient. From this estimated intake, a MoS of greater than 69,000 was calculated for Eucalyptus Oil (FEMA 2466) when used as a flavoring ingredient, based on a NOAEL of 300 mg/kg bw/day determined for eucalyptus oil administered to female rats in an OECD guideline 422 combined repeated dose oral gavage toxicity study with a

reproduction/developmental toxicity screening test (ECHA, 2013g). The FEMA Expert Panel concluded that under conditions of intended use as a flavoring ingredient, consumption of Eucalyptus Oil (FEMA 2466) in food is safe for both adults and children.

In consideration of the group of unidentified constituents, the estimated intake for each NFC was considered to be sufficiently below the TTC for structural class III. Low concentrations of estragole, methyl eugenol and/or elemicin which have a potential genotoxicity concern were present in Bay Sweet Oil (FEMA 2125), Laurel Leaves Extract (FEMA 2613), Marjoram Oleoresin (FEMA 2659), Marjoram Oil Sweet (FEMA 2663) and Rosemary Oil (FEMA 2992). The estimated intakes were far below the TTC of 0.15 μg/person/day for compounds with structural alerts for genotoxicity or had MOEs far greater than 10,000 which indicates a low concern for adults and children.

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 concludes that Bay Sweet Oil (FEMA 2125), Cajeput Oil (FEMA 2225), Eucalyptus Oil (FEMA 2466), Laurel Leaves Extract (FEMA 2613), Marjoram Oleoresin (FEMA 2659), Marjoram Oil Sweet (FEMA 2663), Rosemary Oil (FEMA 2992) and Sage Spanish Oil (FEMA 3003), are affirmed as GRAS under conditions of intended use as a flavoring substance.

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

The major congeneric groups represented in the constituent profiles for the NFCs under consideration are Group 23 (Aliphatic and aromatic ethers), Group 19 (Aliphatic and aromatic hydrocarbons), Group 12 (Aliphatic and aromatic tertiary alcohols and related esters), Group 10 (Alicyclic ketones, secondary alcohols and related esters) and Group 21 (Hydroxyallylbenzenes and hydroxypropenylbenzene derivatives). Summaries of the biological and toxicological information available for all of these groups are included in previously published safety assessments (Adams et al., 1996, 2011; Marnett et al., 2014; Rietjens et al., 2014) and for Group 23, in the Scientific Literature Reviews available via the National Technical Information Service (FDA, 1978). Because the FEMA Expert Panel has not previously published a safety assessment for eucalyptol, the principal constituent of Bay Sweet Oil (FEMA 2125), Cajeput Oil (FEMA 2225), Eucalyptus Oil (FEMA 2466), Marjoram Sweet Oil (FEMA 2663), Rosemary Oil (FEMA 2992) and Sage Spanish Oil (FEMA 3003), data on the metabolism, toxicity and genotoxicity of this flavoring ingredient are presented below. Studies on the NFCs evaluated are also presented below. A summary of the genotoxicity studies reviewed is provided in Table 6.

7.1. Eucalyptol

7.1.1. Absorption, distribution, metabolism and excretion

Alicyclic ethers, such as eucalyptol, as well as aliphatic and aromatic ethers are expected to be rapidly absorbed from the gastrointestinal tract and excreted in the urine by one of two metabolic pathways. In the first, an ether undergoes *O*-dealkylation to form the corresponding alcohol. The alcohol may be further oxidized and/or conjugated and excreted in the feces and urine. The second metabolic route available to ethers is a P450 mediated ring hydroxylation or side-chain oxidation, followed by formation of sulfate or glucuronic acid conjugates which are readily excreted (Smith et al., 2018). In mammalian test species and humans, eucalyptol (1,8-cineole) is oxidized by P450 enzymes to yield polar hydroxylated metabolites, which are conjugated and excreted or further oxidized and excreted (Hiroi et al., 1995; Miyazawa and Shindo, 2001; Miyazawa et al., 2001b, a).
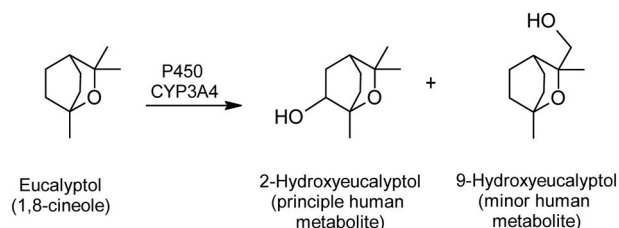

Eucalyptol (1,8-cineole)

2-Hydroxyeucalyptol (principle human metabolite)

9-Hydroxyeucalyptol (minor human metabolite)

Fig. 8. Metabolism of eucalyptol in humans.

The major metabolites of eucalyptol were identified as 2-hydroxyeucalyptol, 3-hydroxyeucalyptol and 9-hydroxyeucalyptol. There were species differences noted: in rats 3-hydroxyeucalyptol was the principal metabolite but in rabbits and humans the primary metabolite identified was 2-hydroxyeucalyptol with minor amounts of 9-hydroxyeucalyptol (Miyazawa et al., 1989, 2001a; Miyazawa and Shindo, 2001; Pass et al., 2001). In humans, the formation of the principal metabolite 2-hydroxyeucalyptol (2-hydroxy-1,8-cineol) is mediated by cytochrome P450 3A4 (See Fig. 8) (Miyazawa et al., 2001b).

The metabolism of eucalyptol was studied in incubations with rat and human liver microsomes and with recombinant P450 enzymes expressed in insect cells, in which human P450 and NADPH-P450 reductase cDNAs had been introduced. Rat and human microsomal CYP3A enzymes rapidly oxidized eucalyptol to 2-hydroxyeucalyptol (Miyazawa and Shindo, 2001; Miyazawa et al., 2001b, a). Earlier studies also indicated that the CYP3A subfamily of enzymes is induced by eucalyptol. Hepatic microsomes isolated from male Sprague-Dawley rats that were intraperitoneally injected with 300 mg eucalyptol/kg bw daily for five days, and then terminated, showed the induction of P450 enzymes 2B1 and 3A2 (Hiroi et al., 1995).

7.1.2. Short-term studies of toxicity

In a 28-day toxicity study, male Wistar rats (10/dose) were administered eucalyptol at doses of 0 (vehicle control), 500 or 1000 mg/kg bw/day by oral gavage (Kristiansen and Madsen, 1995). The vehicle control was soybean oil. Due to the pervasive aroma of eucalyptol, a second control group of 10 males was placed in a separate space. At the end of the study, the animals were terminated and samples from the brain, liver and kidneys were taken for histopathological examination.

There were no clinical effects reported. One animal from the 500 mg/kg bw/day and one animal from the 1000 mg/kg bw/day dose groups and one from the second control group died due to gavage errors and were excluded from the study. Statistically significant decreases in terminal body weight were reported at the 500 and 1000 mg/kg bw/day groups. There was a significant increase in the relative brain weight for the highest dose group, although no histopathological changes attributable to eucalyptol were seen in the brain. In addition, significant increases in the relative liver and kidney weights in all dose groups were observed. In the kidney, the accumulation of renal droplets in the cytoplasm of the proximal tubule cells was observed in all groups using Mallory's Heidenhain staining. A dose-dependent increase in severity was observed and the presence of α_{2u}-globulin in selected slides from all groups was confirmed by immunohistochemistry. α_{2u}-Globulin nephropathy in the kidney of male rats is sex and species specific and not toxicologically relevant to humans (Capen et al., 1999; Cohen et al., 2019; Flamm and Lehman-McKeeman, 1991; Lehman-McKeeman, 2010a; Swenberg et al., 1989; US-EPA, 1991). The study authors did not report any histopathological findings in the liver. The study authors did not determine a NOAEL for this study (Kristiansen and Madsen, 1995).

In a 28-day OECD guideline oral toxicity study conducted in compliance with GLP standards, groups of Wistar Han™:RccHan™: WIST rats (5/sex/dose) were administered eucalyptol at doses of 0 (vehicle control), 30, 300 or 600 mg/kg bw/day in Arachis oil BP via gavage (Fulcher and Watson, 2013).

There were two additional recovery groups of 5 rats per sex that received either the vehicle control or 600 mg/kg bw/day of eucalyptol by gavage. The recovery groups were treated during the 28-day test period and maintained without treatment for an additional 14 days. Signs of clinical toxicology, body weight changes, food and water consumption were routinely monitored. Hematology, clinical chemistry and urine analysis parameters were measured for the animals in the standard treatment and vehicle control groups at the end of the treatment period and for the recovery groups 14 days thereafter.

There were no unscheduled deaths during the study or recovery periods. Post-dosing salivation was observed for 2 males and all females in the 300 mg/kg bw/day group and all males and females in the 600

mg/kg bw/day groups. There were no test substance-related behavioral, functional, food consumption, blood chemistry or urinalysis changes recorded. It was noted that males of the 600 mg/kg bw/day group showed a slightly lower but statistically significant mean body weight which was not seen in females or either sex at the lower dose. Females in the 600 mg/kg bw/day group had lowered water consumption, but males at the same dose and both sexes at lower doses were unaffected. Statistically significant increases in mean platelet counts for the 300 and 600 mg/kg bw/day male groups were observed but without evidence of thrombosis. At necropsy there were no gross lesions.

The males in the 300 and 600 mg/kg bw/day dose groups showed statistically significant increases in absolute and relative kidney weights. Histopathology of the kidneys showed an increase in hyaline droplets in the proximal tubules accompanied by sporadic tubular cell degeneration at the high dose. At the sites of excessive hyaline droplet formation, increased mean severity of multifocal tubular basophilia and interstitial mononuclear cell foci were observed. The severity at the 600 mg/kg bw/day dose decreased after the 14-day recovery period. These renal changes were due to α_{2u}-globulin nephropathy which is a condition specific to male rats and of no toxicological relevance to humans (Flamm and Lehman-McKeeman, 1991; Lehman-McKeeman, 2010b; Swenberg et al., 1989). The FEMA Expert Panel concurs with the U.S. Environmental Protection Agency (US EPA) and International Agency for Research on Cancer (IARC) who have determined that the development of α_{2u}-globulin nephropathy in male rats should not be used to estimate the nephrotoxic or cancer hazard for humans (Capen et al., 1999; Cohen et al., 2019; Flamm and Lehman-McKeeman, 1991; Lehman-McKeeman, 2010a; Swenberg et al., 1989; US EPA, 1991; US-EPA, 1991).

There were significant increases in absolute and relative liver weights for females at 30 mg/kg bw/day dose and both sexes at the two higher doses relative to controls. However, the relative liver weights for both sexes in the 300 mg/kg bw/day dose group and females in the 30 mg/kg bw/day dose group were within the historical control range. For both sexes, the histopathologic examination of the liver tissue showed that for the 300 and 600 mg/kg bw/day groups there was a dose-dependent incidence of centrilobular hypertrophy of the hepatocytes with no further indication of liver damage. In the 600 mg/kg bw/day dose groups examined following a 14-day recovery period, the noted centrilobular hypertrophy of the hepatocytes was not observed following the recovery period in either sex and therefore considered to be of a metabolic nature and adaptive character and not adverse by the study authors. Based on these observations, the study authors determined a NOAEL of 600 mg/kg bw/day for male and female rats (Fulcher and Watson, 2013). In reviewing this study, the FEMA Expert Panel considered dose-dependent incidence of centrilobular hypertrophy of the hepatocytes at the mid- and high dose to be potentially adverse and determined the NOAEL for both male and female rats to be 30 mg/kg bw/day. This study was used to calculate a MoS of greater than 2500 for Group 23 (Aliphatic and aromatic ethers) constituents, of which eucalyptol is the primary constituent, in Rosemary Oil (FEMA 2992) in Step 6 of the safety evaluation.

A short-term study of oral toxicity of eucalyptol designed to compare the administration of encapsulated eucalyptol in the feed to administration by oral gavage in Fischer 344 rats and to determine appropriate dose levels for subsequent subchronic studies was conducted (NTP, 1987b). Groups of Fischer 344 rats (6/sex/group) were exposed to eucalyptol in trioctanoin by gavage or in the feed for a period of 28 days. In the feed study, encapsulated eucalyptol in cellulose was admixed in the feed at dietary concentrations of 3,750, 7,500, 15,000 or 30,000 ppm. These amounts correspond to dose levels of approximately 187, 375, 750 or 1500 mg/kg bw per day, respectively (FDA, 1993). In the gavage study, eucalyptol was administered to rats at doses of 150, 300, 600 or 1200 mg/kg bw per day. For controls, groups of rats (6/sex/group) received the vehicle (trioctanoin) or feed alone, or no gavage treatment. In the gavage administration study, a statistically significant decrease in body weight gain was reported in males only in

the 600 and 1200 mg/kg bw/day dose groups while in the feed study, statistically significant lower body weight gain was reported at the highest dose for males only compared to control animals. No statistically significant differences in body weight gain were observed at any dose level for females in either the feed or gavage study. In all test groups, feed or gavage, there were no reported statistically significant differences in food or water consumption for either sex compared to controls although it was noted that due to the small sample size only large differences could have been detected. Vehicle control rats gavaged with triooctanoin consumed less feed than the untreated controls. Absolute and relative major organ weights were comparable among treated animals and control groups in the feed and gavage studies.

In the male test groups in the feed study, hepatic centrilobular cytoplasmic vacuolization (untreated control: 0/6, vehicle control: 0/6, 187 mg/kg bw per day: 3/6, 375 mg/kg bw per day: 1/6, 750 mg/kg bw per day: 1/6 and 1500 mg/kg bw per day: 4/6) and centrilobular fatty changes in the liver (untreated control: 0/6, vehicle control: 0/6, 187 mg/kg bw per day: 2/6, 375 mg/kg bw per day: 2/6, 750 mg/kg bw per day: 2/6 and 1500 mg/kg bw per day: 6/6) were observed at all dose levels, but not in a dose dependent manner. In the gavage study, hepatic centrilobular cytoplasmic vacuolization was also observed in male rats at the two highest dose levels, 600 and 1200 mg/kg bw/day (untreated control: 0/6, vehicle control: 0/6, 150 mg/kg bw/day: 0/6, 300 mg/kg bw/day: 0/6, 600 mg/kg bw/day: 3/6 and 1200 mg/kg bw/day: 6/6). In female rats, there was no evidence of these types of alterations at any dose level in either the feed or the gavage studies.

In the feed study, a non-dose dependent increase in the severity of epithelial cell regeneration was observed in the kidney of all male test groups, and cytoplasmic alterations in the cortical renal tubules were reported at the two highest dietary levels for male rats, consistent with α_{2u}-globulin nephropathy (Flamm and Lehman-McKeeman, 1991; Lehman-McKeeman, 2010b; Swenberg et al., 1989). Diffuse cytoplasmic changes to the parotid salivary gland without further description were also observed in male rats at the lowest and the two highest levels in the feed study (untreated control: 0/6, vehicle control: 0/6, 187 mg/kg bw per day: 1/6, 375 mg/kg bw per day: 0/6, 750 mg/kg bw per day: 1/6 and 1500 mg/kg bw per day: 6/6). This effect was not observed in the female rats of the dietary study or the rats of either sex in the gavage studies (NTP, 1987b). Due to the limited nature of this study, the NTP did not draw further conclusions from the results of this study.

Following the same protocol as above, groups of B6C3F$_1$ hybrid mice (6/sex/group) were exposed to eucalyptol in trioctanoin by oral gavage or encapsulation in α-cellulose with incorporation into the feed, for a period of 28 days (NTP, 1987a). In the feed study, encapsulated eucalyptol was mixed in the feed at concentrations of 3,750, 7,500, 15,000 or 30,000 ppm. Control groups consisting of a group receiving α-cellulose used for encapsulation in the feed and a group receiving only feed were used. The intake levels correspond to approximately 562, 1,130, 2250 or 4500 mg/kg bw/day, respectively (FDA, 1993). In the gavage study, eucalyptol was administered to mice at doses of 150, 300, 600 or 1200 mg/kg bw per day. Controls consisting of a group administered the vehicle control, trioctanoin and a group receiving no gavage treatment were used. There were no statistically significant dose-related differences in body weight, absolute organ weight and feed or water consumption between any of the treated animals in the feed or gavage studies and the controls. The average relative brain weight of female mice of the 4500 mg/kg bw per day dietary exposure group was significantly higher compared to the control and all other dose groups but no histopathological analysis of the brain was reported. Further, the relative liver weight in the 1,130, 2250 and 4500 mg/kg bw/day dietary exposed group male mice was significantly higher compared to the control and low-dose animals. Histopathological examination of the liver revealed no treatment related effects for the gavage route of exposure. Minimal hypertrophy of the centrilobular hepatocytes was reported in males at all dietary levels except the lowest level (untreated control 0/6; vehicle control 0/6; 562 mg/kg bw per day: 0/6; 1130

mg/kg bw per day: 1/6; 2250 mg/kg bw per day: 5/6; 4500 mg/kg bw per day: 6/6) and in female mice at the lowest and two highest dietary levels of the feed study (untreated control 0/6; vehicle control 0/6; 562 mg/kg bw per day: 1/6; 1130 mg/kg bw per day: 0/6; 2250 mg/kg bw per day: 4/6; 4500 mg/kg bw per day: 6/6). Esophageal and stomach lesions were most likely related to gavage administration reported in treated mice of the gavage study and not due to the test substance. The authors concluded that, when compared to Fischer 344 rats, B6C3F$_1$ hybrid mice are less susceptible to effects in the liver upon administration of eucalyptol, regardless of whether by gavage or encapsulated and mixed in the feed (NTP, 1987a). For the oral gavage part of the study, a NOAEL of 1200 mg/kg bw/day was determined for both male rats and female mice.

In a modified OECD guideline study, Wistar rats (10/sex/group) were administered eucalyptol by gavage at doses of 100, 500 or 1000 mg/kg bw/day for 50 days (Caldas et al., 2016). The vehicle control was a 1% Tween-80 (w/v) aqueous solution. Blood samples were drawn at the end of the study prior to euthanasia. Macroscopic examination of the heart, lung, liver, kidneys, adrenal glands, spleen, stomach, intestine, pancreas, brain and reproductive organs was performed for five randomly chosen male and female rats in each treatment group and histopathological analyses were performed on the remaining animals. The study reported no clinical signs of toxicity. Diarrhea was observed in study animals at the mid- and high doses during the first week of treatment but ceased on the second week and following weeks and did not affect the total weight gain of the animals. Lower body weights were observed between days 7 and 50 of the study in the mid- and high male and female dose groups. Reduced body weight gains were observed for high dose male and female groups and mid-dose males in the first week of treatment that was followed by an increase in body weights of males and females in all treatment groups. All treatment groups showed non-dose related changes in water and food consumption compared to controls.

Hematological analyses showed a significant increase in mean corpuscular volume in high dose males, increase in platelet count and decrease in mean corpuscular hemoglobin concentration in the mid- and high dose males, and decreased mean platelet volume in all male treatment groups. No significant hematological changes were observed in any of the female treatment groups. Biochemical analyses of serum concentrations showed a decrease in alkaline phosphatase in the low dose male treatment group and increase in urea in mid- and high dose female rats.

Macroscopic analysis of the organs showed a decrease in the absolute weight of the lungs and spleen in mid- and high dose male rats. An increase in the relative and absolute liver weights was observed in high dose females. No other significant changes were observed between the treatment and control groups. Histopathological examinations found eosinophilic and lymphocytic infiltrate in the lungs of males and females and the uterus in all the treatment groups, raising concerns that the animals were infected. Lymphocytic infiltrate was observed in the liver of mid- and high dose males, but mild degrees of such infiltrates are common in rats. An increase in the glomerular space in the kidneys was observed in high dose males and mid- and high dose female rats. Based on the reduction of water consumption, mean platelet volume and alkaline phosphatase observed in the low dose group, the study authors did not determine a NOAEL (Caldas et al., 2016). For this study, the FEMA Expert Panel has determined a lowest-observed-adverse-effect-level (LOAEL) of 100 mg/kg bw/day eucalyptol in rats. Additionally, the Panel has concerns regarding infection of the animals reported in all groups including controls that limits the usefulness of this study for risk assessment especially. This concern is supported by the lack of adverse effects observed at dose levels of 8 and 32 mg/kg bw/day eucalyptol in the long-term study by Roe and co-workers using specific pathogen-free (SPF) mice (Roe et al., 1979).

7.1.3. Long-term studies of oral toxicity

In a toxicity study on several ingredients of toothpaste, one of the groups was administered eucalyptol alone at dose levels of 8 or 32 mg/kg bw per day by gavage 6 days weekly for 80 weeks to male specific pathogen-free CFLP mice (52/group) (Roe et al., 1979). Control groups (52/group) included an untreated control group and a vehicle control given a toothpaste base (52/group). At week 80, all surviving animals were terminated and organ weights were recorded for the adrenals, kidneys, liver, lungs and spleen. All macroscopically identified tumors were processed for histopathological examination along with tissue samples from the liver, kidneys, lungs, and brain. There were no eucalyptol related changes observed in food consumption, body weight, organ weights or clinical signs. Histopathological analysis found no differences between the control and eucalyptol groups in the incidence of tumors of the liver, kidney, lung or malignant lymphoma. The FEMA Expert Panel determined the NOAEL to be greater than 32 mg/kg bw/day, the highest dose tested.

7.1.4. Reproductive and developmental toxicity

In a reproductive/developmental study, pregnant rats, 7 to 10 animals per group, were randomly distributed into eight groups (Caldas et al., 2016). Four groups were treated during the preimplantation period (Day 1–6 of pregnancy) and four groups were treated during organogenesis (Day 7–14 of pregnancy). The treatment groups were administered eucalyptol by gavage at doses of 250, 500 or 1000 mg/kg bw/day for 7 days. The vehicle control was a 1% Tween-80 aqueous solution. At day 21, all animals were terminated and the uterine horns, ovaries, fetuses and placentae were removed. The number of implantations, resorptions and live and dead fetuses were recorded. Ovaries were weighed and the number of corpora lutea counted. The fetuses and placentae were weighed (absolute mass) and observed macroscopically for any visible abnormalities.

There were no deaths, changes in food or water consumption or clinical signs observed during the study. There was a significant decrease observed in maternal weight gain during pre-implantation (Days 1–6) at all dose levels for rats treated during the pre-implantation and organogenesis periods. A significant decrease in maternal weight gain during the pregnancy period (Days 1–20) was only observed when eucalyptol was administered at the highest dose during the pre-implantation period. No changes in the ovary and placental mass, the implantation or resorption index and the loss rate of pre-and post-implantation were observed during the pre-implantation or organogenesis periods in all the treated experimental groups. In the high dose pre-implantation period group, dead fetuses and reduction in the mass of fetuses were observed. During the organogenesis period, a single high dose rat presented vaginal bleeding on the 13th day of gestation and laparotomy did not reveal any fetuses (live or dead). There was a reduction in the number of corpora lutea in low dose females of the organogenesis group, when compared to the control group. Based on the reduction of maternal weight gain at all treatment doses of eucalyptol during the pre-implantation and organogenesis periods, the study authors did not determine a maternal NOAEL nor a NOAEL for the progeny (Caldas et al., 2016). This study was performed in the same laboratory and used the same strain of rat used in the repeated dose study summarized above. In the repeated dose study, histopathological examinations found eosinophilic and lymphocytic infiltrate in the lungs of males and females and the uterus in all the treatment groups, raising concerns that the animals were infected. In the reproduction study, histopathological examinations on the lungs and uterus were not reported, but because this study used the same strain of rat, in the same laboratory presumably during the same approximate time period, the FEMA Expert Panel has the same concerns regarding infection, limiting the usefulness of this reproduction study for the risk assessment of eucalyptol, especially considering the results of the OECD guideline study described below.

In an OECD and GLP guideline-compliant reproductive and developmental toxicity study, groups of male and female Wistar rats were administered 0 (vehicle control), 30, 300 or 600 mg/kg bw/day of eucalyptol in arachis oil BP by gavage (ECHA, 2013c). The animals received the test substance daily until females and offspring were terminated on day 5 post-partum except for the 600 mg/kg bw/day group which were mated again. All non-successfully impregnated females were terminated on day 25 post coitum; one female in the high dose group animals who was non-pregnant after a second mating phase was terminated on day 43. Males were terminated immediately after the second pairing.

There were no premature deaths reported for any test or control group animals and only minor clinical observations were observed that were incidental and not associated with test substance administration. Females in the 600 mg/kg bw/day group showed a significantly lower overall body weight gain at the end of the study period when compared to controls. There were no effects on mating at any test dose. For the high dose females that were re-paired with males following an unsuccessful first mating phase, all but one was successfully impregnated during the second mating phase. Gross examination during necropsy of this female rat revealed that the ovaries were encased in fluid filled sacs, an effect that may have been congenital and was not attributed to administration of the test substance. Gestation length for females and male sex organ weights were unaffected by eucalyptol administration. There were no histopathology findings of note. The NOAEL for the parental generation was determined to be 600 mg/kg bw/day, the highest dose level tested.

In the F1 generation there were no effects noted at any concentration on indicators of viability, number of corpora lutea and implantations, pre-and post-implantation loss, number of live off-spring, survival of offspring through weaning at day 4, mean litter size and sex ratio. Clinical observations of the pups were unremarkable. For the 600 mg/kg bw/day groups, there was no effect on initial pup body weights, but by day 4 there was a statistically significant reduction in body and litter weights noted, although this may have been due to the decreased weight of the dams rather than a direct effect on the pups. The FEMA Expert Panel considered this reduction in body weight and litter weight to be an adverse effect. No such differences were observed at the 30 and 300 mg/kg bw/day doses. There were no differences between test and controls in surface righting measurements on Day 1. Based on the reduction in body weights and litter weights in the highest dose group, the Panel determined the NOAEL for reproduction and developmental toxicity to be 300 mg/kg bw/day (ECHA, 2013c).

7.1.5. Genotoxicity

Negative results were reported in Ames assays when *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA1535 and TA1537 were incubated with eucalyptol at concentrations up to 3333 µg/plate, with and without S9 metabolic activation derived from Aroclor 1254-treated rats (Gomes-Carneiro et al., 1998; Haworth et al., 1983). In the rec assay with *Bacillus subtilis* H17 and M45, which detects DNA-damaging activity based on differences in growth inhibition zones, eucalyptol was negative at concentrations ranging from 18 to 20,000 µg/disk (Oda et al., 1978; Yoo, 1986). The OECD notes that indicator tests such as the rec assay should be considered in connection with the results of other assays that measure DNA damage or induction of heritable mutations (OECD, 2015).

In an *in vitro* sister chromatid exchange (SCE) assay in Chinese hamster ovary (CHO) cells, a statistically significant increase ($p < 0.05$) in the incidence of SCEs without metabolic activation was reported at high doses of eucalyptol that induced cell cycle delay (200–500 µg/mL) (Galloway et al., 1987a, 1987b). This finding was not confirmed in a subsequent study with test concentrations up to 333 µg/mL at which a clear precipitation, not present at lower concentrations (3.3, 10, and 33.3 µg/mL), was observed (Sasaki et al., 1989). In the earlier study, there was no increased incidence of SCE found in the presence or absence of metabolic activation at concentrations below 200 µg/mL

eucalyptol (Galloway et al., 1987a). In the subsequent study, no in-duction of chromosomal aberrations was observed when CHO cells were incubated with eucalyptol at either 479–663 μg/mL in the absence or 630–810 μg/mL in presence of a S9 metabolic activation system (Galloway et al., 1987a).

In summary, eucalyptol was negative for mutagenicity in the Ames assay and negative for the induction of chromosomal aberrations at doses below the limit of cytotoxicity (cell cycle delay). Although the *in vitro* SCE assay was removed from the OECD library of standardized assays due to a lack of understanding of the underlying mechanism(s) of action (OECD, 2015), SCE assays performed with eucalyptol in CHO cells were also negative below the limit of cytotoxicity. Based on the results of all the genotoxicity studies, there is no genotoxic concern for eucalyptol.

7.2. Group 19 constituent: d-limonene

In Step 6 of the safety evaluation, the MoS for Group 19 (Aliphatic and aromatic hydrocarbons) constituents of Rosemary Oil (FEMA 2992) was calculated based on the NOAEL for *d*-limonene of 215 mg/kg bw/day (adjusted daily dose from 300 mg/kg bw/day administered 5 days/week) reported for female F344N rats in the 103 week NTP study (NTP, 1990). A brief summary of this study is presented below. This study was reviewed recently by the FEMA Expert Panel as part of their GRAS affirmation of *Citrus*-derived NFCs (Cohen et al., 2019). In addition, the FEMA Expert Panel has re-evaluated the safety of flavoring materials of this congeneric group (Adams et al., 2011).

The chronic effects of *d*-limonene administration were tested in male and female F344/N rats via gavage administration at dose levels of 0, 75 or 150 mg/kg bw/day or 0, 300 or 600 mg/kg bw/day respectively, for 5 days/week for 103 weeks (NTP, 1990). No treatment related clinical signs, other than lower mean body weights in the high dose groups for both sexes and increased mortality in the high dose female group, were reported during the study. The kidneys of the male rats showed dose-related increases in the incidence of mineralization of the renal papilla, focal hyperplasia of the epithelium lining the papilla, a dose-related increase in the severity of nephropathy and increased incidences of tubular cell hyperplasia and neoplasia. A dose-related increase in α_{2u}-globulin in the kidney in conjunction with the tubular cell hyperplasia in male rats with a lack of corollary findings in female rats, is indicative of these tumors happening secondary to α_{2u}-globulin nephropathy. Due to these effects, the NTP concluded that *d*-limonene was carcinogenic for male F344/N rats, but not for female rats at doses up to 600 mg/kg bw/day.

The development of α_{2u}-globulin nephropathy in male rats has been extensively studied and determined to be species- and sex-specific (Webb et al., 1990). Treatment with *d*-limonene causes the accumulation of α_{2u}-globulin and hyaline droplet formation in proximal tubule cells of the male rat (Hard et al., 1993). The buildup of hyaline droplets leads to renal cell damage, and then subsequently to the development of renal tumors (Lehman-McKeeman, 2010b). Both the U.S. Environmental Protection Agency (US EPA) and the International Agency for Research on Cancer (IARC) have determined that the development of α_{2u}-globulin nephropathy in male rats should not be used to estimate the nephrotoxic or cancer hazard for humans (Capen et al., 1999; US-EPA, 1991). Detailed analysis of α_{2u}-globulin nephropathy development in the male rat and additional evidence indicated that this type of nephropathy is doubtful to occur in humans and other species (Flamm and Lehman-McKeeman, 1991). Subsequent evaluations of *d*-limonene as a flavoring ingredient by both the European Food Safety Authority (EFSA) and Joint FAO/WHO Expert Committee on Food Additives (JECFA) have agreed that the NTP observed male rat nephropathy is not of human relevance (EFSA, 2015; JECFA, 2005); the FEMA Expert Panel concurs with these previous evaluations, concluding that the NOAEL for *d*-limonene is 215 mg/kg bw/day (adjusted daily dose from 300 mg/kg bw/day administered 5 days/week) based on the lower mean body

weights and increased mortality in the high dose female group in the 103 week NTP study (NTP, 1990).

7.3. Natural flavor complexes

7.3.1. Bay Sweet Oil

Genotoxicity. In an OECD and GLP guideline-compliant reverse mutation (Ames) assay, bay sweet oil (also known as laurel leaf oil) was tested in *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and *E. coli* WP2 at concentrations up to 150 μg/plate in the absence of S9 metabolic activation system and 500 μg/plate with an S9 metabolic activation system derived from the liver of phenobarbitone/5,6-benzoflavone-induced rats. Bay sweet oil caused a visible reduction in the growth of the bacterial background lawns in all the tester strains, from 50 μg/plate in the absence of S9 and from 150 μg/plate in the presence of S9-mix. Bay sweet oil was non-mutagenic under the conditions tested (ECHA, 2013a).

7.3.2. Cajeput Oil

Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, cajeput green oil was tested in *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and *E. coli* WP2 at concentrations up to 500 μg/plate both with and without an S9 metabolic activation system derived from the liver of phenobarbitone/5,6- benzoflavone-induced rats. Cajeput green oil was non-mutagenic under the conditions tested (ECHA, 2013b).

7.3.3. Eucalyptus Oil

7.3.3.1. Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, eucalyptus oil was tested in *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and *E coli* WP2 at concentrations up to 5000 μg/plate both with and without an S9 metabolic activation system derived from the liver of phenobarbital sodium/5,6-benzoflavone-induced rats. Eucalyptus oils was non-mutagenic under the conditions tested (ECHA, 2013e).

In an OECD and GLP guideline-compliant chromosomal aberration study in human peripheral blood lymphocytes, eucalyptus oil was tested at concentrations up to 350 μg/mL in a 3 h treatment with an S9 metabolic activation system derived from the liver of phenobarbital sodium/5,6- benzoflavone-induced rats, at concentrations up to 100 μg/mL in a 3 h treatment without S9 and at concentrations up to 90 μg/mL in a 21 h treatment also in the absence of S9. Under the conditions tested, eucalyptus oil was non-clastogenic (ECHA, 2013d).

In an OECD and GLP guideline-compliant mutation assay in mouse lymphoma L5178Y cells, eucalyptus oil was tested at concentrations up to 250 μg/mL in a 3 h treatment in the presence of S9 metabolic activation system derived from the liver of phenobarbital/5,6-benzoflavone-induced rats and at concentrations up to 175 μg/mL and 250 μg/mL in 3 h and 24 h treatments, respectively, in the absence of an S9 metabolic activation system. Under the conditions tested, eucalyptus oil was not mutagenic at the TK locus in mouse lymphoma L5178Y cells (ECHA, 2013f).

7.3.3.2. Combined repeated dose toxicity study with the reproduction/ developmental toxicity screening test. In a GLP study conducted under the OECD Guideline 422 combined repeated dose toxicity study with the reproduction/developmental toxicity screening test, eucalyptus oil was administered to Crl:CD(SD) rats (10/sex/dose) by oral gavage at doses of 0 (corn oil vehicle), 100, 300 or 1000 mg/kg per day (ECHA, 2013g). Male rats were treated for 14 days prior to pairing and up to necropsy (minimum of 5 weeks). Female rats were treated for 14 days before pairing, throughout pairing and gestation periods until day 6 of lactation or for females that failed to produce a litter, until day 25 after mating.

During the study, the death of one pregnant female receiving 1000 mg/kg bw/day eucalyptus oil occurred on Day 15 after mating but it was not attributed to treatment. Both high dose male and female groups displayed chin rubbing and salivation and signs of under activity and unsteady muscle reactions. Salivation was also recorded in female rats in the middle dose group. Body weight gain was reduced in high dose males compared to the control group in the Week 0–1 period but were similar to the control group following Week 1. No changes in sensory reactivity, grip strength or motor activity were observed in the test groups compared to controls.

Body weight gain was reduced in high dose females compared to the control group during gestation and the absolute and relative body weight differences were statistically significant from Day 6 of gestation. Absolute body weight of the high dose female group was significantly lower than the control group at Day 1 of lactation but not at Day 7 of lactation. Food consumption appeared slightly lowered for high dose females during Week 1 of study, and significantly lowered during Days 6–19 of gestation and Days 4–6 of lactation. In the male groups, there were no differences detected in food consumption.

Biochemical and hematological analyses performed during Week 2 of treatment showed high lymphocyte, basophil, monocyte and large un-stained cell counts (resulting in an associated increase in total white blood cell counts) in females in the middle and high dose groups compared to the control group. These findings lacked any dose-relationship trend, were not apparent in males and were considered not to be adverse by the study authors. Activated partial thromboplastin time was lowered in the high dose male rats but because no clear dose response was apparent and this difference was not seen in females, it was not considered to be an adverse effect by the study authors. High alanine aminotransferase activity and bile acid concentration were recorded in the blood plasma of high dose females. High urea and low triglyceride concentrations were recorded in high dose males and may be associated with the microscopic changes observed in the liver and kidneys.

At necropsy, dose related higher kidney weights were observed in the male rats. Depressed areas were present in the kidneys of four males in the high dose group that were correlated with foci of tubular degeneration/regeneration that were related to test article administration. All treated male groups had hyaline droplets in the proximal tubules of the cortex, with a dose-related incidence and severity. Multifocal tubular degeneration/regeneration was also noted in all treated male groups, but without a dose-relationship. Tubular casts of cell debris, seen at 100 and 1000 mg/kg bw/day in males, were considered to originate from the degenerating tubules, causing the occasional dilation of the lumens of tubules at the corticomedullary junction. These effects are consistent with hyaline droplet nephropathy caused by accumulation of α_{2u}-globulin, produced by the male rat liver, in the proximal tubules. This effect was also seen in *d*-limonene studies discussed above and is an effect specific to the male rat that is not relevant to human health (Capen et al., 1999; US-EPA, 1991).

A dose related increase in liver weights was recorded for both male and female treatment groups. Histopathological analysis found a dose-related occurrence of centrilobular hepatocyte hypertrophy in the male treatment groups that the study authors attributed to an adaptive change associated with microsomal enzyme induction. A slight increase in the incidence and severity of glycogenic vacuolation was found in the livers of treatment group females but not in control females. The study authors did not consider this effect adverse. They also note that although centrilobular hepatocyte hypertrophy was not recorded in the females, a minimal diffuse hypertrophy, that is difficult to detect histologically, may have accounted for the liver weight increase in this sex.

An increase in adrenal weights was observed in the high dose female treatment group compared to the control group and the adjusted spleen weights were lower than in controls and attained statistical significance for all female treatment groups although this effect was not dose-related. In addition, there were no microscopic observations correlated to the observed decreases in spleen weight and the increase in adrenal weight

of the 1000 mg/kg/day females.

The uterus (including uterine cervix and oviducts) weights in the high dose female treatment group were statistically higher compared to the control group though this was considered not adverse by the study authors. Treatment with eucalyptus oil did not result in significant changes in estrous cycles, mating performance and fertility, gestation length and parturition observations or reproductive performance. There were no significant differences in the male reproductive organ weights between the control and treatment groups. In the offspring, there were no significant effects of the test material on litter size, survival indices or sex ratio. Bodyweights for the low and middle dose group offspring on Day 1 of age were similar to those of the control group offspring. For offspring of the high dose group, body weight gains of male and female offspring were low and by Day 4 of age, absolute body weights of this group were also significantly lower than control offspring. Macropathological screening of the offspring reported no findings attributable to eucalyptus oil.

A NOAEL of 300 mg/kg bw/day for systemic toxicity was determined for female rats based on lowered body weight gains and lowered food consumption observed in the high dose treatment group. A NOAEL of 1000 mg/kg bw/day for systemic toxicity was determined for male rats, the highest dose tested.

7.3.3.3. Additional considerations. There are reports of accidental poisonings from direct oral consumption of eucalyptus oil in its neat or concentrated liquid form by children and adults and from the consumption of vapor rub ointments and vaporizing liquids by children (Cosmetic Ingredient Review Expert Panel, 2018; Day et al., 1997; Flaman et al., 2001; Ittyachen et al., 2019; Paul et al., 2010; Sitaraman and Rao, 2019; Spoerke et al., 1989; Tibballs, 1995; Webb and Pitt, 1993). These reports describe the accidental ingestion of eucalyptus oil, with amounts ranging from a "taste" to several milliliters of neat eucalyptus oil resulting in effects such as vomiting and depression of the central nervous system that resolved over time. The FEMA Expert Panel considered whether poisoning from Eucalyptus Oil (FEMA 2466) from use as a flavoring ingredient could occur as a result of its ingestion from food. Pertinent to this context, there are no reports of eucalyptus oil poisoning from food. Compared to use in massage therapy and in over-the-counter (OTC) drugs, Eucalyptus Oil (FEMA 2466) is used at much lower concentrations as a flavoring ingredient in foods, ranging from approximately 4 to 2000 ppm (0.2%) with the highest use level occurring in hard candies. In the OECD guideline 422 repeated dose oral gavage toxicity study combined with a reproduction/developmental toxicity screening test summarized above, a NOAEL of 300 mg/kg bw/day was determined for eucalyptus oil administered to female rats (ECHA, 2013g). Based on this NOAEL and the estimated *per capita* intake reported in Table 1 from use of Eucalytptus Oil (FEMA 2466) as a flavoring ingredient, a MoS of greater than 69,000 was calculated.

7.3.4. Laurel leaf extract

Genotoxicity. An ethanolic extract of laurel was tested in a screening mutagenicity experiment. Strains TA98 and TA100 of *S. typhimurium* were treated with 10, 30 or 50 mg/plate of the laurel extract, which was non-mutagenic under the conditions tested (Namiki et al., 1984). Another screening assay was also negative when a methanol-chloroform extract of laurel leaves was tested in TA98 and TA100 in the presence of S9 activation, obtained from the livers of Aroclor 1254-induced rats (Rockwell and Raw, 1979).

Laurel leaf extract was non-genotoxic in an *in vitro* micronucleus assay using primary rat hepatocytes (Turkez and Geyikoglu, 2011). In this study, concentrations of 50, 100, and 200 mg/L of the ethanolic extract were evaluated in the absence of an exogenous metabolic activation system and did not increase the frequency of micronucleated hepatocytes (Turkez and Geyikoglu, 2011).

7.3.5. Marjoram oil

Genotoxicity. Marjoram oil, isolated by steam distillation of *O. marjorana*, was tested in an Ames reverse mutation assay to evaluate its mutagenic potential. The chemical composition, determined by gas chromatography, of the essential oil was 26% *p*-mentha-1,4-diene, 17% 4-carvomenthenol, 17% *p*-mentha-1,3-diene, 11% sabinene and other minor components. Concentration ranges of 2.2–35 μg/plate and 4.4 and 71 μg/plate[7] were tested in the absence and presence of an Aroclor 1254-induced S9 metabolic activation system from rat liver. Marjoram oil did not increase the number of revertant colonies in *S. typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535 (Dantas et al., 2016). In an OECD and GLP guideline-compliant Ames assay, marjoram sweet oil was tested in *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and *E coli* WP2 at concentrations up to 5000 μg/plate both with and without an S9 metabolic activation system derived from the liver of phenobarbital/5,6-benzoflavone-induced rats. Marjoram oil was non-mutagenic under the conditions tested (ECHA, 2017). No induction in the formation of micronuclei in binucleated cells was reported for marjoram oil in a micronucleus assay in Chinese hamster lung fibroblasts (V79 cells) in the absence of S9 (Dantas et al., 2016).

7.3.6. Rosemary Oil

Genotoxicity. In an OECD and GLP guideline-compliant study, rosemary oil (composition not provided but certificate of analysis indicates that it was consistent with FEMA 2992) was not mutagenic in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2*uvrA* using the plate incorporation method up to 5000 μg/plate in both the presence and absence of an Aroclor 1254-induced rat liver S9 metabolic activation system. Initial and confirmatory assays in tester strain TA98 showed an increase in revertant counts, 1.7- and 1.9-fold, respectively; however, these changes did not meet the criteria to be considered indicative of mutagenicity (Dakoulas, 2014).

In an OECD and GLP guideline-compliant *in vitro* micronucleus study with human peripheral blood lymphocytes, rosemary oil (composition not provided; item listed as FEMA 2992) showed no induction of micronuclei either with or without an Aroclor 1254-induced rat liver S9 metabolic activating system (Roy, 2015). The 50 μg/mL S9-activated 4-h exposure group had a statistically significant increase in micronucleated cells when compared to the control, but a statistically significant dose-response was not observed. Therefore, this increase was not considered to be biologically relevant and rosemary oil was concluded to be negative for the induction of micronuclei in human peripheral blood lymphocytes in the presence or absence of S9 metabolic activation (Roy, 2015).

In a combined *in vivo* chromosomal aberration and micronucleus assay, three groups of six Wistar rats (3 male and 3 female) were administered a single dose of 6.43, 100 or 200 mg/kg bw of a *Rosmarinus officinalis* Linn aqueous ethanolic solution (constituent profile not provided). The test substance was prepared by maceration of the dried leaves and stems of *R. officinalis* Linn followed by extraction into a hydroalcoholic solution at room temperature for 15 days, filtering of the extract and evaporation of the solvent to dryness. No significant increases in either the induction of micronucleated polychromatic erythrocytes or chromosomal aberrations in bone marrow cells was reported (Gaiani et al., 2006).

A combined *in vivo* alkaline comet and micronucleus assay in albino Swiss mice yielded positive results in both arms at doses of 300, 1000 or 2000 mg/kg bw of laboratory prepared rosemary oil (Maistro et al., 2010). The FEMA Expert Panel reviewed this study and determined it

not to be helpful for safety evaluation for the following reasons: the starting material lacked compositional data to determine its similarity to the FEMA recognized rosemary oil and the testing and evaluation in both assays deviated significantly from OECD and GLP guidelines. Significant DNA damage was noted at all three test doses in the liver cells and leukocytes, however, a dose-response was not observed and evaluation of animal histology and cytotoxicity was not reported as part of the micronucleus assay, thereby limiting a complete evaluation of the study.

7.3.7. Spanish Sage Oil

Genotoxicity. In an OECD and GLP guideline-compliant Ames assay, Spanish sage oil was non-mutagenic when tested in *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and *E coli* WP2 at concentrations up to 5000 μg/plate both with and without S9 metabolic activation system (ECHA, 2018). In another Ames assay the essential oil of *Salvia officinalis* L. var. *lavandulaefolia,* commonly known as Spanish sage oil, was not mutagenic in an Ames reverse mutation assay in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 at three concentrations up to 1 μL/plate, in the absence or presence of metabolic activation (Zani et al., 1991). The composition of the essential oil was analyzed by gas chromatography and determined to be 31% eucalyptol, 12% camphor, 7% β-pinene, 6% humulene and other minor components.

The essential oil of *Salvia officinalis* L. var. *lavandulaefolia* was also negative in the *Bacillus subtilis* rec assay at concentrations up to 6 mg/mL of the prepared essential oil (Zani et al., 1991). As previously mentioned, the OECD notes that indicator tests such as the rec assay should be considered in connection with the results of other assays that measure induction of DNA damage or heritable mutations (OECD, 2015).

7.3.8. Summary

Long and short-term toxicity studies as well as reproductive and developmental toxicity assays on the primary constituent eucalyptol presented sufficient margins of safety when reported NOAELs are compared to the estimated daily intake of each of the NFCs under consideration for use as flavoring. Additionally, eucalyptol was negative for genotoxicity in several OECD guideline and non-guideline studies.

Although one genotoxicity assay found a positive result for rosemary oil, the Panel determined this study not to be helpful for the safety evaluation due to testing irregularities and lack of specification for the sample tested, and therefore did not include it in the safety evaluation of rosemary oil. Other available genotoxicity assays on bay sweet oil, eucalyptus oil, cajeput oil, rosemary oil, marjoram oil, laurel leaf extract and Spanish sage oil yielded negative results. Based on the available studies on the NFCs and their respective constituents including primarily Group 19 constituents and eucalyptol, there is no concern for genotoxicity for the NFCs under consideration.

8. Recognition of GRAS status

The NFCs listed in Table 1 were determined to be GRAS under conditions of intended use by FEMA in 1965. The safety evaluation of these NFCs has indicated that their constituents are absorbed, metabolized to innocuous metabolites and excreted. Long and short-term toxicity studies on eucalyptol provide sufficient margins of safety for the estimated daily intake of the eucalyptol-containing NFCs. In addition, there is no genotoxicity concern for eucalyptol and the NFCs under consideration based on the available studies on the NFCs and major constituents. Exposure to allylalkoxybenzene constituents at estimated intakes below the TTC of 0.15 μg/person/day in Bay Sweet Oil (FEMA 2125), Laurel Leaves Extract (FEMA 2613), Marjoram Oil Sweet (FEMA 2663) and Marjoram Oleoresin (FEMA 2659) were determined not be a safety concern. In Rosemary Oil (FEMA 2992), Bay Sweet Oil (FEMA 2125) and Marjoram Oil Sweet (FEMA 2663) where the estimated intake of estragole or methyl eugenol exceeded the TTC for compounds with a

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[7] Based on median density of 0.898 g/mL (Source: Food Chemical Codex 12th Edition, United States Pharmacopeia (USP), Rockville, MD, USA).

Table 7
NFCs affirmed FEMA GRAS.

FEMA No.	Name
2125	Bay Sweet Oil (*Laurus nobilis* L.)
2225	Cajeput Oil (*Melaleuca leucadendron* L.)
2466	Eucalyptus Oil (*Eucalyptus globulus* Labille)
2613	Laurel Leaves Extract (*Laurus nobilis* L.)
2657	Marjoram Oleoresin (*Majorana hortensis* Moench (*Origanum majorana* L.))
2663	Marjoram Oil Sweet (*Majorana hortensis* Moench (*Origanum majorana* L.))
2992	Rosemary Oil (*Rosmarinus officinalis* L.), Garden rosemary oil
3003	Sage Spanish Oil (*Salvia lavandulaefolia* Vahl.)

structural alert for genotoxicity, a sufficient MOE was determined, thereby not raising a concern.

After review of the relevant scientific data on the NFCs listed in Table 7 for use as flavoring ingredients, the FEMA Expert Panel affirmed their GRAS status under intended conditions of use.

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 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.fct.2021.112357.

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