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FEMA GRAS assessment of natural flavor complexes: *Citrus*-derived flavoring ingredients

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ABSTRACT

In 2015, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) initiated a re-evaluation of the safety of over 250 natural flavor complexes (NFCs) used as flavoring ingredients. This publication is the first in a series and summarizes the evaluation of 54 *Citrus*-derived NFCs using the procedure outlined in Smith et al. (2005) and updated in Cohen et al. (2018) to evaluate the safety of naturally-occurring mixtures for their intended use as flavoring ingredients. The procedure relies on a complete chemical characterization of each NFC intended for commerce and organization of each NFC's 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 members of the congeneric groups and the NFC under evaluation. As a result of the application of the procedure, 54 natural flavor complexes derived from botanicals of the *Citrus* genus were affirmed as generally recognized as safe (GRAS) under their conditions of intended use as flavoring ingredients based on an evaluation of each NFC and the constituents and congeneric groups therein.

Abbreviations: CF, Correction factor; CFR, Code of federal regulations; CHO, Chinese hamster ovary (cells); CYP, Cytochrome P450 (enzymes); DFG, Deutsche Forschungsgemeinschaft; DTC, Decision tree class; EFFA, European Flavour Association; EFSA, European Food Safety Authority; EMEA, European Medicines Agency; ERS/USDA, Economic Research Service/U.S. Department of Agriculture; FAO, Food and Agriculture Organization; FDA, Food and Drug Administration; FEMA, Flavor and Extract Manufacturers Association; FID, Flame ionization detector; GC, Gas chromatography; GC-MS, Gas chromatography-mass spectrometry; GEF, Global evaluation factor; GLP, Good laboratory practice; GRAS, Generally recognized as safe; IARC, International Agency for Research on Cancer; IFEAT, International Federation of Essential Oils and Aroma Trades; IOFI, International Organization of the Flavor Industry; JECFA, Joint FAO/WHO Expert Committee on Food Additives; JFFMA, Japan Fragrance and Flavor Materials Association; LC-MS, Liquid chromatography-mass spectrometry; LD_{50}, Median lethal dose; MF, Mutant frequency; MLA, Mouse lymphoma assay; MoS, Margin of safety; MSD, Mass spectrometric detector; NAS, National Academy of Sciences; NFC, Natural flavoring complex; NOAEL, No observed adverse effect level; NTP, National Toxicology Program; OECD, Organization for Economic Co-Operation and Development; OSOM, Outer stripe of the outer medulla; PCI, Per capita intake; SCE, Sister chromatid exchange; SD, Sprague-Dawley (rat); SKLM, Senate Commission on Food Safety (Germany); TTC, Threshold of toxicological concern; UDS, Unscheduled DNA synthesis (assay); USDA, U.S. Department of Agriculture; US-EPA, U.S. Environmental Protection Agency; WHO, World Health Organization

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1. Introduction

For over fifty years, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) has served as the primary, independent body evaluating the safety of flavoring ingredients for use in human food. The Expert Panel evaluates flavoring ingredients to determine if they can be considered "generally recognized as safe" (GRAS) for their intended use as flavoring ingredients consistent with the 1958 Food Additive Amendment to the Federal Food Drug, and Cosmetic Act (Hallagan and Hall, 1995, 2009). Currently, the FEMA Expert Panel has determined that over 2700 flavoring ingredients have met the criteria for GRAS status under conditions of intended use as flavoring ingredients.

A key part of the FEMA GRAS program is the cyclical re-evaluation of the GRAS status of flavoring ingredients determined to be GRAS by the FEMA Expert Panel. Flavoring ingredients are generally divided into two broad categories, chemically-defined flavoring materials and natural flavor complexes (NFCs). The chemically defined flavoring materials are typically single chemical substances whereas the NFCs are naturally occurring mixtures typically derived from botanical materials. The Panel has previously completed two re-evaluations of the chemically defined flavoring ingredients and in 2015 expanded the re-evaluation program to include more than 250 NFCs on the FEMA GRAS list and other relevant NFCs using a scientifically-based procedure for the safety evaluation of NFCs (Cohen et al., 2018; Smith et al., 2005). The procedure describes a step-wise evaluation of the chemical composition of an NFC. Since many NFCs are products of common plant biochemical pathways (Schwab et al., 2008), the constituents can be organized into a limited number of well-established chemical groups referred to as congeneric groups. The safety of the intake of each congeneric group from consumption of the NFC is evaluated in the context of data on absorption, metabolism, and toxicology of members of the congeneric group. Groups of NFCs of similar chemical composition or taxonomy have been assembled to facilitate the re-evaluation of all the NFCs. The first group re-evaluated comprises flavoring ingredients derived from the *Citrus* genus and is the subject of the present report.

In 2015, the FEMA Expert Panel issued a call for data requesting complete chemical analyses and physical properties for ~50 *Citrus*-derived NFCs known to be used globally by the flavor industry. Members from the International Organization of the Flavor Industry (IOFI), including FEMA (United States), the Japan Fragrance and Flavor Materials Association (JFFMA), the European Flavour Association (EFFA), in addition to the International Federation of Essential Oils and Aroma Trades (IFEAT), responded, providing data on *Citrus* oils currently in commerce for the purpose of flavoring food and beverage products. The *Citrus* flavoring materials re-evaluated by the Expert Panel are listed in Table 1 and are grouped based on the source of the flavoring ingredient.

The *Citrus* NFCs listed in Table 1 have been divided into six general types: 1) peel oils; 2) essence oils and water phase essence; 3) terpenes; 4) petitgrain, and neroli oils; 5) terpeneless peel and essence oils; and 6) extracts. For several major *Citrus* fruit crops including sweet oranges, lemons, grapefruits and limes, two types of *Citrus* oils are produced for use as flavoring materials, as outlined in Fig. 1. Essential "peel" oils are collected by cold-expression from the peels of these fruits (left) while "essence" or "aroma" is collected in the concentration step following the juicing of the whole fruit (right). The essence collected is separated into the oil and water phases, resulting in *Citrus* essence oils and *Citrus* essence water phase. Both peel oils and essence oils recovered directly from *Citrus* fruit without further concentration are considered to be unfolded and termed single fold (1X). Single fold *Citrus* oils may be concentrated by fractional distillation to produce "folded oils" which are also commonly used as flavoring materials. Highly concentrated oils in which the monoterpene hydrocarbon content has been greatly reduced are termed "terpeneless". Orange, lemon, lime and grapefruit terpenes are flavoring materials derived from the distillate of the folding process. These materials are rich in monoterpenes, particularly *d*-limonene, a major constituent in *Citrus* essential oils. The collection of *Citrus* essential oils by distillation of *Citrus* peels and/or *Citrus* juices is generally not practiced since distillation produces lower quality oils (Di Giacomo and Di Giacomo, 2002). The exception is lime oil. Both distilled and cold-expressed lime oils are currently in commerce. The sharp flavor of distilled lime oil is desirable for some products and remains an important flavoring ingredient.

The remaining categories of *Citrus* flavoring materials are the petitgrain/neroli oils and *Citrus* extracts. Petitgrain oils are prepared by steam distillation of the buds and/or leaves of the *Citrus* plant. Neroli oil is prepared by the steam distillation of the flowers of *C. aurantium*. Finally, *Citrus* extracts are prepared from the fruit peel or the peel oil for use as flavoring ingredients.

2. History of food use

The *Citrus* genus includes a variety of fruits commonly found in markets for fresh consumption such as sweet oranges, lemons, grapefruits, tangerines, mandarins and limes. Juice products from sweet orange, lemon and grapefruit are high volume products in western consumer markets. Other *Citrus* fruits, such as bergamot and bitter orange are usually cultivated for their essential oils. In Japan, popular *Citrus* fruits include iyokan, hassaku, sikuwasya, natsumikan, mikan, yuzu, sudachi, kabosu and ponkan.

Despite the wide variety of *Citrus* fruits available today, genetic analysis of *Citrus* trees indicate that all *Citrus* varieties known today originated from only a few types, the pummelo (*C. maxima*), the citron (*C. medica*), the mandarin and the uncultivated papeda (*C. papeda*) (Carbonell-Caballero et al., 2015; Velasco and Licciardello, 2014; Wu et al., 2014). All *Citrus* species are believed to have originated in southeast China and the Malaysian archipelago. East-west trade routes facilitated the introduction and eventual cultivation of *Citrus* into western territories (Calabrese, 2002). Archeological excavations indicate that citron trees were cultivated in Persia around ~4000 BC. Citrons are a small round fruit that are typically eaten whole. Both lemon and lime arose from the hybridization of citron with papeda, a wild, uncultivated *Citrus* species. Alexander the Great brought citrons to the ancient Greeks and Romans by ~300 BC and the fruits are described in both Greek and Roman literature of that time as the "fruit of Persia" or the "fruit of Media". The Greeks are thought to have taken citrons into Palestine around 200 BC and a Jewish coin minted in 136 BC depicts a citron on one side. The citron is mentioned in the Old Testament of the Bible and is part of the Jewish autumn Feast of the Tabernacles. During the time of the Roman Empire, citron, lemon and lime were cultivated throughout the territory as evidenced by their appearance in the artwork from Rome, Carthage, Sicily, Northern Africa (Algeria and Tunisia) and Spain (Calabrese, 2002; Laszo, 2007).

The pummelo (*C. maxima*), also called shaddock, is similar in overall size to grapefruit and is grown in southern Asia where it remains a popular food. Grapefruit, a popular food in western markets, is a hybrid of the pummelo that first appeared in the Caribbean *Citrus* groves in the 17th century (Laszo, 2007). In Japan, hassaku (*C. hassaku)* and natumikan (*C. natsudaidai*) are similar in size and consumed similarly to grapefruit and are probably genetically related to the pummelo (Hirai et al., 1986).

Recent genetic analysis of the sweet orange (*C. sinensis*) genome indicates that it is derived from a yet undetermined series of crosses between pummelo and mandarin species (Wu et al., 2014). Sweet oranges are cultivated primarily for their sweet fruit and juice for sale in food markets and the essential oil is a valuable flavoring ingredient. Due to a lack of references to the sweet orange in historical texts and artwork, the history of the cultivation of the sweet orange (*C. sinenesis*) is not clear. While it appears that the sweet orange originated in China, there is little reference to this *Citrus* fruit until it was recorded as being grown around Lisbon in 1520 (Laszo, 2007). During the Renaissance,

Table 1
Citrus NFCs evaluated by the Expert Panel.

Name[a]	FEMA No.[b]	Fold	Intake (μg/person/day)[c]	Most recent annual volume (kg)[d]
Peel Oils:				
Orange Peel Sweet Oil (*Citrus sinensis* (L.) Osbeck) (1X)	2825A	1X	10,100	944,000
Orange Peel Sweet Oil (*Citrus sinensis* (L.) Osbeck) (5X)	2825B	5X	1800	163,000
Blood Orange Oil (*Citrus sinensis* (L.) Osbeck 'Blood orange')	4856	1X	170	1,570[e]
Lemon Oil (*Citrus limon* (L.) Burm. F.) (1X)	2625A	1X	6800	637,000
Lemon Oil (*Citrus limon* (L.) Burm. F.) (5X)	2625B	5X	610	56,800
Lime Oil, Distilled (*Citrus aurantifolia* (Christman) Swingle) (1X)	2631A	1X	2500	234,000
Lime Oil, Distilled (*Citrus aurantifolia* (Christman) Swingle) (5X)	2631B	5X	520	4820
Mexican Lime Oil, Expressed (*Citrus aurantifolia, Citrus medica* var. *acida*)	4743	1X	110	1070
Persian Lime Oil, Expressed (*Citrus latifolia*)	4744	1X	1000	9450
Mandarin Oil (*Citrus reticulata* Blanco 'Mandarin') (1X)	2657A	1X	1430	13,400
Mandarin Oil (*Citrus reticulata* Blanco 'Mandarin') (5X)	2657C	5X	33	310
Tangerine Oil (*Citrus reticulata* Blanco 'Tangerine') (1X)	3041A	1X	700	65,800
Tangerine Oil (*Citrus reticulata* Blanco 'Tangerine') (5X)	3041B	5X	200	1810
Clementine Oil (*Citrus clementina* hort. ex Tanaka)	4855	1X	0.22	2
Tangelo Oil (*Citrus paradisi Macf.* × *Citrus tangerine* hort. ex Tanaka)	4854	1X	1	0.1
Orange Peel Bitter Oil (*Citrus aurantium* L.) (1X)	2823A	1X	550	5180
Orange Peel Bitter Oil (*Citrus aurantium* L.) (5X)	2823B	5X	10	96[f]
Bergamot Oil (*Citrus aurantium* L. subsp. bergamia Wright et Am.)	2153	1X	1650	15,400
Curacao Peel Oil (*Citrus aurantium* L.)	2345	1X	0.01	0.1
Daidai Peel Oil (*Citrus aurantium* L. subspecies *cyathifera* Y.)	3823	1X	96	820[e]
Grapefruit Oil (*Citrus paradisi* Macf.) (1X)	2530A	1X	1100	100,000
Grapefruit Oil (*Citrus paradisi* Macf.) (5X)	2530B	5X	350	3300
Sarcodactylis Oil (*Citrus medica* L. var. *Sarcodactylis* Swingle)	3899	1X	17	160
Iyokan Oil (*Citrus iyo*)	4857	1X	33	110[g]
Hassaku Oil (*Citrus hassaku* hort. ex Tanaka)	4858	1X	20	65[g]
Sikuwasya Oil (*Citrus depressa*)	4859	1X	35	120[g]
Natumikan Oil (*Citrus natsudaidai*)	4860	1X	112	390[g]
Mikan Oil (*Citrus unshiu*)	4861	1X	162	570[g]
Yuzu Oil (*Citrus junos* (Sieb.) c. Tanaka)	4862	1X	1230	4,340[g]
Sudachi Oil (*Citrus sudachi* hort. ex Shirai)	4863	1X	51	180[g]
Kabosu Oil (*Citrus sphaerocarpa*)	4864	1X	59	200[g]
Ponkan Oil (*Citrus reticulata* Blanco 'Ponkan')	4865	1X	22	79[g]
Essence Oils and Essence Water Phase:				
Orange Essence Oil (*Citrus sinensis* (L.) Osbeck) (1X)	2821A	1X	4510	422,000
Orange Essence Oil (*Citrus sinensis* (L.) Osbeck) (5X)	2821B	5X	1200	11,000
Orange Essence Water Phase (*Citrus sinensis* (L.) Osbeck)	4866	1X	8400	787,000
Grapefruit Essence Oil (*Citrus paradisi* Macf.)	4846	1X	2300	21,700
Lemon Essence Oil (*Citrus limon* (L.) Burm. F.)	4852	1X	1740	11,100
Terpenes:				
Grapefruit Terpenes (*Citrus paradisi* Macf.)	4851	1X	630	59,200
Lemon Terpenes (*Citrus limon* (L.) Burm. f.)	4848	1X	1840	172,000
Lime Terpenes (*Citrus aurantifolia* Swingle, *Citrus medica* var. *acida, Citrus latifolia*)	4849	1X	2800	263,000
Orange Terpenes (*Citrus sinensis* (L.) Osbeck)	4850	1X	39,000	3,650,000
Petitgrain/Neroli Oils:				
Petitgrain Lemon Oil (*Citrus limon* L. Burm. F)	2853	1X	80	780
Petitgrain Mandarin Oil (*Citrus reticulata* Blanco var. *Mandarin*)	2854	1X	105	990
Petitgrain Oil (*Citrus aurantium* L.)	2855	1X	430	4050
Petitgrain Oil, Terpeneless (*Citrus auranthium* L.)	4853		1	7
Neroli Bigarade Oil (*Citrus aurantium* L.)	2771	1X	11	99
Terpeneless Peel and Essence Oils:				
Grapefruit Oil, Terpeneless (*Citrus paradisi* Macf.)	4847		800	7510
Lemon Oil, Terpeneless (*Citrus limon* (L.) Burm. F.)	2626		460	43,200
Lime Oil, Terpeneless (*Citrus aurantifolia* (Christman) Swingle)	2632		370	34,300
Orange Essence Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck)	2822		2230	209,000
Orange Peel Sweet Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck)	2826		300	26,600
Extracts:				
Curacao Peel Extract (*Citrus aurantium* L.)	2344		28	250[h]
Lemon Extract (*Citrus limon* (L.) Burm. F.)	2623		970	9020
Orange Peel Sweet Extract (*Citrus sinensis* (L.) Osbeck) (1X)	2824		1200	110,000

[a] Federal Code 21 CFR 182.20 (Essential oils, solvent-free oleoresins, and natural extractives, including distillates) lists as FDA GRAS *Citrus* peels, botanical name: *Citrus* spp. All the flavoring materials listed can be classified under this CFR listing.

[b] FEMA numbers correspond to the National Academy of Sciences (NAS) identification number. The suffixes "A" and "B" indicate single fold (1X) and five-fold (5X) oils, respectively.

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

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

[e] Private communication.

[f] Harman, C.L., Lipman, M.D. and Hallagan, J.B. (2013) 2010 Poundage and Technical Effects Survey. Flavor and Extract Manufacturers Association of the United States (FEMA), Washington DC, USA.

[g] Source: Japanese Flavor and Fragrance Materials Association (JFFMA) The population of Japan (120 million) was used to calculate intake.

[h] Harman, C.L., Lipman, M.D. and Hallagan, J.B. (2013) 2010 Poundage and Technical Effects Survey. Flavor and Extract Manufacturers Association of the United States (FEMA), Washington DC, USA.

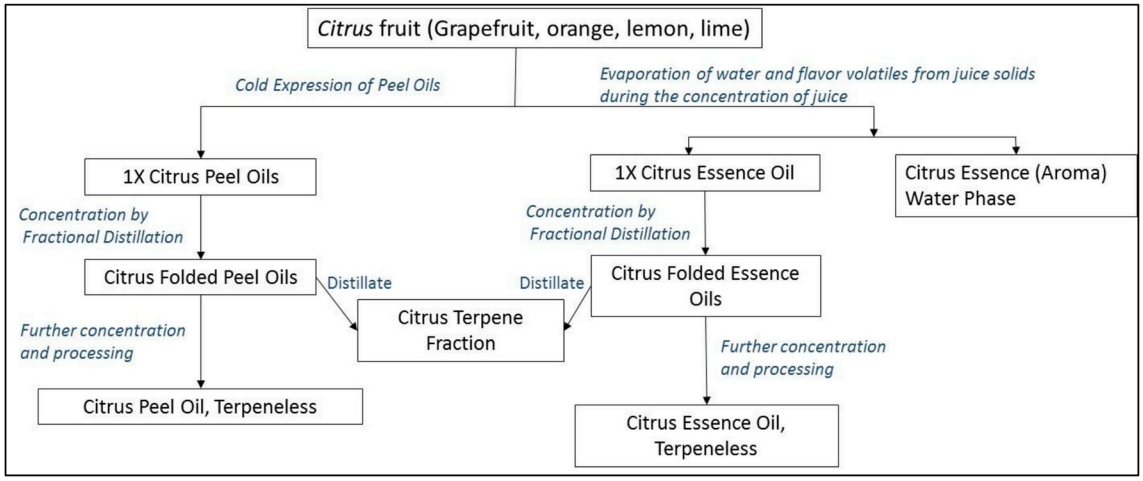

Fig. 1. Summary of the processes used in the production of *Citrus* NFCs derived from the fruit. The essential oils of *Citrus* fruits are isolated from both the peel (peel oil) and the fruit juice (essence oil and essence water phase). The unconcentrated, single fold (1X) oils are used directly as flavoring ingredients or may be concentrated by distillation, in which a fraction of the monoterpene hydrocarbon fraction is removed, yielding a folded oil. When the monoterpene hydrocarbon fraction is almost completely removed, the resulting oil is considered terpeneless. Single fold, folded and terpeneless *Citrus* oils, *Citrus* essence water phase as well as the terpene fraction, are used as flavoring ingredients. In addition, the family of *Citrus* NFCs also include extracts prepared from the peel, peel oil or essence oil.

the cultivation of *Citrus* trees continued to expand northward from the Mediterranean basin. *Citrus* fruits were so desirable that in colder northern areas of Europe wealthy families built “orangeries”, some with furnaces, to maintain proper conditions for the growth and maintenance of *Citrus* trees on their estates (Calabrese, 2002).

Bitter orange (*C. aurantium*), also called sour orange, appears to have arisen from a simple F1 cross between pummelo and mandarin parents (Wu et al., 2014). Unlike sweet oranges that are primarily cultivated for their sweet fruit and juice, bitter oranges are primarily cultivated for their flowers, which are steam distilled for neroli oil and the fruit from which the essential oil is expressed for flavor and perfumery applications. Petitgrain oils, produced by the steam distillation of buds and leaves from the bitter orange tree are also used as flavoring materials. The essential peel oil of bergamot orange, a subspecies of the bitter orange, is also an important flavoring ingredient and best known as the flavoring in Earl Grey tea (Guenther, 1949; Laszo, 2007). The rind of the bitter orange is also commonly used in the preparation of orange marmalade (Laszo, 2007). While the cultivation of bitter oranges is not evident during the Roman empire, Arab literature from the Middle Ages (500-1500 AD) is rich in references to the planting of bitter orange trees in gardens and mosques for their fragrant blossoms and the cultivation of a variety of *Citrus* trees (Calabrese, 2002). The production of neroli oil by steam distillation of the blossoms of bitter orange trees dates to 16th century Italy and this oil remains a valuable flavoring and perfumery ingredient (Govindasamy et al., 2011; Guenther, 1949).

Many varieties of mandarins, including tangerines and clementines, are also prevalent in fresh food markets worldwide (Morton, 1987). The term “mandarin” is used within the citrus industry to refer to the easily peeled, small and sweet *Citrus* fruit. Genetic analysis shows that currently some cultivated mandarins have some pummelo genetic material and differ from the ancient traditional wild mandarin (*C. reticulata*) from which the sweet and bitter orange were derived (Wu et al., 2014). Tangerines (*C. reticulata*), tangelos (*C. paradisi Macf.* × *C. tangerine* hort. ex Tanaka) and clementines (*C. clementina*) are all considered mandarin hybrids and each have distinct origins and flavor profiles for both the fruit and peel oils (Nicolosi, 2007; Reeve and Arthur, 2002). In Japan, mandarins are among the most popular *Citrus* fruits consumed. The trade of *Citrus* from China also extended eastward to Japan and references to *Citrus* trees and fruit appeared in Japanese literature around 710 A.D. The satsuma mandarin, also known as Unshu mikan (*C. unshiu*), was introduced into Japan from China in 1500 AD and is a widely consumed fruit in Japan. Approximately 896,000 metric tons of satsuma were harvested in Japan in 2013, of which 90 percent went to markets for fresh consumption and 10 percent were processed into juice and canned products (Sugimoto, 2014). The ponkan mandarin (*C. reticulata* Blanco) and mandarin-like iyokan (*C. iyo*) are sweet in nature and also popular in Japanese fresh markets. Several other well-known *Citrus* fruits in Japan-yuzu (*C. junos*), sikuwasya (*C. depressa*), kabosu (*C. sphaerocarpa*) and sudachi (*C. sudachi*)- are hybrids derived from mandarin and papeda that are more acidic in taste and are primarily consumed as juice (Siebert and Kahn, 2009). The essential oils of these fruits are also used as flavoring materials.

As the popularity of *Citrus* expanded into Europe during the Renaissance period, European explorers were expanding trade routes to the far-east and discovering the Americas. Spanish and Portuguese explorers introduced *Citrus* trees, including sweet orange, to the Antilles, Mexico, Florida and Brazil (Calabrese, 2002). Catholic missionaries introduced *Citrus* trees to Mexico and California. Currently, Florida, Brazil and California are high producers of *Citrus* products, particularly *Citrus* juices.

At present, *Citrus* fruits are cultivated worldwide although the highest production occurs in subtropical and tropical areas on both sides of the equator. While many of these fruits are consumed fresh, it is estimated that about a third of *Citrus* fruits grown worldwide is processed into fruit juices (Liu et al., 2012). Concurrent with the popularity of *Citrus* fruits, essential oils of *Citrus* fruits, produced by cold expression from the peel of the fruit or as a by-product of the juicing process as well as other *Citrus* derived flavoring materials, also have a long history of use in food as flavoring materials and are currently used extensively as flavoring ingredients across a wide variety of food categories.

3. Current usage

Citrus flavoring materials are used in a variety of foods including beverages, candies, gelatins, frozen dairy products, baked goods and sauces. The most recent annual poundage (Harman and Murray, 2018) and exposure calculations for each material are listed in Table 1. Of the peel and essence oils, sweet orange oils derived from *C. sinensis* (L.) Osbeck have the highest usage with an estimated *per capita* consumption of 10.1 mg/person/day for single fold Orange Peel Sweet Oil (FEMA 2825) and 4.5 mg/person/day for single fold Orange Essence Oil (FEMA 2821). When consumed fresh, the peel of the sweet orange is usually first removed and only the pulp is eaten and thus the essential

Table 2
Estimation of total *Citrus* oils consumed in the USA from juices and fresh fruit in 2014.[a]

	Juices				Fresh Fruit				
	Orange	Grapefruit	Lemon	Lime	Orange	Grapefruit	Lemon	Lime	Mandarin family
USDA *per capita* (g/person/day)	33.1	1.9	1.6	0.4	23.0	6.0	8.3	7.5	12.2
Total Volume *Citrus* oil (ug/person/day)	4960	280	242	60	1150	298	416	373	609

[a] Market data for orange, grapefruit, lemon, lime, and mandarin fresh fruit and fruit juices obtained from ERS/USDA based on data from various sources (see http://www.ers.usda.gov/data-products/food-availability-(per-capita)-data-system/food-availability-documentation.aspx). Data last updated Feb. 1, 2016. Information was downloaded on March 14, 2017. Annual volume naturally occurring in foods calculated from the *per capita* consumption of each in 2014 multiplied by the estimated population for the United States.

oils within the peel are not consumed. However, processed sweet orange juice is estimated to contain 0.015–0.025% total oil that derives from the peel (66–80%) and the juice sacs (20–30%) (Kimball, 1991; Moshonas and Shaw, 1994). In Table 2, the estimation of the annual volume of sweet orange, grapefruit, lemon, lime and mandarin oils consumed from juice and fresh fruit is shown based on *per capita* data gathered by the United States Department of Agriculture (USDA) for 2014. The total essential oil consumed from each juice is calculated based on the conservative estimate that they contain 0.015% essential oils by weight and that the origin of the oil is 30:70 juice sacs:peel. For calculation of the amount of essential oils consumed from the whole fruits, only the oil from the juice sacs of the fruit, estimated to be approximately 0.005%, was considered since the peels of these fruits are usually removed prior to consumption of the carpel or the inner fruit (Rice et al., 1952). On a *per capita* basis, sweet oranges and their juice have the highest consumption of the *Citrus* fruits with a concomitant estimated consumption of 6.1 mg/person/day of sweet orange oil per year from these foods. The *per capita* consumption of lime, lemon, grapefruit and mandarin oils from the consumption of juice and fresh fruit ranges from 0.43 to 0.66 mg/person/day.

4. Manufacturing methodology

Peel oils are harvested from the oil glands of the flavedo, which is the outermost, colored part of the *Citrus* fruit. Historically, peel oils were manually cold-pressed from *Citrus* peels by the Sponge or Ecuelle processes, both of which required manual pressure to break the oil glands and express the essential oil into a collection device. In the early twentieth century, machines were developed to mimic the manual processes (Guenther, 1949). In contemporary high processing systems, mechanical pressure or cutting is used to open the oil glands as water is sprayed onto the surface to wash the expressed oil into a collection container. This process is usually done at room temperature and termed “cold expression”. The resulting water-oil emulsion, also called the “cream”, is separated and polished by centrifugation. Polished oil is then put into cold storage to precipitate and separate out the waxy constituents. The oil is decanted from the waxy precipitate into a separate container and stored under refrigeration (Di Giacomo and Di Giacomo, 2002; Johnson, 2001).

Citrus juice producers have engineered systems that simultaneously extract and process the juice of the fruit and express the volatile oil from the peel, channeling each into its separate processing stream. Following extraction of the *Citrus* juice and the peel oils into separated processing streams, the peel oils are polished, de-waxed and stored as described previously. In the second processing stream, the juice is ‘finished’ to remove juice sacs. The finished juice may then be centrifuged to reduce the pulp prior to concentration by evaporation. In the early stages of the evaporation process in which water is removed to concentrate the juice, the “essence” or “aroma” vapor fraction of the juice, consisting of *d*-limonene, esters, aldehydes, ketones and alcohols, is collected in a de-oiling step. Removal of this essence oil from *Citrus* juice is often essential to maintain the quality of the juice. The oil and water phases of the essence are separated resulting in essence oil and essence water phases. Sweet orange, lemon and grapefruit juice are all valuable, relatively high volume commodities and their production provides opportunities for the collection of high volumes of essence oil for use by the flavor industry and others (Bates et al., 2001; Di Giacomo, 2002).

Most of the other *Citrus* oils listed in Table 1 are peel oils, including mandarin, bitter orange, tangerine, tangelo, bergamot, curacao, iyokan, hassaku, sikuwasya, natsumikan, mikan, yuzu, sudachi, kabosu and ponkan oils. Exceptions to this paradigm are the lime oils. While Mexican lime oil and Persian lime oil are prepared by cold expression from the fruit peels, distilled lime oil is obtained from the distillation of a macerated fruit slurry (Haro-Guzman, 2002).

Citrus oils collected by cold expression of peel oils from the juicing process or by steam distillation that have not been concentrated are considered to be single fold (1X) oils. The constituent profiles of single fold (1X) *Citrus* peel and essence oils are characterized by high concentrations of monoterpenes, particularly *d*-limonene. Single fold oils are often “folded” or concentrated by distillation during which the monoterpene fraction is fully or partially removed yielding a monoterpene-rich distillate and the concentrated, folded oil. The degree of folding is measured by weight. For example, 100 g of 1X *Citrus* oil concentrated to 20 g results in a five fold (5X) *Citrus* oil. A variety of folded oils, ranging from 2X to 20X, are used as flavoring ingredients. Highly concentrated oils in which the terpene hydrocarbons have been almost completely removed are termed terpeneless. The distillate resulting from the folding process, termed orange, lemon, lime or grapefruit terpenes, depending on the type of *Citrus* oil being concentrated, is also a valuable flavoring material.

There are several additional flavoring materials isolated from sweet orange essence oil by fractional distillation. A terpeneless aldehyde fraction, also called orange carbonyl, is the essence oil enriched in octanal, nonanal and decanal that is prepared by fractional distillation. Fractions of orange essence oil enriched in ethyl butyrate or valencene are also prepared by fractional distillation and used as flavoring materials.

Another group of *Citrus* flavoring materials listed in Table 1 are the petitgrain oils. Petitgrain oils are obtained by the steam distillation of the twigs, buds and leaves of a particular *Citrus* tree. Petitgrain lemon, petitgrain mandarin and petitgrain (*C. aurantium* or Paraguay) oils are used as flavoring materials. Neroli bigarade oil is produced by steam distillation of the flowers of the *C. aurantium* tree, the same tree that produces bitter orange fruit. The last group in Table 1 are the extracts of lemon, sweet orange and curacao orange. These extracts can be prepared by solvent extraction of the peels or a previously isolated peel or essence oil. The extract may be further processed to remove the solvent, yielding a concentrated flavoring material, or in the case of some water/ethanol extracts, may be used in the diluted form.

The majority of the flavoring ingredients listed in Table 1 were determined to be FEMA GRAS under their conditions of intended use in 1965 (Hall and Oser, 1965) and the names and descriptions of these *Citrus* materials have not changed much over time, with the exception of the sweet orange oils. Although the single fold peel and essence oils of sweet orange (*C. sinensis*) are both high in *d*-limonene content, they

differ in flavor and in the profile of their minor constituents such as octanal and decanal. In past volumes of use surveys conducted by FEMA and the National Academy of Sciences, three sweet orange oil flavoring materials were surveyed:

FEMA 2821: Orange Oil Distilled (*Citrus sinensis* (L.) Osbeck)
FEMA 2825: Orange Peel Sweet Oil (*Citrus sinensis* (L.) Osbeck)
NAS 6706: Orange Essence Oil

A review of current industry practices revealed that there are only two types of sweet orange oil from which flavoring materials are derived, peel oil and essence oil. Currently, essence oil is recovered during juice production using modern evaporators, as discussed above, but in the past the process was more similar to a distillation. Presently, distillation techniques are not used to capture orange oils from the peel, fruit or juice but are applied in subsequent processing and concentration of *Citrus* oils. To accurately reflect materials currently in commerce, the FEMA Expert Panel has updated the description of FEMA 2821 to Orange Essence Oil (*Citrus sinensis* (L.) Osbeck). For the 2015 FEMA Poundage Survey, usage for NAS 6706 and FEMA 2821 are combined under FEMA 2821.

In addition, in past FEMA Poundage Surveys, two terpeneless sweet orange oil flavoring materials were surveyed:

FEMA 2822: Orange Oil Terpeneless (*Citrus sinensis* (L.) Osbeck)
FEMA 2826: Orange Peel Sweet Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck)

To more accurately describe that FEMA 2822 is derived from orange essence oil while FEMA 2826 is derived from orange peel oil, the FEMA Expert Panel has updated the description for FEMA 2822 to Orange Essence Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck). This change will be reflected in the 2015 FEMA poundage survey. Finally, updated FEMA names are listed in Table 1 for the *Citrus* materials to reflect conventional industry terms. Because it is understood that *Citrus* peel oils are prepared by expression rather than distillation and essence oils are derived from the juicing process, additional wording regarding preparation is not added, with the exception of lime oils. Because lime oils can be prepared by distillation or expression resulting in flavoring materials with different characteristics and constituent profiles, “distillation” or “expression” is included in the FEMA name.

5. Chemical composition

Complete analyses of the *Citrus* flavoring materials listed in Table 1 were collected. For this evaluation, data were collected for 1X and 5X folds and terpeneless *Citrus* oils. It is expected that these three concentrations represent a sufficient and reasonable range of *Citrus* oils that are in commerce. *Citrus* flavoring materials are characterized by their volatile constituents and are typically analyzed by gas-chromatography (GC) using a mass spectrometric detector (MSD) to identify constituents by comparison to a standardized library and a flame ionization detector (FID) for quantitation of each chromatographic peak. Identified and unidentified GC peaks are reported as the Area % of the chromatogram. A summary of the constituent data for each NFC *Citrus* flavoring material has been compiled in Appendix A. Each constituent of an NFC is classified into its proper congeneric group and its Cramer decision tree class (Cramer et al., 1978) is determined, both of which are based on the chemical structure and the functional groups of each constituent. Under Step 5 in Appendix A, the constituents present in each NFC with a mean % greater than 1% are reported, organized by congeneric group and subtotals for the mean percentage concentration (%) for each congeneric group present with a mean % greater than 1% are reported. The congeneric grouping scheme is provided in the procedure for NFC evaluation (Cohen et al., 2018), and is consistent with the chemical groups used by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its evaluation of chemically defined flavoring materials (JECFA, 1997, 1998, 1999, 2000a; b, 2001, 2002, 2004, 2005). The Cramer decision tree class assigned to each congeneric group is determined by assigning the most conservative class for the constituents within each group.

The analytical results for each *Citrus* NFC were reviewed and several trends emerged. The congeneric group distribution for single fold Lemon Oil (FEMA 2625A) is depicted in a pie chart in Fig. 3. For many of the single fold peel and essence oils, Group 19 constituents (Aliphatic and aromatic hydrocarbons) are a large percentage of the total composition. These constituents include *d*-limonene, β-pinene, and *p*-mentha-1,4-diene. Group 1 (Saturated aliphatic acyclic, linear primary alcohols, aldehydes, carboxylic acids and related esters) compounds such as octanal, nonanal and decanal and Group 3 (Aliphatic linear and branched-chain alpha, beta-unsaturated aldehydes and related alcohols, acids and esters) compounds, such as citral are often present at lower concentrations in single fold *Citrus* NFCs. The constituent profile of Bergamot Oil (FEMA 2153) contains relatively high percentages of Group 12 (aliphatic and aromatic tertiary alcohols and related esters) compounds such as linalool and linalyl acetate compared to other single fold peel oils (Fig. 4, right). The constituent profile of single fold Lime Oil, Distilled (FEMA 2631A) is characterized by higher levels of alpha-terpineol (Group 12) in comparison to cold expressed lime oils and other *Citrus* oils. Structures of common constituents of *Citrus* oils are shown in Fig. 2.

A number of *Citrus* oils are folded or concentrated by distillation, in which the monoterpene hydrocarbons are removed. A single fold (1X) *Citrus* oil can be concentrated to any degree in this manner from a slightly concentrated two-fold (2X) oil to a more concentrated five to ten fold (5-10X) citrus oil to a highly concentrated terpeneless oil, each with distinguishing flavor character. The change in the constituent profile with folding is illustrated in Fig. 3 for single fold Lemon Oil (FEMA 2625A). In the single fold oil, the terpene hydrocarbon content (Group 19) is approximately 95%, with approximately 3% citral and other Group 3 (Aliphatic linear and branched-chain α, β-unsaturated aldehydes and related alcohols, acids and esters) constituents. The single fold *Citrus* peel and essence oils reviewed here all contain

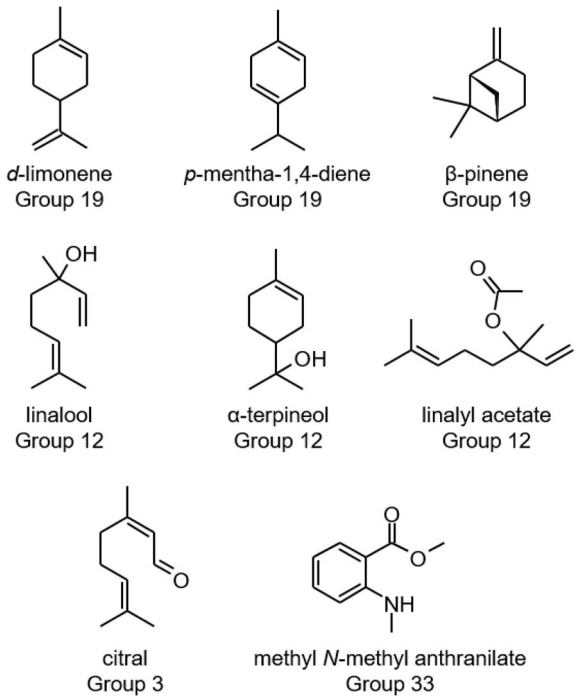

Fig. 2. Some commonly reported constituents of *Citrus* natural flavor complexes and their respective congeneric groups.

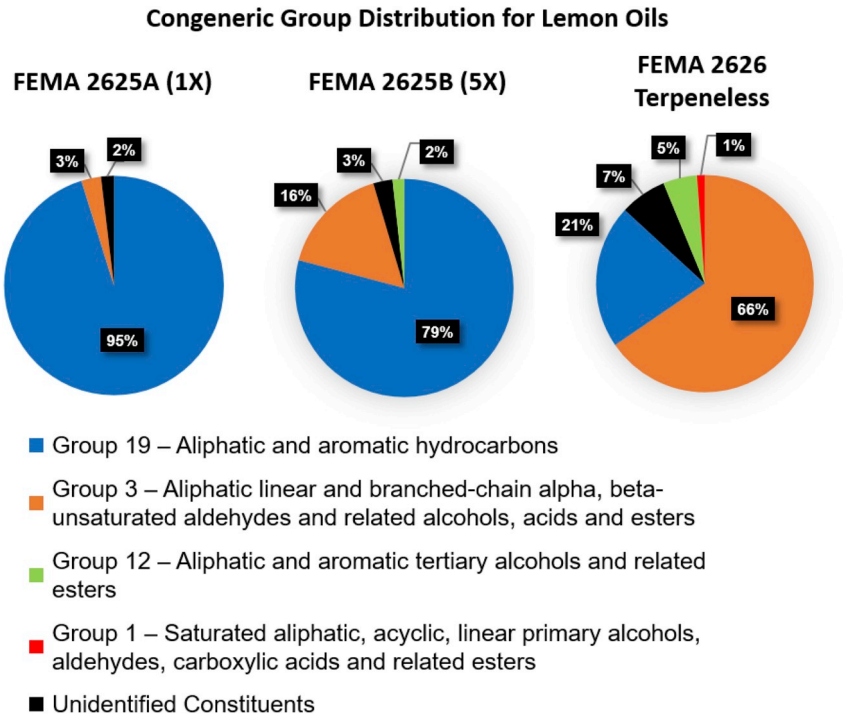

Fig. 3. Constituent profiles for 1X and 5X folds of Lemon Oil (FEMA 2625) and Lemon Oil, Terpeneless (FEMA 2626).

significant amounts of *d*-limonene, which has a low odor threshold (Ahmed et al., 1978). The characteristic flavor of orange peel and essence oils is contributed by the minor oxygenated constituents while minor oxygenated constituents and grapefruit mercaptan are important to the flavor profile of grapefruit oils (Buccellato, 2017). The less abundant but more flavor impactful constituents of a *Citrus* oil are concentrated upon removal of *d*-limonene and other monoterpene hydrocarbons by the folding process. For example, upon folding of (1X) Lemon Oil (FEMA 2625A), the concentration of monoterpene hydrocarbons (Group 19) decreases and the relative percentage of citral and other Group 3 constituents is increased, as shown by the center pie chart in Fig. 3 for 5X Lemon Oil (FEMA 2625B). The monoterpene hydrocarbon concentration in the oil can be further reduced until the oil is considered "terpeneless". Terpeneless oils contain minimal amount of monoterpene hydrocarbons. The total concentration of Group 19 constituents is further reduced while the concentrations of Group 3, Group 12 and Group 1 constituents are increased in Lemon Oil, Terpeneless (FEMA 2626) as shown in Fig. 3 (right). A continuum of folded oils are commonly produced from Orange Peel Sweet Oil (FEMA 2825), Orange Essence Oil (FEMA 2821), Grapefruit Oil (FEMA 2530), Lime Oil, Distilled (FEMA 2631), Mandarin Oil (FEMA 2657) and Tangerine Oil (FEMA 3041) for use as flavoring ingredients.

The constituent profiles for Petitgrain Lemon Oil (FEMA 2853), Petitgrain Mandarin Oil (FEMA 2854), Petitgrain Oil (FEMA 2855) and Neroli Bigarade Oil (FEMA 2771) are distinct in comparison to the peel oils. These oils are obtained from the steam distillation of the leaves, bud and twigs and flowers of *Citrus* trees, respectively. Petitgrain Mandarin Oil (FEMA 2854) is distinguished by a large percentage of methyl-*N*-methyl anthranilate, a Group 33 Anthranilate derivatives constituent, specific to the mandarin type. As depicted by the pie charts in Fig. 4, Bergamot Oil (FEMA 2153), Petitgrain (Paraguay) Oil (FEMA 2855), Neroli Bigarade Oil (FEMA 2771) and Petitgrain Oil, Terpeneless (FEMA 4853) (not shown) are characterized by a large percentage of Group 12 constituents (linalool and linalyl acetate) and are all derived from *C. aurantium*.

The last set of *Citrus* NFCs are the extracts, Lemon Extract (FEMA 2623), Orange Peel Sweet Extract (FEMA 2824) and Curacao Peel Extract (FEMA 2344). These extracts are prepared by solvent extraction of the *Citrus* peel or peel oils. For some, the solvent has been completely removed, while others are ethanolic extracts containing a high percentage of ethanol and water. The constituent profiles for these NFCs mirror those reported in their respective peel oils.

6. Safety Evaluation

The procedure for the safety evaluation for NFCs, outlined in Fig. 5, is guided by a set of criteria initially outlined in two publications (Smith et al., 2004, 2005) and updated recently (Cohen et al., 2018), applying the threshold of toxicological concern (TTC) concept in addition to data on absorption, metabolism, and toxicology of members of the congeneric groups and the NFC under evaluation. Briefly, the NFC passes through a 14-step process; Step 1 requires the gathering of data and assesses the consumption of the NFC as a flavor relative to 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 scientific data on metabolism and toxicity; Steps 6-12 address the potential toxicity, including genotoxicity of the unidentified constituents; Step 13 evaluates the overall safety along with considerations of potential biologically relevant synergistic or antagonistic interactions among constituents; lastly, in Step 14, the final determination of GRAS status is made. Below, the safety evaluation is presented in which each step of the procedure (Cohen et al., 2018) (provided in italics), is considered and answered for the *Citrus* NFCs.

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

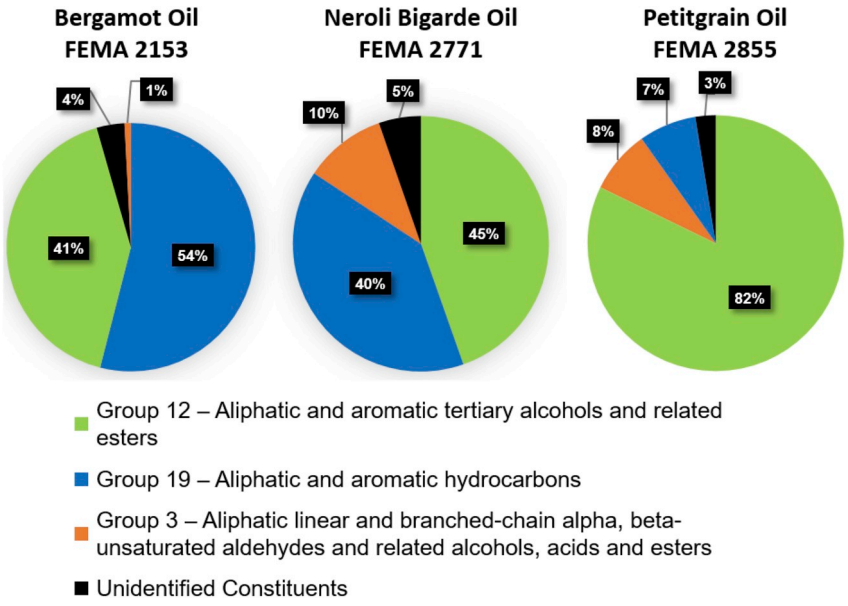

Fig. 4. Constituent profiles for Bergamot Oil (FEMA 2153), Neroli Bigarade Oil (FEMA 2771) and Petitgrain Oil (FEMA 2855).

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.

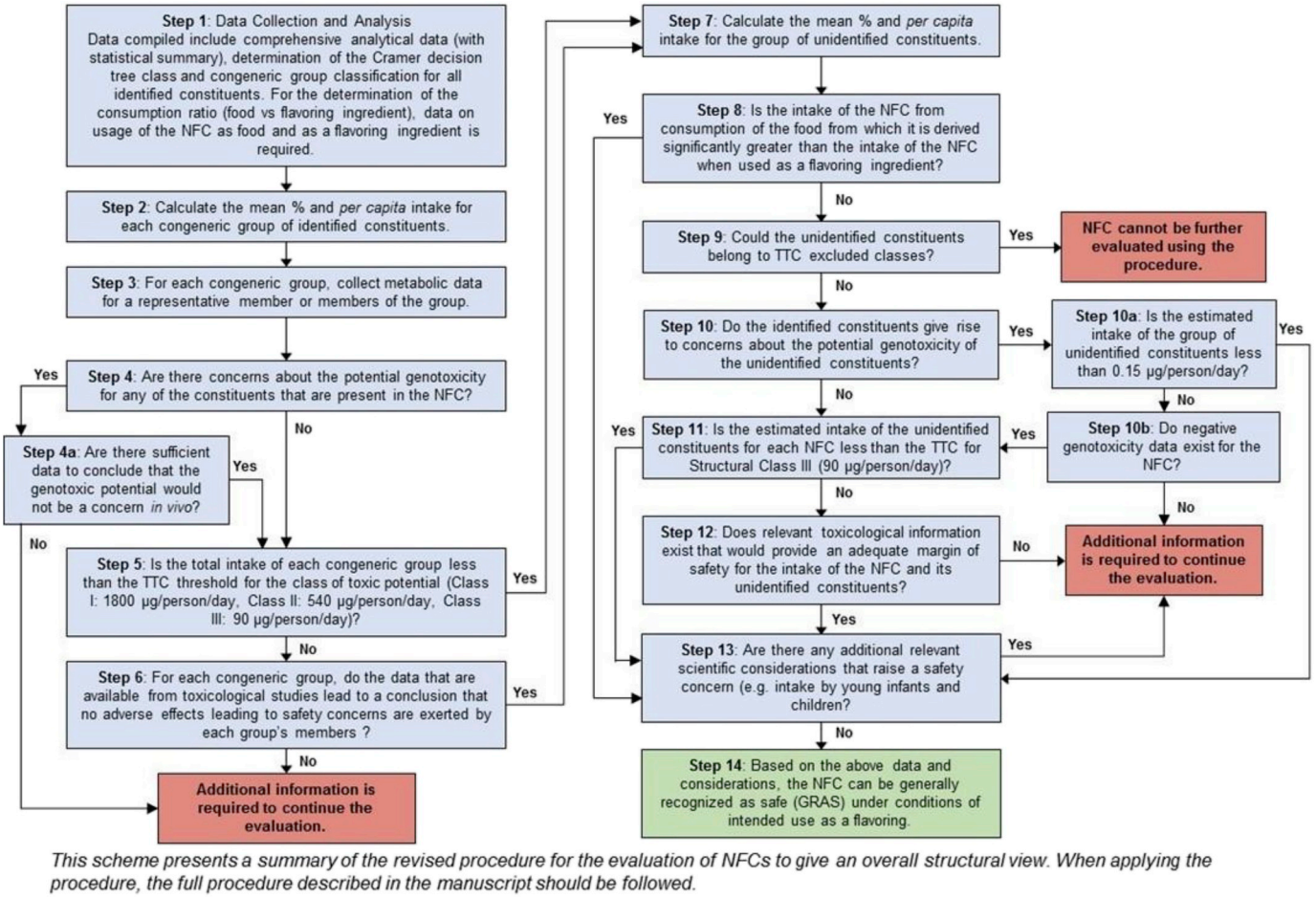

Fig. 5. Procedure for the Safety Evaluation of NFCs (Cohen et al., 2018).

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:

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

where:

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

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

If applicable, estimate the intake resulting from consumption of the commonly consumed food from which the NFC is derived. The aspect of food use is particularly important. It determines whether intake of the NFC occurs predominantly from the food of which it is derived, or from the NFC itself when it is added as a flavoring ingredient (Stofberg and Grundschober, 1987)[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.*

As discussed earlier, the *Citrus* NFCs under consideration in this evaluation are derived from the peel of the fruit or flowers, twigs and buds of the *Citrus* tree, not the inner fruit which is commonly consumed as food. Some *Citrus* species such as the bitter orange (*C. aurantium*) are not typically consumed as juice or whole fruit but cultivated for their flowers and peel oils. Therefore, a direct comparison of whole fruit consumption to the consumption of the related NFC as flavor in food is not applicable for the *Citrus* NFCs. However, measurable amounts of essential oil are present in the *Citrus* juice sacs comprising the inner fruit and in *Citrus* juices. Table 2 contains an estimation of the *per capita* intake of sweet orange, lemon, lime, grapefruit and mandarin oils, which are consumed in the USA, from juices and fresh fruit in 2014. In Table 3, the estimated intake of essential oils from fruit and juice consumption is compiled with the estimated *per capita* consumption of the NFCs derived from sweet orange (*C. sinensis*), lemon (*C. limon*), lime (*C. aurantifolia* and *latifolia*), grapefruit (*C. paradisi*) and mandarin types (includes tangelo, clementine) (*C. reticulata, C. paradisi Macf.* × *C. tangerine* hort. ex Tanaka)*, C. clemenina*). The intake of sweet orange oil from juice and fresh fruit is estimated to be 6.1 mg/person/day. In comparison, the *per capita* intakes for sweet orange NFCs Orange Peel Sweet Oil (1X) – FEMA 2825A and Orange Essence Oil (1X) – FEMA 2821A were calculated to be 10.1 and 4.5 mg/person/day, respectively. The *per capita* consumption of lime, lemon, grapefruit and mandarin oils from the consumption of juice and fresh fruit ranges from 0.43 to 0.66 mg/person/day. In general, these intakes are typically lower than those estimated for the related lime, lemon, grapefruit and mandarin NFCs. For example, the *per capita* intake for Lemon Oil (1X) (FEMA 2625A) and Lemon Essence Oil (FEMA 4852) was calculated to be 6.8 and 1.7 mg/person/day, respectively compared to an intake of 0.66 mg/person/day from fresh fruit and juice.

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 for each constituent the Cramer Decision Tree Class (DTC) is determined (Cramer et al., 1978).

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

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

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

Proceed to Step 2.

All reported constituents in 54 NFCs were organized by congeneric group and a summary report for each NFC is shown in Appendix A. In Appendix A, the congeneric groups with constituents with a mean% greater or equal to 1% of the NFC are listed in order of highest to lowest mean%. For each congeneric group listed, the constituents with a mean % equal or greater than 1% are also shown and the minor constituents (< 1%) are summed and reported. The total mean% for each congeneric group is subtotaled and reported with the DTC for the group.

Step 2

D intake[3] *of each congeneric group. (a) is calculated by summing the mean percentage of each of the constituents within a congeneric group, and (b) is calculated from consumption of the NFC and the mean percentage.*

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

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

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

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

Proceed to Step 3.

In the summary report for each NFC provided in Appendix A, the total mean% for each congeneric group is subtotaled and reported with the DTC and intake (PCI × 10 or PCI, as appropriate) for each congeneric group listed.

Step 3

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

[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.

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

Table 3
Intakes of select *Citrus* oils from food (highlighted) and NFC intakes as flavor.

		Intake(μg/person/day)
Orange	**Orange Oil from whole fruit and juice (USDA 2014)**	**6108**
	2821A – Orange Essence Oil (*Citrus sinensis* (L.) Osbeck) (1X)	4510
	2825A – Orange Peel Sweet Oil (*Citrus sinensis* (L.) Osbeck) (1X)	10,100
	4856 – Blood Orange Oil (*Citrus sinensis* (L.) Osbeck 'Blood Orange')	170
Lemon	**Lemon Oil from whole fruit and juice (USDA 2014)**	**660**
	2625A – Lemon Oil (*Citrus limon* (L.) Burm. F.) (1X)	6800
	4852 – Lemon Essence Oil (*Citrus limon* (L.) Burm. F.)	1740
Lime	**Lime Oil from whole fruit and juice (USDA 2014)**	**430**
	2631A – Lime Oil, Distilled (*Citrus aurantifolia* (Christman) Swingle) (1X)	2500
	4743 – Mexican Lime Oil, Expressed (*Citrus aurantifolia*, *Citrus medica* var. *acida*)	110
	4744 – Persian Lime Oil, Expressed (*Citrus latifolia*)	1000
Grapefruit	**Grapefruit Oil from whole fruit and juice (USDA 2014)**	**580**
	2530A – Grapefruit Oil (*Citrus paradisi* Macf.) (1X)	1100
	4846 – Grapefruit Essence Oil (*Citrus paradisi* Macf.)	2300
Mandarin	**Mandarin Family Oil from whole fruit and juice (USDA 2014)**	**610**
	2657A – Mandarin Oil (*Citrus reticulata* Blanco 'Mandarin') (1X)	1430
	3041A – Tangerine Oil (*Citrus reticulata* Blanco 'Tangerine') (1X)	700
	4854 – Tangelo Oil (*Citrus paradisi* Macf. x *Citrus tangerine* hort. ex Tanaka)	1
	4855 – Clementine Oil (*Citrus clementina* hort. ex Tanaka)	0.2

Table 4
Consideration of Group 19 for *Citrus* NFCs where Intake > TTC for Group 19, aliphatic and alicyclic hydrocarbons.

Name (FEMA No.)	Mean % Grp 19	Intake for Group 19 (μg/p/d)	NOAEL (mg/kg bw/day)	MoS[a]
Lemon Oil (*Citrus limon* (L.) Burm. F.) (FEMA 2625A)	94.9	6470	215	> 1900
Lime Oil, Distilled (*Citrus aurantifolia* (Christman) Swingle) (FEMA 2631A)	82.4	2060	215	> 6200
Orange Peel Sweet Oil (1X) (*Citrus sinensis* (L.) Osbeck) (FEMA 2825A)	98.4	9940	215	> 1200
Grapefruit Essence Oil (*Citrus paradisi* Macf.) (FEMA 4846)	93.0	2160	215	> 5900
Orange Essence Oil (*Citrus sinensis* (L.) Osbeck) (FEMA 2821A)	97.3	4380	215	> 2900
Lemon Terpenes (*Citrus limon* (L.) Burm. F.) (FEMA 4848)	98.5	1810	215	> 7100
Lime Terpenes (*Citrus aurantifolia* Swingle, *Citrus medica* var. *acida, Citrus latifolia*) (FEMA 4849)	94.6	2660	215	> 4800
Orange Terpenes (*Citrus sinensis* (L.) Osbeck) (FEMA 4850)	98.9	38,500	215	> 300
Orange Peel Sweet Oil (5X) (*Citrus sinensis* (L.) Osbeck) (FEMA 2825B)	93.8	1640	215	> 7800
Orange Essence Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck) (FEMA 2822)	25.5	560	215	> 23,000

[a] MoS calculation based on a NOAEL of 215 mg/kg bw/day for limonene (adjusted daily dose from 300 mg/kg bw/day administered 5 days/week) reported for a two-year toxicity study of *d*-limonene in female F344N rats (National Toxicology Program, 1990).

additional considerations at Step 13 of the procedure.

Proceed to Step 4.

For each congeneric group, metabolic data exist for one or more representative members of the group. For more detailed descriptions of the studies and extensive discussion and interpretation of the findings see the related FEMA Expert Panel safety assessments for the primary two congeneric groups (Adams et al., 2011; Marnett et al., 2014) and previously published assessments of other groups or individual constituents (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).

For all of the congeneric groups listed, a summary of metabolic data, organized by the congeneric group number (see Appendix B), is available that indicates that members of their respective groups are metabolized to innocuous products.

Step 4

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

If Yes, proceed to Step 4a.

If No, proceed to Step 5.

No, examination of *in vitro* and *in vivo* genotoxicity studies on several *Citrus* oils and the congeneric groups present in *Citrus* NFCs indicate no concerns for potential genotoxicity of the constituents that are present for the *Citrus* NFCs.

Step 4a

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

If Yes, proceed to Step 5.

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

Not required.

Step 5

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

If Yes, proceed to Step 7.

If No, proceed to Step 6.

With exception of 10 *Citrus* NFCs, the total intake for each of the congeneric groups present in each NFC is below the corresponding TTC for the group (see Appendix A). These NFC materials proceed to Step 7 of the evaluation procedure. However, the estimated intake of the congeneric Group 19, the aliphatic and alicyclic hydrocarbons, exceeds the relevant TTC in ten *Citrus* NFC materials. The mean percentage (%) and intake of Group 19 constituents for these ten NFCs are shown in Table 4 for further evaluation in 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.

A review of relevant toxicological studies on *Citrus* oils and Group 19 constituents *d*-limonene, β-myrcene, β-caryophyllene and *p*-mentha-1,3-diene are summarized later in this manuscript. It is noted that *d*-limonene is the major Group 19 constituent reported for these NFCs. The margin of safety (MoS) was calculated for the ten NFCs listed in Table 4, for which the intake of Group 19 constituents exceeds the TTC threshold, based on 215 mg/kg bw/day NOAEL (adjusted daily dose from 300 mg/kg bw/day administered 5 days/week) reported for a two-year toxicity study of *d*-limonene in female F344N rats (National Toxicology Program, 1990). With the determination of an adequate MoS, these NFCs proceed to Step 7.

Step 7

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

Proceed to Step 8.

The mean % was determined and the daily *per capita* intake for the group of unidentified constituents is reported in Appendix A.

Step 8

Using the data from Step 1, is the intake of the NFC from consumption of the food from which it is derived significantly greater than the intake of the NFC when used as a flavoring ingredient.[4]

If Yes, proceed to Step 13.

If No, proceed to Step 9.

No – As discussed in Step 1, the available data does not allow for the presumption that essential oil intake via food is the predominant manner of consumption. Therefore, intake is assumed to be predominantly from the flavor added to foods. Proceed to Step 9.

Step 9

Could the unidentified constituents belong to TTC excluded classes?[5] *The excluded classes are defined as high potency carcinogens, certain inorganic substances, metals and organometallics, certain proteins, steroids known or predicted bio-accumulators, nanomaterials, and radioactive materials* (EFSA, 2016; Kroes et al., 2004).

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

If No, proceed to Step 10.

No – Unidentified constituents are not suspected to belong to TTC excluded classes.

Based on the identified constituents, the unidentified constituents are most likely monoterpenoid and sesquiterpenoid products of the isoprene pathway. Because the production process for these oils includes one or more distillation steps and collection of only the volatile constituents, the presence of the TTC excluded classes in the unidentified constituents is unlikely. Finally, the literature on *Citrus* oils does not indicate the presence of TTC excluded class compounds (Dugo and Mondello, 2011). 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 - Based on the composition of the identified constituents of each NFC and standard Ames assays performed with *Citrus* oils, there is no indication that the unidentified substances have structural alerts for genotoxicity. Steps 10a and 10b are not required. Proceed to Step 11.

Step 10a

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

If Yes, proceed to Step 13.

If No, proceed to Step 10b.

This Step is not required.

Step 10b

Do negative genotoxicity data exist for the NFC?

If Yes, proceed to Step 11.

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

This Step is not required. However, a review of *in vitro* and *in vivo* genotoxicity studies on *Citrus* oils and major individual *Citrus* constituents is presented later in this manuscript.

Step 11

Is the estimated intake of the unidentified constituents (calculated in Step 7) less than the TTC (Kroes et al., 2000; Munro et al., 1996*) for Structural Class III (90 μg/person/day)?*[6]

If Yes, proceed to Step 13.

If No, proceed to Step 12.

Yes, as calculated in Appendix A for 52 *Citrus* NFCs, the estimated intake of the unidentified constituent fraction is less than 90 μg/person/day. For these NFCs, proceed to Step 13.

No, for two NFCs, Lemon Oil (*Citrus limon* (L.) Burm. F.) (FEMA 2625A) and Orange Essence Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck) (FEMA 2822) the intake of the unidentified constituents exceeds the TTC for Structural Class III (90 μg/person/day). For these NFCs proceed to Step 12.

Step 12

Does relevant toxicological information exist that would provide an

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

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

[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).

Table 5
Consideration of *Citrus* NFCs with an intake of unidentified constituents in excess of the TTC for Structural Class III.

Name	FEMA No.	NOAEL (mg/kg bw/day)	Intake of NFC (μg/p/day)	MoS[a] (NFC)
Lemon Oil (*Citrus limon* (L.) Burm. F.)	2625A	600	6800	> 5000
Orange Essence Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck)	2822	600	2230	> 16,000

[a] MoS calculation based on NOAEL determined for sweet orange oil in rats of 600 mg/kg bw/day from a 28-day oral gavage study (Serota, 1990a, 1990b).

adequate margin of safety for the intake of the NFC and its unidentified constituents?

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

If Yes, proceed to Step 13.

If No, perform appropriate toxicity tests or obtain further analytical data to reduce the fraction of unidentified constituents. Resubmit for further evaluation.

Yes, the NOAEL for sweet orange oil in rats is 600 mg/kg bw/day from a 28-day oral gavage study (Serota, 1990a) and provides an adequate margin of safety for the *Citrus* NFCs Lemon Oil (*C. limon* (L.) *Burm.* F. (FEMA 2625A) and Orange Essence Oil, Terpeneless (*C. sinensis* (L.) Osbeck) (FEMA 2822) with a total intake of unidentified constituents above TTC for Structural Class III (Table 5). Proceed to Step 13.

Step 13

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

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

If No, proceed to Step 14.

A further evaluation to consider possible exposure to children and infants, given their lower body weights and the potential for differences in toxicokinetics and toxicodynamics as compared to adults, was conducted. Table 4 lists the congeneric groups that exceed TTC threshold and Table 5 lists two NFCs for which the intake of the unknown constituent fraction exceeds the TTC thresholds for Class 3. In each instance, the margin of safety remains > 100 using a body weight of 20 kg. For Orange Essence Oil, Terpeneless (FEMA 2822), the intake of congeneric Group 12 (aliphatic and aromatic tertiary alcohols and related esters) was below but close to the TTC threshold. When compared to the NOAEL for linalool, the principal constituent of this congeneric group in orange essence oil, terpeneless, a margin of safety of greater than 1900 (based on 20 kg) was determined from a 12 week study in mice (Oser, 1967).

Furocoumarin compounds are a well-known group of natural food constituents occurring mainly in plants belonging to the *Rutaceae (*e.g. *Citrus)* and *Umbelliferae* (e.g. parsnips, carrots, parsley, celery) (Dolan et al., 2010). Considered to be natural pesticides, plants produce furocoumarins to defend against various viruses, bacteria, and insects (Wagstaff, 1991). While furocoumarins have been shown to be present in *Citrus* oils, the NFC *Citrus* oils are often processed in a manner that reduces their furocoumarin content compared to the freshly harvested peel oil (Frérot and Decorzant, 2004). For bergamot oil, methods for the reduction of bergapten include an alkaline treatment, vacuum fractional distillation techniques and fractionation using super critical fluid technology (Gionfriddo et al., 2004). In general, NFC *Citrus* oils are often further processed by distillation for the purpose of concentrating or folding the oil or remove higher molecular weight compounds that color the oil. Because of their lower volatility, the furocoumarin content of distilled oils is reduced compared to the raw essential oil.

Furocoumarins have both phototoxic and photomutagenic properties following exposure to UV light and thus the use of furocoumarin-containing materials in skin-care and cosmetic products is regulated (Cosmetic Ingredient Review Expert Panel, 2016; Scientific Committee on Consumer Products, 2005). In the European Medicines Agency (EMEA) Committee on Herbal Medicines draft report on the risks associated with furocoumarins contained in preparations of *Angelica archangelica* L., a daily intake of 15 μg/day furocoumarins in herbal medicinal preparations was considered not to pose an unacceptable risk to consumers (European Medicines Agency, 2007).

In consideration of the limited information on the typical intake of furocoumarin compounds from food and their potential effects, regulatory bodies have not regulated dietary exposure to furocoumarin content from food. In “Furocoumarins in Plant Foods” published in 1996 by the Nordic Council of Ministers, the Nordic Working Group on Natural Toxins presents a risk assessment on toxicological effects that may occur with the consumption of furocoumarins at levels present in fruits and vegetables (Nordic Working Group on Natural Toxins, 1996). While gaps in knowledge regarding the occurence, intake and bioavailability of furocoumarins consumed with food exist, the working group concluded that the average daily intake of furocoumarins in food is unlikely to elicit a phototoxic response or increase the cancer risk in internal organs not exposed to UVA ultraviolet light. The group acknowledges that repeated consumption of furocoumarin-rich foods could result in higher concentrations in the serum that are correlated with a phototoxic response (Nordic Working Group on Natural Toxins, 1996). In a 1996 report, the Committee on Toxicity, Mutagenicity, Carcinogenicity of Chemicals in Food, Consumer Products and the Environment, Department of Health, Britian concluded “that the likelihood of any risk to health from dietary intakes of furocoumarins was very small” (COT, 1998). In 2004, the DFG Senate Commission on Food Safety – Germany (SKLM) published its toxicological assessment on furocoumarins in foods, concluding that “additional risk of skin cancer arising from the consumption of typical quantities of furocoumarin-containing food, which remain significantly below the range of phototoxic doses, is regarded as insignificant” (SKLM, 2004). In 2010, the SKLM published an update that included an analysis of estimated furocoumarin intakes from non-flavored and flavored foods which determined that intakes from non-flavored food such as grapefruit juice was a much higher source of furocoumarins than flavored foods, such as drinks flavored with lime oils (Gorgus et al., 2010; SKLM, 2010). In this updated report, the SKLM confirmed its 2004 opinion that furocoumarins in food do not present a significant risk for phototoxic effects (SKLM, 2010). The FEMA Expert Panel concurs with these opinions and concludes that the potential additional safety concerns arising from the extremely low level of furocoumarins present in *Citrus*-derived NFCs used as flavor ingredients does not present a safety concern under conditions of intended use.

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.

Based on the above assessment and the application of the judgment of the FEMA Expert Panel that the current FEMA GRAS *Citrus* NFCs are affirmed as GRAS under conditions of intended use as flavor substances and are listed in Table 6. *Citrus* flavor materials that were not previously evaluated by the Panel have been determined to be GRAS under conditions of intended use and are listed in Table 7.

Table 6
FEMA GRAS *Citrus* flavor materials affirmed (GRAS).

FEMA No.	Name
2153	Bergamot Oil (*Citrus auranthium* L. ssp. Bergamia)
2344	Curacao Peel Extract (*Citrus aurantium* L.)
2345	Curacao Peel Oil (*Citrus aurantium* L.)
2530	Grapefruit Oil (*Citrus paradisi* Macf.)
2623	Lemon Extract (*Citrus limon* (L.) Burm. F.)
2625	Lemon Oil (*Citrus limon* (L.) Burm. F.)
2626	Lemon Oil, Terpeneless (*Citrus limon* (L.) Burm. F.)
2631	Lime Oil, Distilled (*Citrus aurantifolia* (Christman) Swingle)
2632	Lime Oil, Terpeneless (*Citrus aurantifolia* (Christman) Swingle)
2657	Mandarin Oil (*Citrus reticulata* Blanco 'Mandarin')
2771	Neroli Bigarade Oil (*Citrus aurantium* L.)
2821	Orange Essence Oil (*Citrus sinensis* (L.) Osbeck)
2822	Orange Essence Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck)
2823	Orange Peel Bitter Oil (*Citrus aurantium* L.)
2824	Orange Peel Sweet Extract (*Citrus sinensis* (L.) Osbeck)
2825	Orange Peel Sweet Oil (*Citrus sinensis* (L.) Osbeck)
2826	Orange Peel Sweet Oil, Terpeneless (*Citrus sinensis* (L.) Osbeck)
2853	Petitgrain Lemon Oil (*Citrus limon* L. Burm. F)
2854	Petitgrain Mandarin Oil (*Citrus reticulata* Blanco var. *mandarin*)
2855	Petitgrain Oil (*Citrus aurantium* L.)
3041	Tangerine Oil (*Citrus reticulata* Blanco 'Tangerine')
3823	Daidai Peel Oil (*Citrus aurantium* L. subspecies *cyathifera* Y.)
3899	Sarcodactylis Oil (*Citrus medica* L. var. *Sarcodactylis* Swingle)
4743	Mexican Lime Oil, Expressed (*Citrus aurantifolia, Citrus medica* var. *acida*)
4744	Persian Lime Oil, Expressed (*Citrus latifolia*)

Table 7
New FEMA GRAS *Citrus* flavoring materials.

FEMA No.	Name
4846	Grapefruit Essence Oil (*Citrus paradisi* Macf.)
4847	Grapefruit Oil, Terpeneless (*Citrus paradisi* Macf.)
4848	Lemon Terpenes (*Citrus limon* (L.) Burm. F.)
4849	Lime Terpenes (*Citrus aurantifolia* Swingle, *Citrus medica* var. *acida, Citrus latifolia*)
4850	Orange Terpenes (*Citrus sinensis* (L.) Osbeck)
4851	Grapefruit Terpenes (*Citrus paradisi* Macf.)
4852	Lemon Essence Oil (*Citrus limon* (L.) Burm. F.)
4853	Petitgrain Oil, Terpeneless (*Citrus aurantium* L.)
4854	Tangelo Oil (*Citrus paradise* Macf. x *Citrus tangerine* hort. ex Tanaka)
4855	Clementine Oil (*Citrus clementina* hort. ex Tanaka)
4856	Blood Orange Oil (*Citrus sinensis* (L.) Osbeck 'Blood Orange')
4857	Iyokan Oil (*Citrus iyo*)
4858	Hassaku Oil (*Citrus hassaku* hort. ex Tanaka)
4859	Sikuwasya Oil (*Citrus depressa*)
4860	Natumikan Oil (*Citrus natsudaidai*)
4861	Mikan Oil (*Citrus unshiu*)
4862	Yuzu Oil (*Citrus junos* (Sieb.) c. Tanaka)
4863	Sudachi Oil (*Citrus sudachi* hort. ex Shirai)
4864	Kabosu Oil (*Citrus sphaerocarpa*)
4865	Ponkan Oil (*Citrus reticulata* Blanco'Ponkan')
4866	Orange Essence Water Phase (*Citrus sinensis* (L.) Osbeck)

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

As the constituent analyses of the *Citrus* NFCs have demonstrated, the Aliphatic and alicyclic hydrocarbons (Group 19) and to a much lesser extent, the Aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances (Group 12) are the two primary congeneric groups that account for the majority of the NFC composition (Appendix A). As noted in Step 5 of the safety evaluation procedure, the TTC for the congeneric group is exceeded for the Aliphatic and alicyclic hydrocarbons group (Group 19) for ten *Citrus* NFCs. The Aliphatic and alicyclic hydrocarbons group, which is comprised of 17 FEMA GRAS chemically defined flavoring ingredients, was reaffirmed as GRAS for use as chemically defined flavor materials in 2011 (Adams et al., 2011). The major constituents from the Aliphatic and alicyclic hydrocarbons group (Group 19) found in *Citrus* NFCs include *d*-limonene (FEMA 2633), *p*-mentha-1,4-diene (FEMA 3559), α-pinene (FEMA 2902), β-pinene (FEMA 2903), β-myrcene (FEMA 2762), terpinolene (FEMA 3046) and β-caryophyllene (FEMA 2252). This section provides metabolic and toxicological information relevant to the evaluation of *Citrus* NFCs via this procedure (Cohen et al., 2018).

7.1. Absorption, distribution, metabolism and excretion

Hydrocarbons are highly lipophilic and may cross biological membranes (gastrointestinal tract, skin, respiratory epithelia, etc.) by passive diffusion, driven by concentration gradients between the gastrointestinal tract and portal blood, or by one or more active transport mechanisms. Therefore, chemicals in this group are rapidly absorbed and widely distributed. Following oral administration, *d*-limonene is first distributed to the liver, kidney and blood (C_{max} at 2 h) (Igimi et al., 1974). Hydrocarbons are slowly eliminated, primarily in the urine, as polar conjugates of oxidized metabolites (> 60% of oral *d*-limonene) (Del Toro-Arreola et al., 2005).

In oxidative metabolism, the biotransformation of *d*-limonene, β-myrcene and α- and β-pinenes, as well as the other group members is catalyzed by cytochrome P450 enzymes. The oxidative metabolites are then conjugated and excreted mainly in the urine. The metabolic profiles are remarkably similar, with the primary pathways including side chain oxidation or epoxidation of double bonds. Oxidation of alkene functional groups and alkyl substituents, primarily by allylic or benzylic hydroxylation, is followed by either conjugation with glucuronic acid and urinary excretion or further oxidation to yield the corresponding carboxylic acids. In addition, sterically unhindered alkenes, such as myrcene and limonene also undergo epoxidation, unlike sterically hindered structures (e.g.bicyclic monoterpenes pinene and camphene). Epoxide metabolites are either hydrolyzed to yield diols or conjugated with glutathione to subsequently yield mercapturic acid derivatives. The diols may also be conjugated with glucuronic acid and excreted in the urine. Of the acyclic hydrocarbons, β-myrcene primarily undergoes epoxidation followed by hydrolytic epoxide ring opening to yield myrcene-3,10-glycol and to a lesser degree myrcene-1,2-glycol, as shown in Fig. 6 (Ishida et al., 1981; Madyastha and Srivatsan, 1987). A minor metabolic pathway for myrcene involves cyclization and subsequent formation of limonene as a transient intermediate which undergoes rapid oxidation to form uroterpenol (*p*-menth-1-en-8,9-diol) (Ishida et al., 1981).

Of the monocyclic hydrocarbons, *d*-limonene undergoes either allylic oxidation of the exocyclic methyl group yielding perillic acid and dihydroperillic acid, or epoxidation and hydrolysis to yield limonene-1,2-diol and limonene-8,9-diol, as shown in Fig. 7 (Crowell et al., 1994; Poon et al., 1996; Vigushin et al., 1998). In humans, allylic oxidation is the dominant metabolic pathway of *d*-limonene (Poon et al., 1996) and is followed by glucuronic acid conjugation of all major and minor metabolites (Kodama et al., 1974; Poon et al., 1996). In the rat, *d*-limonene is primarily oxidized to perillic acid (~85%) that is either excreted in the urine unchanged or conjugated with glycine or glucuronic acid. Further oxidation to perillic acid-8,9-diol or 2-hydroxy-*p*-menth-8-en-7-oic acid also occurs.

A similar metabolic profile has been reported with microsomal incubation of *d*-limonene *in vitro* (with the 8,9-diol as the primary metabolite) (Watabe et al., 1981). Sex related differences have been reported in the formation of alcohol metabolites of *d*-limonene in rats by CYP2C11 oxidation (Miyazawa et al., 2002).

Of the bicyclic hydrocarbons, (+)-α-pinene (Falk et al., 1990) exposure in humans results in the rapid excretion of urinary metabolites *cis*- and *trans*-verbenol at a ratio of 1:10, that are largely eliminated within 20 h. As summarized in Fig. 8, two diols, *cis*- and *trans*-4-hydroxymyrtenol, formed by methyl group hydroxylation of *cis*- and *trans*-verbenol and *trans*-4-hydroxymyrtenal were also detected (Eriksson and Levin, 1996; Schmidt and Goen, 2017). At high exposure in humans

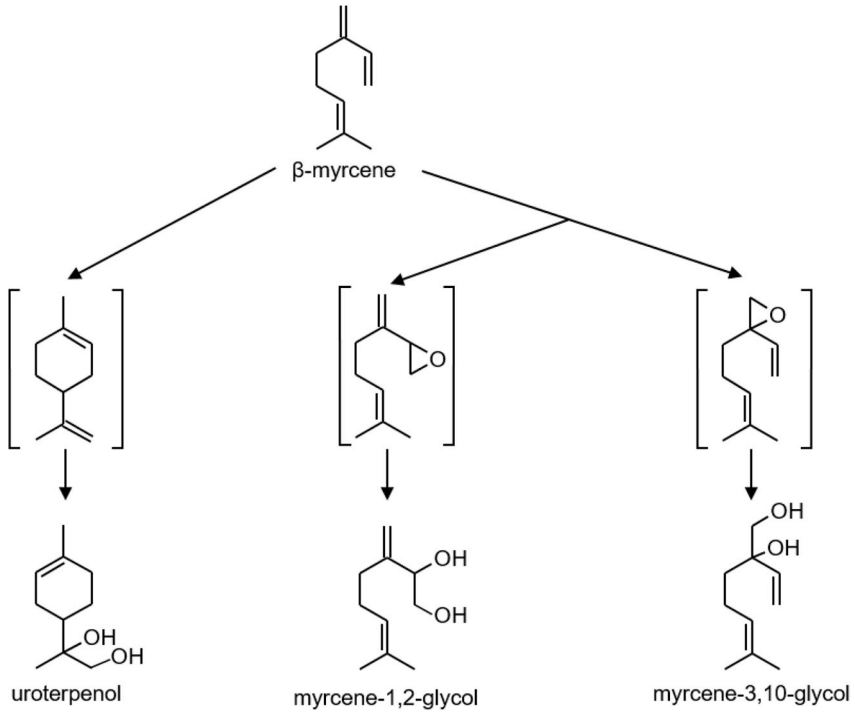

Fig. 6. Metabolism of β-myrcene in rats.

(suicide attempt) myrtenol, verbenol, and borneol were also detected in the urine (Koppel et al., 1981). However, α-pinene, β-pinene, 3-carene, and camphene were also detected in the urine of normal humans (Zlatkis et al., 1973). In male albino rabbits (6/dose) greater than 80% of the dose of each (+)-α-pinene, (−)-α-pinene, (±)-α-pinene, (−)-β-pinene, (−)-*cis*-pinane, or (+)-*d*-3-carene was found as glucuronic acid conjugates in the urine (Ishida et al., 1981). Verbenol is the principal metabolite of α-pinene (Ishida et al., 1981). In rabbits, β-caryophyllene undergoes epoxidation of the endocyclic 5,6-double bond, hydroxylation at the *gem*-dimethyl group and epoxidation of the exocyclic 2,12-double bond (See Fig. 9) (Asakawa et al., 1981; Ishida et al., 1979).

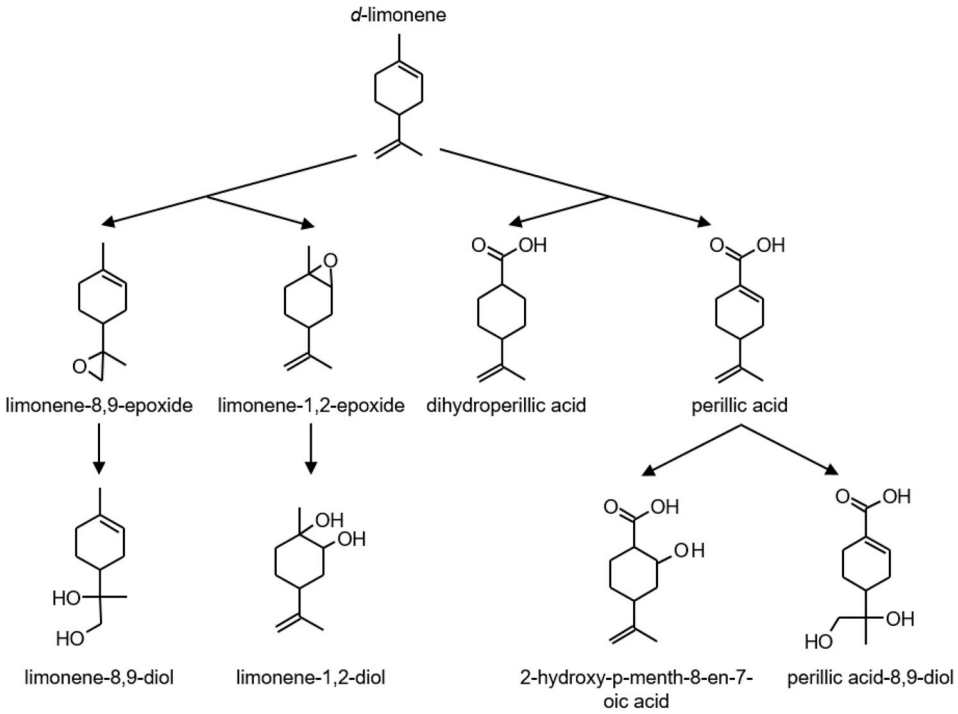

Fig. 7. Summary of *d*-limonene metabolism.

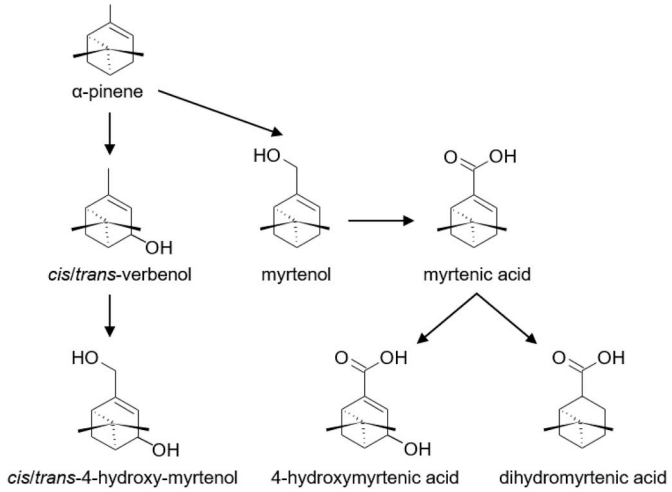

Fig. 8. α-pinene metabolism in humans.

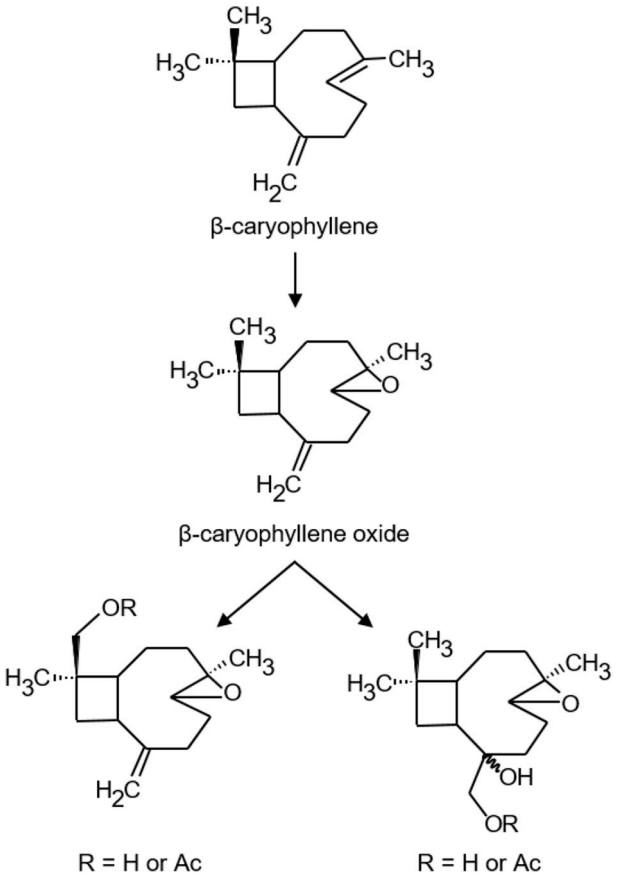

Fig. 9. β-caryophyllene metabolism in rabbits.

7.2. Enzyme induction

At high dose levels, acyclic, monocyclic, bicyclic, and aromatic terpene hydrocarbons induce an array of cytochrome P450 enzymes that are responsible for the hydroxylation of the hydrocarbons eventually leading to more polar metabolite conjugates. In rats, *d*-limonene (Austin et al., 1988; Maltzman et al., 1991; Miyazawa et al., 2002), β-myrcene (De-Oliveira et al., 1997) and α- and β-pinenes (Austin et al., 1988; White Jr. and Agosin, 1980), are inducers and competitive inhibitors of the CYP2B enzymes, specifically CYP2B1, the CYP2C enzymes and epoxide hydrolase. Evidence of liver microsomal enzyme induction is also reported for β-caryophyllene in rats and mice (Ambrose, 1983).

7.3. Short-term and long-term studies of toxicity

7.3.1. Acute oral toxicity

The LD_{50} in male and female rats of the sweet orange oil was determined to be 1122 mg/kg bw (Serota, 1984). Aliphatic and aromatic hydrocarbons exhibit very low acute oral toxicity with oral LD_{50} values ranging from 1590 to greater than 8000 mg/kg bw in rats, and from 2000 to greater than 13,360 mg/kg bw in mice (Adams et al., 2011).

7.3.2. Subchronic oral toxicity

7.3.2.1. Sweet orange oil. A GLP-compliant oral toxicity study has been conducted for sweet orange oil in male and female SD rats (10/sex/dose). The composition of the sweet orange oil was determined to be 97.5% limonene, 1.6% β-myrcene, 0.2% α-pinene, 0.2% sabinene and 0.1% δ-3-carene by chromatographic analysis. The animals received doses of 0 (vehicle), 240, 600 or 1500 mg/kg bw/day of sweet orange oil in methyl cellulose via gavage for 28 days (Serota, 1990b). Treatment did not have any effect on survival, clinical parameters, body weight, or food consumption. Dose-related decreases in blood glucose levels were observed at the highest dose in animals of both sexes and in the mid-dose group females. Female animals at all doses and males of the high-dose group also showed dose-related increases in the total serum protein and serum albumin levels. Gross pathology findings of the non-glandular stomach were reported in both males and females of the high dose group and correlated with histopathology findings, including rough, thickened and/or filmy material in the mucosa, increased incidence of squamous epithelial hyperplasia (5/10 males and 7/10 females), and subacute inflammation (3/10 males and 4/10 females). A modest incidence of thickened mucosa was also found in the mid-dose females. Gross pathology also revealed dark areas and/or thickened mucosa in the non-glandular region of the stomach in high-dose rats of both sexes. The non-glandular stomach changes are indicative of an irritation effect by the administered chemical and are not relevant to humans (Adams et al., 2008; Proctor et al., 2007). There was also an increased incidence of pale or dark areas of the kidneys of male animals in the mid and high dose groups. Incidence of hydronephrosis (dilatation and/or fluid in the kidney pelvis) was observed in 4/10 low-dose male animals and 4/10 high dose females with isolated cases in low and middle dose females (1/10, each). Hydronephrosis is highly variable in these rats and does not produce adverse renal effects. The males exhibited a high incidence of renal hyaline droplet degeneration with a low to moderate incidence of renal tubular necrosis and regeneration of the renal tubular epithelium at all doses, characteristic of α_{2u}-globulin-mediated effects, a male rat-specific phenomenon that is not relevant to humans (Capen et al., 1999) that is discussed later in the manuscript. Indeed, although high-dose females showed increased kidney weights, there was no corresponding histopathologic alteration. Increased absolute liver weights in all male animals and in high-dose females were also without corresponding histopathological findings and most likely was related to induction of metabolic enzymes. The latter is also supported by the ability of sweet orange oil to induce liver microsomal *p*-nitroanisole *O*-demethylase in male and female rats and hepatic aniline hydroxylase in females after 4 days exposure to 2500 mg/kg bw/day (Thomas, 1981). Based on these observations, including the non-relevance of changes in the non-glandular stomach to humans, the no-observed-adverse-effect-level (NOAEL) for sweet orange oil in rats is 600 mg/kg bw/day. This NOAEL value was used to assess the MoS for two *Citrus* NFCs, as described in Table 5 above.

The α_{2u}-globulin-associated renal effects seen in male rats in the sweet orange oil study described above are consistent with similar effects reported for flavoring substances that belong to congeneric Group 19, aliphatic and aromatic hydrocarbons, in several short-term and

long-term toxicity studies, including studies on β-myrcene, *d*-limonene, α-pinene, β-pinene, and camphene. A comprehensive review of toxicity studies is available in the recent safety re-evaluation of the group of Aliphatic and aromatic hydrocarbons (Adams et al., 2011). The relevant studies are described briefly below.

7.3.2.2. d-Limonene. In a National Toxicology Program (NTP) subchronic study with male and female F344/N rats (10/sex/dose), *d*-limonene was administered via gavage at dose levels of 0, 150, 300, 600, 1200 or 2400 mg/kg bw/day in corn oil, 5 days/week for 13 weeks (National Toxicology Program, 1990). Mortality (90%) was reported at 2400 mg/kg bw/day in both males (5/10) and females (9/10) and signs of toxicity were observed in both sexes at the dose levels 1200 and 2400 mg/kg bw/day. Lower final mean body weights were reported for male animals at doses of 600 mg/kg bw/day and above and the one surviving female animal at the top dose. In addition, a dose-related increase in severity of nephropathy was noted in males only, characterized by epithelial degeneration in the convoluted tubules; granular casts with tubular lumens, primarily in the outer stripe of the outer medulla (OSOM) and regeneration of the tubular epithelium; and hyaline droplets in the epithelium of the proximal convoluted tubules. Hyaline droplets were observed in all animals including vehicle controls, but aggregation of the droplets was only seen in the treated male rats. A NOAEL of 1200 mg/kg bw/day was selected for female rats based on low survival at the highest dose. Due to renal effects at all dose levels, no NOAEL could be assigned for male rats. The male rat specific kidney effects were later determined to be not relevant to human toxicity (Swenberg and Lehman-McKeeman, 1999).

In a study using B6C3F$_1$ mice (10/sex/dose), *d*-limonene was administered in doses of 0, 125, 250, 500, 1000 or 2000 mg/kg bw/day in corn oil by gavage, 5 days/week for 13 weeks (National Toxicology Program, 1990). One male and 2 females at 2000 mg/kg bw/day and 1 female at 500 mg/kg bw/day died before the end of the study. Lower final mean body weights were reported at the two highest dose levels for males (up to 10%) and females (up to ~2%), along with clinical signs of toxicity at the 1000 and 2000 mg/kg bw/day levels and one female animal with alveolar cell adenoma, a common tumor in this strain of mice, was reported at the highest dose level (2000 mg/kg bw/day). Based on lower body weights and mortality at higher levels, the NOAEL in this study is 500 mg/kg bw/day for males and 1000 mg/kg bw/day for females.

7.3.2.3. β-Myrcene. In a 90-day study conducted according to OECD Testing Guideline 408, CRL SD CD®IGS rats (10/sex/dietary intake level) were fed a diet containing 0 (dietary control), 700, 2100 or 4200 ppm of β-myrcene daily designed to provide target dose levels of 50, 150, or 300 mg/kg bw/day (Bastaki et al., 2018). The neat test material was stable under conditions of storage; however, stability was reduced to 45.1, 43.6 or 42.9% of target (7-day average) when incorporated into the diet, with measured concentrations of β-myrcene of 316, 916 or 1802 ppm in the low, middle and high dietary concentrations, respectively. Homogeneity studies revealed that the test material was evenly dispersed in the feed. Therefore, the measured dietary concentrations corresponded to estimated daily intakes of 20, 59 or 115 mg/kg bw/day for males and 24, 70 or 136 mg/kg bw/day for females. No mortalities, clinical signs of toxicity or ophthalmological changes associated with the presence of β-myrcene in the diet were reported. In this study, there were no statistically significant changes in any of the parameters evaluated that were associated with β-myrcene in the diet when compared to the concurrent control group. Parameters evaluated included body weight, body weight gain, food consumption and food efficiencies, clinical pathology parameters, macroscopic and microscopic findings, and organ weight measurements. Although a few changes in hematology and clinical chemistry values reached statistical significance when compared to concurrent controls, they were all within historical control ranges and did not correlate with macroscopic or microscopic findings. Therefore, none of these were determined to be of toxicological relevance. All histopathological findings were considered incidental, spontaneous and not related to the presence of β-myrcene in the diet. NOAEL values of 115 and 136 mg/kg bw/day for males and females, respectively, were determined based on the lack of adverse effects (Bastaki et al., 2018).

In an NTP study, β-myrcene was administered to male and female F344N Fischer rats (10/sex/dose) at doses of 0, 250, 500, 1000, 2000 or 4000 mg/kg bw/day by oral gavage for 13-weeks (National Toxicology Program, 2010). The same parameters were evaluated as described above. In addition, the left kidneys of male rats were processed for Mallory-Heidenhain staining for investigation of α_{2u}-globulin effects. High mortality was reported at the top dose group and additionally 2 males and 4 females at 2000 mg/kg bw/day, 1 male and 1 female at 1000 mg/kg bw/day and 1 male at 500 mg/kg bw/day groups died. Significantly lower mean body weights and body weight gains were reported in males at 500, 1000 and 2000 mg/kg bw/day and females at 2000 mg/kg bw/day. Significant decreases in blood leukocytes and lymphocytes in males and females of the 2000 mg/kg bw/day and significantly decreased serum creatinine levels at all dose levels in females and at 1000 and 2000 mg/kg bw/day in males were also observed. Several statistically significant changes were reported at all dose levels. These included significant increases in mean absolute and relative liver and kidney weights in male and female rats, which were dose dependent except for mean absolute liver weight in males, and significant decreases in mean absolute and relative thymus weights. Prominent kidney effects were also reported, including renal tubular hyaline droplet formation starting at 250 mg/kg bw/day in all males except the 2000 mg/kg bw/day group; significantly increased renal tubule necrosis in all treatment groups of males and females; significantly higher incidence of nephrosis in males and females at 1000 or 2000 mg/kg bw/day; evidence of nephropathy was found at similar incidences (7–10 animals/dose) in males of all groups including the control animals, and evidence of porphyrin pigmentation of the Harderian gland was significant at 500 mg/kg bw/day and higher. Additional inflammatory and degenerative effects were reported in other tissues at 1000 and 2000 mg/kg bw/day. In a follow-up pathology study of the kidney lesions observed in the β-myrcene bioassay, another pathology was identified in both male and female rats involving the OSOM (Cesta et al., 2013). On this basis, a NOAEL could not be assigned for male rats. For females, a NOAEL of 250 mg/kg bw/day is assigned based on reduced body weights at higher dose levels.

In a parallel NTP study, β-myrcene was tested in male and female B6C3F$_1$ mice by gavage at doses of 0, 250, 500, 1000, 2000 or 4000 mg/kg bw/day (10/sex/dose) for 13 weeks (National Toxicology Program, 2010). High mortality was reported in the top two dose groups. The remaining groups were evaluated for clinical signs of toxicity, body weight, sperm morphology, vaginal cytology evaluations, clinical chemistry, hematology, organ weights and histopathological examination of a wide variety of tissues. A NOAEL could not be assigned because of significantly increased mean weight of the right kidney relative to body weight in females at 250, 500 and 1000 mg/kg bw/day. There were also increased absolute and/or relative weights of other organs (liver and/or kidney) in females at 500 mg/kg bw/day and in both sexes at 1000 mg/kg bw/day and additional effects at the 1000 mg/kg bw/day dose level, including reduced body weight gains in males and females, significantly lower mean body weights in females, a significant decrease in hematocrit (males), hemoglobin (males) and erythrocyte count (both sexes). However, no abnormalities were found in histopathological evaluation up to 1000 mg/kg bw/day in both sexes.

7.3.2.4. β-Caryophyllene. In an OECD Section 4 (part 408) compliant 90-day study, male Crl: SD CD IGS rats (10/sex/group) were maintained on diets containing 0, 3500, 7000 or 21,000 ppm β-caryophyllene, calculated to provide an average daily intake of 0, 222, 456 or 1367 mg/kg bw/day, respectively. Female rats (10/sex/

group) were maintained on diets containing 0, 3500, 14,000 or 56,000 ppm β-caryophyllene, calculated to provide an average daily intake of 0, 263, 1033 or 4278 mg/kg bw/day, respectively (Bauter, 2013).

Stability of test substance in the diet was evaluated by analyzing dietary levels of β-caryophyllene on Day 0, 4, 7 and 10 after preparation and showed that the test article was homogeneously distributed and stable. The eyes of all rats were examined by focal illumination and indirect ophthalmoscopy before beginning the study and on Day 91. During the study, animals were observed for viability, signs of gross toxicity and behavioral changes at least once daily and weekly for a battery of detailed clinical observations. Body weights were recorded twice during acclimation and prior to test initiation and together with food consumption, approximately weekly thereafter and prior to terminal sacrifice. Urine and blood samples were collected on Day 85 from all study animals for urinalysis, hematology and clinical chemistry determinations. Coagulation assessments were performed on Day 94 or 95, prior to necropsy. Gross necropsies and histological evaluation of selected organs and tissues were performed on all study animals.

At the end of the study, there were no mortalities, clinical or ophthalmological changes attributable to dietary intake of β-caryophyllene. At the highest dose level in both the male and female study groups, statistically significant concentration-dependent reductions in body weight, body weight gain, food consumption and food efficiency were reported and attributed to the possible decrease in test substance palatability at high dietary levels.

Statistically significant changes in hematology, clinical chemistry, coagulation and urinalysis parameters were observed in mid and high-dose male and female rats. Most of the observed changes were not concentration dependent, within the range of historical values and had no direct correlation with a histological evaluation. However, concentration-dependent decreases in glucose and increases in triglyceride concentrations, correlating with changes in liver weights in females were considered the possible metabolic result of test substance administration. Also, fine granular casts, potentially related to α_{2u}-globulin accumulation, were found in the urine of some males in all the test groups as well as the control group.

Macroscopic findings included enlarged livers in the high dose female group that corresponded to histological evidence of concentration dependent hepatocellular hypertrophy. In the high dose male group, enlarged kidneys were reported that correlated with an increase in relative kidney weights and microscopic findings of nephropathy and tubular cytoplasmic droplets in the kidneys. This most likely is related to the α_{2u}-globulin nephropathy that was reported in the histopathologic examination of the male rats. Kidney cells of affected males were reported to have necrotic nuclei and an increase in eosinophilic cytoplasm. Consistent with this spontaneous nephropathy, there were also increases in the number and size of hyaline droplets present in the kidneys. This was confirmed by positive Mallory-Heidenhain staining of the kidney sections from all male rats. The staining showed a concentration-dependent increase in intensity correlated to increasing dietary levels of β-caryophyllene (Garlick, 2013). Based on the International Agency for Research on Cancer (IARC) criteria defining the occurrence of α_{2u}-globulin nephropathy, these observations are consistent with previous studies that define α_{2u}-globulin nephropathy in the male rat. The development of α_{2u}-globulin nephropathy has been shown to be specific to the male rat and as discussed in the analysis of the *d*-limonene study in rats, this effect is not considered relevant to human health.

A no-observed-adverse effect level (NOAEL) of 3500 ppm (equivalent to approximately 222 mg/kg bw/day for males and 263 mg/kg bw/day in females) is determined for β-caryophyllene based on histologic evidence of hepatocyte hypertrophy reported at the mid and high male and female dose groups.

7.3.3. Long term studies of toxicity and carcinogenicity

Chronic toxicity and carcinogenicity studies have been conducted for representatives of the congeneric group of hydrocarbons, *d*-limonene and β-myrcene, in both mice and rats.

7.3.3.1. d-Limonene. $B6C3F_1$ mice (50/sex/dose) were administered *d*-limonene either at 0, 250 or 500 mg/kg bw/day (males) or 500 or 1000 mg/kg bw/day (females) in corn oil by gavage, 5 days/week for 103 weeks (National Toxicology Program, 1990). Lower final mean body weights were reported in high-dose female mice after week 28 to the study's termination. No other clinical signs of toxicity were reported. Mortality was not dose related, with higher incidence in the low-dose male group than the high-dose male mice. The incidence of multinucleated and cytomegalic hepatocytes in the high dose male mice was significantly higher than in control mice but the incidences of hepatocellular adenomas or carcinomas (combined) in *d*-limonene treated mice were not significantly different from vehicle controls. No other chemical-related neoplasms were reported in any of the mice treated with *d*-limonene and it was concluded that there was no evidence of carcinogenic activity of *d*-limonene for male or female $B6C3F_1$ mice under the conditions of this 2-year gavage study.

In F344/N rats, the effects of chronic *d*-limonene administration at dose levels of 0, 75 or 150 mg/kg bw/day (males) or 0, 300 or 600 mg/kg bw/day (females) in corn oil by gavage, 5 days/week for 103 weeks were evaluated (National Toxicology Program, 1990). Lower mean body weights were observed at the high dose in male rats after 2 weeks of treatment and in female rats after week 28 to study termination. Increased mortality was observed in the high dose female group after week 39, relative to both controls and male rats. No other chemical-related clinical signs were reported for the duration of the study. Observations in the kidneys of male rats showed dose-related increases in the incidence of mineralization of the renal papilla and 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 consistent with findings in sub-chronic studies with this and other related hydrocarbons. The incidences of tubular cell adenoma and of tubular cell adenoma with tubular cell adenocarcinomas combined in treated male rats were significantly higher (0/50, 8/50 and 7/50, respective to dose) than the incidence of the control group. The NTP review concluded that under the conditions of this 2-year study, there was clear evidence of carcinogenic activity of *d*-limonene for male F344/N rats, based on the increased incidences of tubular cell hyperplasia, adenomas, and adenocarcinomas of the kidney, but no evidence of carcinogenic activity of *d*-limonene in female rats up to 600 mg/kg bw/day. A significant dose-related increase in α_{2u}-globulin in the kidney accompanied by a significant increase in renal tubular cell hyperplasia relative to controls and a lack of these findings in female rats is consistent with these tumors occurring secondary to α_{2u}-globulin nephropathy. No other treatment-related lesions were found.

7.3.3.2. α_{2u}-globulin nephropathy in male rats. Responding to the findings of renal toxicity reported in male rats but absent in female rats by the NTP study, several concurrent and subsequent studies were conducted to further characterize and understand the mechanisms of renal toxicity presented by *d*-limonene. A short-term study was performed with young adult male F344/N rats (5/dose), in which *d*-limonene was administered via gavage at dose levels of 0, 75, 150 or 300 mg/kg bw/day, 5 days a week for up to 27 days (Kanerva et al., 1987). The effects observed in the male rats were similar to those previously seen in comparable studies on the effects of decalin, in which the dose-related formation of hyaline droplets, accumulation of a specific protein in renal cortical tissues, accumulation of dose-related granular cast formation in the outer zone of the medulla and cortical

alterations that were collectively classified as nephrosis were observed. At the time this study was reported, the authors noted the emerging pattern of nephropathy in male rats, with similar effects observed in sub-chronic studies of other hydrocarbons such as unleaded gasoline, norbornadiene and cyclopentadiene (Kanerva et al., 1987).

The emergence of renal toxicity in male rats with the administration of lower doses of *d*-limonene was the focus of a sub-chronic study with male Fischer 344 rats in which *d*-limonene was administered via gavage at dose levels of 0, 2, 5, 10, 30 or 75 mg/kg bw/day, 5 days/week for 13 weeks (Webb et al., 1989). In this study, interim sacrifices were performed on subgroups of animals (5/dose/time point) on days 8 and 15 for the 10 mg/kg bw/day dose group and on days 8, 15, 22, and 29 for the control and high dose groups. The remaining animals (10/dose) were terminated on day 91. Findings included significantly increased relative kidney and relative liver weights in high dose rats; hyaline droplet formation in the kidneys, granular casts and multiple cortical changes, diagnosed as chronic nephrosis, starting at 10 mg/kg bw/day and from day 8 of treatment. There were no histopathological changes noted in the livers of treated rats.

Subsequently, the specific protein found to accumulate in the renal cortical tissues was identified as α_{2u}-globulin by amino acid sequencing following its isolation from male rat kidney tissues collected following the administration of [^{14}C]-*d*-limonene. Solvent extraction of the α_{2u}-globulin protein fraction and chromatographic analysis found both *d*-limonene and its metabolite *d*-limonene-1,2-oxide to be associated with α_{2u}-globulin, with *d*-limonene-1,2-oxide to be the major form. Dialysis experiments determined both *d*-limonene and *d*-limonene-1,2-oxide are reversibly bound to this protein (Lehman-McKeeman et al., 1989).

When tested orally in male and female beagle dogs (3/sex/dose) at dose levels of 0, 0.4, 1.2, or 3.6 mL/kg bw/day (approximately, 0, 340, 1000 and 3000 mg/kg bw/day, respectively) for approximately 6 months, *d*-limonene caused frequent vomiting, a decrease in body weight, and decreased total cholesterol and blood sugar levels at the top two doses (statistical significance was not reported) (Tsuji et al., 1975a). Protein casts in the renal tubules of female and male dogs were noted at dose levels of 340 and 1000 mg/kg bw/day, respectively. No other histological changes were reported.

In another study in male and female adult beagle dogs (5/sex/dose), *d*-limonene was administered by gavage for 6 months (Webb et al., 1990) at doses of 0, 100 or 1000 mg/kg bw/day divided in two daily doses. Diarrhea and emesis occurred periodically with the same frequency in the high and low-dose groups. A 35% increase in serum cholesterol and a 2-fold increase in serum alkaline phosphatase occurred at the high dose in both sexes. Unlike the findings reported earlier by Tsuji et al., 1975a, 1975b, no significant changes in body weights, feed consumption, or significant changes in organ weights were observed, except for a positive trend and a statistically significant increase at the high dose in relative kidney weights in males and females and absolute kidney weights in females. At the end of the study, a full histopathological analysis found no significant alterations. No evidence of hyaline droplets or kidney histological abnormalities were reported. In the absence of nephrotoxicity effects in either male or female dogs, the authors concluded that the effects of *d*-limonene on the male rat kidney were species- and sex-specific.

Upon review, a number of substances including *d*-limonene were identified that induce the development of nephropathy in the male rat by the formation of hyaline droplets in proximal tubule cells due to the accumulation of α_{2u}-globulin (Hard et al., 1993). The accumulation of hyaline droplets leads to renal cell injury, and in response, cell proliferation in the kidney leads to the development of renal tumors (Lehman-McKeeman, 2010). Extensive analyses on the development of α_{2u}-globulin nephropathy in the male rat and evidence that this type of nephropathy is unlikely to occur in humans and other species (Flamm and Lehman-McKeeman, 1991; Swenberg et al., 1989) led the U.S. Environmental Protection Agency (US-EPA) and IARC to conclude that the development of α_{2u}-globulin nephropathy in male rats should not be used to estimate the nephrotoxic or cancer hazard for humans (US-EPA, Capen et al., 1999, 1991). These agencies also developed criteria for identifying agents that induce this effect.

The evidence collected in the NTP and other studies on *d*-limonene indicate that the renal nephrotoxicity observed in male rats is due to α_{2u}-globulin nephropathy and meets the IARC criteria that includes: 1) a lack of genotoxic activity, 2) male rat specificity for nephropathy and renal tumors, 3) observation of the accumulation of protein droplets and the induction of characteristic histopathological changes in shorter-term studies, 4) identification of α_{2u}-globulin as the protein that accumulation in tubule cells, 5) demonstration of the reversible binding of the substance or metabolite to α_{2u}-globulin, 6) induction of sustained increased cell proliferation in the renal cortex and 7) similarities in dose-response relationship of the tumor outcome with the histopathological end-points (Capen et al., 1999). IARC determined that *d*-limonene meets these criteria (Swenberg and Lehman-McKeeman, 1999). Because *d*-limonene fully meets the IARC criteria defining the occurrence of α_{2u}-globulin nephropathy, it was used as a positive control for studies examining renal effects upon the administration of methyl isobutyl ketone in male rats (Borghoff et al., 2015).

In its reviews of *d*-limonene for use as a flavoring ingredient, both the European Food Safety Authority (EFSA) and JECFA concurred that the male rat nephropathy observed in the NTP studies is not relevant to humans (EFSA, 2015a; JECFA, 2005). The FEMA Expert Panel, concurs with EFSA in assessing that the NOAEL for *d*-limonene is 215 mg/kg bw/day based on the NOAEL observed for female rats in the 103 week NTP study, (adjusted daily dose from 300 mg/kg bw/day administered 5 days/week) (National Toxicology Program, 1990). In addition, this NOAEL value was used to assess the MoS for ten *Citrus* NFCs, as described in Table 4 above.

7.3.3.3. β-Myrcene. B6C3F$_1$ mice (50/sex/dose) were administered 0, 250, 500 or 1000 mg β-myrcene/kg bw/day in corn oil by gavage, 5 days/week for 104 or 105 weeks (National Toxicology Program, 2010). Increased mortality was noted at 1000 mg/kg bw/day in males (21/50) and females (17/50) surviving to the end of the study and overall mean survival of 577 and 552 days, respectively, and lower body weight gain at all dose levels were reported. The liver was the primary target organ of toxicity in both males and females. Due to increased mortality at the top dose, no data on neoplastic lesions were reported in that group. Male mice were more susceptible to neoplastic effects than female mice and showed significant ($p < 0.001$) increases in the incidences of hepatocellular adenomas at 250 and 500 mg/kg bw/day (41/50 and 43/50, respectively), hepatocellular carcinomas (20/50 and 28/50, respectively), hepatoblastomas (6/50 and 11/50, respectively), and hepatocellular adenomas or carcinomas (combined) (44/50 and 48/50, respectively). However, the incidence in the control group for these tumors was also high, with hepatocellular adenomas (26/50), hepatocellular carcinomas (14/50) and combined hepatocellular adenomas or carcinomas (33/50).

In female mice, smaller increases relative to male mice were reported at the same dose levels in the incidences of hepatocellular adenoma (13/50 and 6/50, respectively), hepatocellular carcinoma (7/50 and 2/50, respectively), and hepatocellular adenoma or carcinoma (combined) (18/50, 36% and 8/50, 16%, respectively). The respective incidences in the control group were also lower (6/50, 1/50 and 7/50, respectively). The above findings were significant at the 250 mg/kg bw/day but not the 500 mg/kg bw/day group. Other effects included dose dependent hepatocellular hypertrophy, increased incidences of bone marrow atrophy (mid-dose females), lymphoid follicle atrophy of the spleen (significant for mid-dose females and dose-related for males), atrophy in the mandibular lymph node (mid-dose females), forestomach inflammation and squamous epithelial hyperplasia (mid-dose females), decreased incidences of pancreatic islet hyperplasia (mid-dose males) and of uterine endometrial hyperplasia (low- and mid-dose females).

The NTP review concluded that there was clear evidence of carcinogenic activity in male mice and equivocal evidence in female mice under the conditions of this 2-year study based on the liver tumors. However, the relevance of mouse hepatocellular tumors to predict human cancer risk has been questioned previously because of lack of human relevance of the mode-of-action, the wide difference in exposure in the human compared to the mouse, and the high background incidence in many mouse strains indicating much greater susceptibility in mice compared to humans (Carmichael et al., 1997; Holsapple et al., 2006; Velazquez et al., 1996). The sensitivity of the B6C3F$_1$ male mouse liver to toxicity and secondary neoplastic changes was widely recognized long before this NTP study (Haseman et al., 1984, 1986, 1990; Maronpot, 2009; Maronpot et al., 1987), and by the NTP itself (King-Herbert and Thayer, 2006). First, male B6C3F$_1$ mice consistently demonstrate a high background incidence of hepatocellular adenomas, carcinomas and adenomas and carcinomas (combined). The NTP program reports a high historical spontaneous incidence of liver neoplasms in male B6C3F$_1$ mice, with combined hepatocellular adenoma and carcinomas rates of 60% for males and 32% for females reported as of 2007 (Maronpot, 2009). The background incidence in vehicle control mice of combined hepatocellular adenomas or carcinomas in the β-myrcene study was 66% for males and 14% for females (National Toxicology Program, 2010). Second, it is recognized that hepatocellular neoplasms seen in 2-year bioassays in B6C3F$_1$ mice with non-genotoxic chemicals are typically secondary to chronic toxicity and regenerative cellular proliferation or secondary to direct mitogenicity as a function of dose (Allen et al., 2004; Boobis et al., 2009; Cohen, 2010; Meek et al., 2003; Ring and Eskofier, 2015), and evidence of hepatic effects in short duration studies is a good predictor of hepatic neoplasia in chronic studies and the higher susceptibility of the male mouse (Allen et al., 2004; Cohen, 2010; Holsapple et al., 2006; Ring and Eskofier, 2015). In fact, the NTP has delisted the status of substances as liver carcinogens on this basis (e.g. p-nitrosodiphenylamine, in 5th and 6th Annual Report on Carcinogens). Critical reviews of the overall pattern of hepatocellular tumors in the mouse model and new understanding of non-genotoxic modes of action in the development of rodent tumors have led to the conclusion that mouse hepatocarcinogenicity in the absence of genotoxicity is not predictive for human cancer risk assessment at expected human exposure levels (Billington et al., 2010; Cohen, 2010; Corton et al., 2014; Elcombe et al., 2014; Holsapple et al., 2006; Kobets and Williams, 2018; Osimitz et al., 2013). Because of the high sensitivity of B6C3F$_1$ mice to liver tumors, in the absence of genotoxicity (see evidence discussed below), the effects observed in the 2-year β-myrcene study were not regarded as relevant to humans (EFSA, 2011).

In a similar chronic 2-year bioassay in F344/N rats (50/sex/dose), β-myrcene was administered at 0, 250, 500 or 1000 mg/kg bw/day in corn oil by gavage, 5 days/week for 104 weeks (National Toxicology Program, 2010). As with mice, significant mortality was reported in male rats, with no animals surviving to the end of the study at the high dose group. Mortality was also seen in females but on a lower scale with 33 animals surviving to the end of the study in the top dose group. Decreased body weights were reported for both males and females at the top dose. Unlike the findings of hepatic toxicity in mice, kidneys were the target organ of toxicity in rats exposed to β-myrcene, with higher susceptibility in male animals compared to female.

Incidence of pathology findings was not reported for the top dose group of males due to extensive mortality. Male rats showed dose-related increases in levels of renal papillary mineralization (1/50, 2%, 48/50, 96% and 40/50, 80%) and nephrosis (0/50, 0%, 42/50, 84% and 46/50, 92%), respectively, at 250 and 500 mg/kg bw/day. There was little or no renal tubule atypical hyperplasia (0/50, 0%, 0/50, 0% and 2/50, 4%), but significant increases in incidences of renal adenomas (0/50, 0%, 12/50, 24% and 13/50, 26%) and lower incidences (not statistically significant) of carcinomas (0/50, 0%, 3/50, 6% and 1/50, 2%) were observed in the treated groups. The incidence of chronic progressive nephropathy was extensive and similar in all male groups including the control (45–48 animals/dose). Epithelial hyperplasia of the lining of renal papilla was significantly higher in the 250 and 500 mg/kg bw/day treated groups (42% and 38%, respectively), compared to control (0%). However, this epithelial hyperplasia involves the lining of the papilla, not the kidney pelvis urothelium and is a manifestation of chronic progressive nephropathy, not a direct effect of the chemical on the epithelium. In females, the incidence and severity of renal effects were less pronounced. Nephropathy was reported also in females across all groups including the control, albeit at a lower incidence compared to males, and was statistically significantly higher in treated groups (52%, 86%, 82% and 88% in control, low, middle, and high dose groups, respectively). Renal tubule nephrosis was significantly increased in the 500 and 1000 mg/kg bw/day groups (54% and 90%, respectively, compared to 0% in the control group) and epithelial hyperplasia of the lining of the papilla was increased in all female dose groups (2%, 24%, 30%, and 38%, respectively). Renal tubule adenomas were present in 4% of 1000 mg/kg bw/day females which was higher than the incidence reported in historical controls. Time-to-first-tumor was also longer in females (689 days) than males (551 days). Another renal pathology was revealed in the OSOM of male and female rats in a follow up investigation where the kidney slides were reviewed (Cesta et al., 2013). This nephrosis was characterized by dilation of the S3 tubules, nuclear enlargement and luminal pyknotic cells of the outermost OSOM was minimal in the 90-day study, but in the 2-year study it was more pronounced and showed a direct dose-correlation (Cesta et al., 2013). The authors suggested that further study is needed to clarify the mechanism of action and show the relation of this pathology to humans (Cesta et al., 2013).

Other effects reported included decreases in basophilic foci and mixed cell foci of the liver, increased eosinophilic foci of the liver, decreased chronic inflammation of the liver, increased chronic inflammation of the nose, increased chronic active inflammation of the forestomach, increased thyroid gland C-cell adenoma, and increased cystic endometrial hyperplasia of the uterus.

The NTP review concluded that there was clear evidence of carcinogenic activity in male rats, based on increased incidences of renal tubule neoplasms, and equivocal evidence in female rats, based on increased incidences of renal tubule adenoma, under the conditions of this 2-year study.

In the review of the renal pathology of the NTP study of β-myrcene (Cesta et al., 2013; National Toxicology Program, 2010), it was concluded that the tumors in the low dose males were due to α_{2u}-globulin nephropathy and thus are not relevant to humans. However, they concluded that the tumors in the mid and high dose males and incidences reported in high dose females were due to other factors in addition to the α_{2u}-nephopathy.

Chronic progressive nephropathy is common in rats, including in the F344 strain used in the NTP study (Baetche et al., 1991; Hard et al., 2012, 2013; Lock and Hard, 2004; Travlos et al., 2011) with the effects more common and more severe in males than females (Hard and Khan, 2004; Hard and Seely, 2005; Haseman et al., 2003; Seely et al., 2002; Swenberg and Lehman-McKeeman, 1999). The reason for this sex difference is unknown but differences between sexes have been observed in various tubular functions, such as the higher expression of organic anion transporter type 1 in the proximal tubule of males (Buist et al., 2002) and higher production and excretion of α_{2u}-globulin from the liver (Swenberg, 1993).

Chronic progressive nephropathy in rats is recognized as not relevant to humans (Hard et al., 2009). Chronic progressive nephropathy is known to be associated with an increased incidence of renal tubular neoplasms, but renal tumors arise secondary to chronic progressive nephropathy only in its most severe form. These tumors are also not considered relevant to humans (Baetche et al., 1991; Capen et al., 1999; Hard et al., 2012, 2013; Lock and Hard, 2004; Travlos et al., 2011). However, in the review by Cesta et al. (2013), the severity of the chronic progressive nephropathy in the β-myrcene-treated rats with

renal neoplasms was not considered of the severity usually associated with development of such neoplasms. Cesta et al. (2013) concluded that the renal tumors in the mid and high dose males and those few in females were related to the unusual nephrosis identified in β-myrcene-treated rats. While it is likely that this nephrosis is specific to the rat and appears to be a high dose effect given the unique lesions were not observed in the Bastaki study (Bastaki et al., 2018), it is not possible to conclude that the lesions are not relevant to humans. However, the lesions haven't been observed previously in humans, which also supports the likely species specificity and potential interaction with both CPN and the α_{2u}-globulin effect in the rat given high doses of β-myrcene. Further, even if the lesions are considered relevant to humans, β-myrcene is non-genotoxic allowing a consideration of a threshold for carcinogenicity using the NOAEL determined in the Bastaki et al. study (EFSA, 2015b). The renal tumors occurring in the rats treated with β-myrcene are likely not relevant to humanssince the chemical is non-genotoxic, neither the renal effects nor tumors were detected in mice, the rat is known to be highly susceptible to these renal effects, especially males, and the renal effects of α_{2u}-globulin nephropathy and chronic progressive nephropathy, and possibly the nephrosis, are not relevant to humans.

7.4. Reproductive and developmental toxicity

7.4.1. Sweet orange oil

In a reproductive/developmental screen, sweet orange oil was administered by oral gavage to virgin female Crl:CD® rats at doses of 375, 750 or 1500 mg/kg bw/day (10 animals/dose) for seven days prior to mating, through cohabitation/mating, gestation, delivery and for four days post-parturition/lactation (Hoberman, 1989). No dam mortality was reported during the study. Observations of excessive salivation for all test groups during pre-mating and gestation were noted as well as during lactation but only for the mid and high-dose groups. Decreased motor activity and urine staining of the abdominal fur were observed during the pre-mating period for mid and high-dose rats. Body weight gain reduction in all dose groups and a statistically significant weight loss and associated significantly reduced feed consumption in the high dose group were reported during the pre-mating period but were transient and did not result in overall dose-related or statistically significant differences in average maternal weights, body weight gain or feed consumption when compared to controls over the test period. Mating performance and fertility were not affected at any dose. A significantly increased incidence of stillborn pups, pup mortality and lower body weight gains of surviving pups were reported in the high dose group. A significantly higher pup mortality in the low and mid-dose groups was attributed to the death of one whole litter in each group. There were no other adverse effects at doses up to 1500 mg/kg bw/day, such as the average duration of cohabitation or gestation, implantations, or pup sex ratios. The pups showed no malformations or gross lesions attributable to sweet orange oil administration. The authors of the study determined the maternal NOAEL for sweet orange oil to be less than 375 mg/kg bw/day based on the clinical signs of toxicity and lower body weight gain, despite the temporary nature of the effects. The NOAEL for the pups is 750 mg/kg bw/day based on the increased numbers of stillbirths, mortality and decreases in body weight gain at the highest dose. Overall, sweet orange oil was not considered to be hazardous to female rat reproductive performance or on development and pup growth.

7.4.2. Group 19: Aliphatic and aromatic hydrocarbons

d-Limonene administered to pregnant ICR mice from days 7–12 of gestation at dose levels of 0, 591 or 2363 mg/kg bw/day resulted in adverse effects for dams at the high dose level and at both dose levels for offspring (Kodama et al., 1977). Maternal toxicity was limited to significantly reduced body weight gains at the high dose. Toxicity in the offspring included significantly lower body weight in males, significantly decreased relative thymus weights, increased incidences of lumbar and fused ribs, and delayed ossification which recovered during post-natal development. In addition, significant differences in absolute and relative organ weights were found at both dose levels in female offspring, but no other significant differences in organ weights of male offspring were observed. Therefore, the maternal NOAEL is 591 mg/kg bw/day but a NOAEL for the offspring could not be determined.

Similar effects were reported for Wistar rats after oral administration of 0, 591 or 2869 mg/kg bw/day of *d*-limonene on days 9–15 of gestation (Tsuji et al., 1975b). Maternal toxicity was noted in the high dose only, including mortality. Signs of toxicity including organ weight effects and delayed ossification were seen in both male and female offspring at both dose levels.

d-Limonene was administered to pregnant Japanese white rabbits from gestational days 6–18 at dose levels of 0, 250, 500 or 1000 mg/kg bw/day (Kodama et al., 1976). Maternal toxicity including mortality and a significant, transient decrease in food consumption and body weight gain were reported at the middle and high dose. No evidence of toxicity, effect on growth or any significant visceral or skeletal abnormalities were found in the offspring. The maternal NOAEL is 250 mg/kg bw/day and the NOAEL for offspring toxicity is greater than 1000 mg/kg bw/day.

β-Myrcene, administered via gavage to female Wistar rats on gestation days 6–15 at doses of 0, 250, 500 or 1200 mg/kg bw/day, resulted in maternal and fetal toxicity only in the high-dose group and was limited to transient decreased maternal body weight gain, mortality in one dam, lower numbers of visible implantation sites and of live fetuses, lower fetal weights and an increased incidence of fetal skeletal malformations. The maternal and fetal NOAEL is determined to be 500 mg/kg bw/day (Delgado et al., 1993a). In a follow-up study, β-myrcene was administered to pregnant Wistar rats via gavage at dose levels of 0, 250, 500, 1000 or 1500 mg/kg bw/day from gestational day 15 to postnatal day 21 to test peri- and post-natal developmental toxicity in rats (Delgado et al., 1993b). Adverse effects were observed at 500 mg/kg bw/day and above, including a dose-related decrease in birth weight, increased perinatal and postnatal mortality, and delayed developmental landmarks. Impaired fertility in female offspring was also reported at the highest two dose levels. Maternal toxicity was evident only in the high-dose group; longer labor duration in the two highest dose groups, and significantly higher number of stillbirths in the high-dose group were also reported. The developmental toxicity NOAEL for this study was reported to be 250 mg/kg bw/day.

In another study, 60 rats (15 male, 45 female) were given β-myrcene via gavage at doses of 0, 100, 300 or 500 mg/kg bw/day (Paumgartten et al., 1998). Males were treated for 91 days prior to mating, and during mating, while females were treated for 21 days prior to mating until 21 days after birth (weaning). Males were terminated at the end of the mating period and one-third of the females in each group were terminated on day 21 of pregnancy. The remaining females were terminated after weaning (postnatal day 21). A slight increase in the relative and absolute liver and kidney weights of males in the high dose group were the only significant difference observed between control and test animals. A significantly higher resorption rate and lower number of live fetuses per implantation site were noted in the high-dose group. The slightly increased frequency of skeletal malformations noted also occurred in the controls and were attributed to strain-specific effects. The NOAEL for this study is determined to be 300 mg/kg bw/day, based on the slight fetotoxic effects observed at 500 mg/kg bw/day.

The developmental toxicity of *p*-mentha-1,3-diene was evaluated in SD rats following daily oral doses of 0, 30, 60, 125 or 250 mg/kg bw/day on days 6–15 of gestation (Araujo et al., 1996). Decreased maternal body weight gain was observed at 125 mg/kg bw/day and higher and but reduced fetal body weights were observed at the highest (250 mg/kg bw/day) dose. Delayed ossification and minor skeletal malformations were reported in pups at doses of 60 mg/kg bw/day and higher but later research has indicated that these types of changes are transient variations and do not represent malformations (DeSesso and Scialli,

2018) and these changes were not statistically significant. The only change at 125 mg/kg that was statistically significant was a delay in ossification that is unlikely to represent an adverse effect since it self corrects by the time of weaning (DeSesso and Scialli, 2018). These effects are considered to be secondary to maternal toxicity, are transient and appear to recover completely postnatally (Kimmel et al., 2014). Based on these observations, there were no developmental adverse effects in this study so a developmental toxicity NOAEL cannot be determined. Based on the observed maternal toxicity at the two highest dose levels, the NOAEL is 60 mg/kg bw/day. However, weight changes have not been detected at similar test doses in other studies, raising concerns about the reliability of this study.

7.5. Genotoxicity

In vitro and *in vivo* genotoxicity studies relevant to the safety evaluation of *Citrus* oils are summarized briefly below. Additional information on the Aliphatic and alicyclic hydrocarbons group is available in Adams et al. (2011).

7.5.1. Sweet orange oil

Among the *Citrus* oils, sweet orange oil has been tested in several genotoxicity assays. Sweet orange oil was negative for mutagenicity in a standard reverse mutation assay, with *S. typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100, at concentrations up to 5000 μg/plate in the absence and presence of a S-9 bioactivation system, derived from Aroclor 1254-induced male SD rat livers (DeGraff, 1983; Heck et al., 1989). Sweet orange oil also showed no evidence of mutagenic activity when tested in an unscheduled DNA synthesis (UDS) assay in rat liver hepatocytes for 18–20 h at concentrations of 0.002–0.5 μL/mL (Curran, 1987; Heck et al., 1989). The top concentration resulted in excessive cytotoxicity in this assay. Weak evidence of mutagenicity was detected for sweet orange oil in the mouse lymphoma forward mutation assay with L5178Y TK $^{\pm}$ mouse lymphoma cells (Cifone, 1983). In this assay, the L5178Y TK $^{\pm}$ mouse lymphoma cells were incubated with 1.25–50 nL/mL of sweet orange oil in the absence of S-9 and with 5–120 nL/mL in the presence of S-9. Mutagenicity was observed at the top two concentrations (40–50 nL/mL) in the absence of S-9 where excessive toxicity also occurred (> 96%). Increased mutant frequencies were also seen in a semi-concentration dependent manner from 60 to 120 nL/mL in the presence of S-9, which was directly related to the degree of cytotoxicity (mutant frequency was higher in cultures with higher toxicity) (Cifone, 1983; Heck et al., 1989). The increased mutant frequencies in the presence of S-9 in this assay would not meet current standards for a biologically relevant result according to the International Workgroup on Genotoxicity Tests for the Mouse Lymphoma Assay (MLA) (Kirkland et al., 2007b). It is also worth noting that the L5178Y mouse lymphoma cells are deficient in p53 function (Kirkland et al., 2007a; Storer et al., 1997) and therefore would fail to undergo cell cycle arrest and apoptosis as a result of DNA damage. The p53 deficiency results in increased mutation rates compared to those in p53-competent cells. Therefore, considering the small magnitude of mutant frequency increases and the scale of cytotoxicity associated with larger mutant frequencies, as well as the clearly negative results in the Ames and other genotoxicity assays, the results of this MLA assay are considered to be not biologically relevant.

7.5.2. Other Citrus NFCs

Lime oil (20 mg/disk) and grapefruit oil (25 mg/disk) showed mutagenic activity in the spore-*rec* assay only in the absence of S-9 metabolic activation, although the degree of cytotoxicity was unclear in this study (Ueno et al., 1984). In another *rec* assay, lemon and orange oils showed no indication of mutagenic activity in the absence or presence of S-9, in either the spore plate or liquid methods (Kuroda et al., 1989). In an OECD compliant Ames study using *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Esherichia coli* strain WP2 *uvrA*, petitgrain paraquay oil demonstrated no mutagenicity in either the presence or absence of S-9 activation at dose levels up to 5000 μg/plate. In a chromosomal aberration study in Chinese hamster fibroblast cells, grapefruit oil (up to 63 μg/mL), lemon oil (up to 125 μg/mL), lime oil (up to 40 μg/mL) and orange oil (up to 125 μg/mL) showed no ability to induce polyploidy (Ishidate et al., 1984). In an OECD compliant *in vitro* mammalian cell micronucleus test using human peripheral blood lymphocytes performed in both the presence and absence of an Aroclor-induced S-9 activation system, petitgrain paraquay oil (up to 400 μg/mL) failed to show an increase in micronucleus induction relative to the vehicle control (Roy, 2015).

7.5.3. Group 19: Aliphatic and aromatic hydrocarbons

Genotoxicity studies on hydrocarbons, the primary constituent group, are summarized below. The overall evidence for this group of substances indicates absence of genotoxicity.

7.5.3.1. d-Limonene. No evidence of genotoxicity has been reported for *d*-limonene in several *in vitro* assays. It was negative for mutagenicity in several Ames assays in multiple *S. typhimurium* strains (TA97, TA98, TA100, TA1535, TA1537, TA1538, and/or TA102, UTH8413, or UTH8414) with and without S-9 metabolic activation at concentrations up to 5000 μg/plate or up to 150,000 nL/plate (Connor et al., 1985; DeGraff, 1983; Florin et al., 1980; Haworth et al., 1983; Heck et al., 1989; Müller et al., 1993). It was also negative for genotoxicity in the chromosomal aberration assay with Chinese hamster ovary (CHO) cells at concentrations ranging from 10 to 500 μg/mL (Anderson et al., 1990); in the sister chromatid exchange (SCE) assay in CHO cells at concentrations ranging from 1.4 to 162 μg/mL (Anderson et al., 1990; Kauderer et al., 1991; Sasaki et al., 1989); and in the MLA in L5178Y cells at concentrations up to 100 μg/mL with and without S-9 metabolic activation (Heck et al., 1989; Myhr et al., 1990). These findings are consistent with lack of mutagenicity *in vivo* in the mammalian spot test in 126 mouse embryos of C57BL/6JHan and T-stock crossed animals exposed *in utero*, following intraperitoneal injection of dams with 215 mg/kg bw/day of *d*-limonene during gestation days 9–11 (Fahrig, 1984). In the *umu* test in which induction of the *umuDC-lacZ* genes by DNA damage is measured, DNA damage was not detected when *d*-limonene was incubated with *S. typhimurium* TA 1535/pSK1002 at concentrations up to 500 μg/mL both in the presence and absence of an S-9 metabolic system. In this study, the S-9 fraction was prepared from livers of SD rats treated with sodium phenobarbital and 5,6-benzoflavone (Yasunaga et al., 2004).

In an *in vivo* mutagenicity assay, limonene was administered at a concentration of 1% in the food of ten (10) male Big Blue™ rats. Analysis of the diet indicated that the concentration of limonene decreased over time from a dose of ~522 mg/kg bw/day at the start to ~360 mg/kg bw/day at the end of the 10-day feeding period. All animals were sacrificed 14 days after the final dose of limonene. DNA was extracted from the liver and kidneys, transformed into *E. coli* and the mutant frequency was determined. The mutant frequency in rats administered limonene was not increased in comparison to rats on the control diet (Turner et al., 2001).

In a series of comet assays, *d*-limonene, administered orally to mice and rats at a single dose of 2000 mg/kg, was not found to induce DNA damage in stomach, colon, liver, kidney, bladder, lung, brain and bone marrow tissues (Sekihashi et al., 2002). In an alkaline comet assay (pH > 13) *d*-limonene, administered to four (4) male SD rats by oral gavage, did not induce DNA damage at concentrations up to 2000 mg/kg bw (Nesslany et al., 2007).

7.5.3.2. β-Myrcene. No evidence of mutagenicity has been reported for β-myrcene when tested in the Ames assay in several *S. typhimurium* strains (TA97, TA98, TA100 and TA1535) with and without S-9 metabolic activation at concentrations up to 10,000 μg/plate (Connor et al., 1985; DeGraff, 1983; Florin et al., 1980; Gomes-Carneiro et al.,

2005; Haworth et al., 1983; Heck et al., 1989; Jagannath, 1984; Müller et al., 1993; National Toxicology Program, 2010; Rockwell and Raw, 1979). β-Myrcene was also negative for mutagenicity in *Escherichia coli* strain WP2 *uvr*A/pKM101 with and without S-9 metabolic activation (National Toxicology Program, 2010). β-Myrcene did not induce mutagenesis in *E. coli* WP2 IC185 and *E. coli* WP2 IC185 *oxyR* mutant IC202 at concentrations up to 1500 μg/plate (Mitic-Culafic et al., 2009) and did not induce DNA damage when incubated with human hepatoma HepG2 cells or human lymphoma NC-NC cells at concentrations up to 7.34 μM (Mitic-Culafic et al., 2009).

β-Myrcene was negative for genotoxicity in the SCE assay using human lymphocytes at concentrations up to 1000 μg/mL with and without metabolic activation (Kauderer et al., 1991) and in a separate SCE assay using V79 and hepatic tumor Chinese hamster cells at concentrations up to 500 μg/mL with and without metabolic activation (Röscheisen et al., 1991).

The lack of genotoxicity for β-myrcene has been confirmed in two *in vivo* studies: a chromosomal aberration (clastogenicity) assay in the bone marrow of male and female Wistar rats (2 or 4/sex/dose) at doses of 0, 100, 500 or 1000 mg/kg bw (Zamith et al., 1993); and a more recent micronucleus assay in the peripheral blood of male and female mice (5 or 2/sex/dose) 0, 250, 500, 1000 or 2000 mg/kg bw as part of an NTP study (National Toxicology Program, 2010).

7.5.3.3. α-Pinene and β-Pinene. No evidence of mutagenicity has been reported for α-pinene and β-pinene when tested in the Ames assay in several *S. typhimurium* strains (TA97, TA97a, TA98, TA100, TA1535, TA1537, TA1538, UTH8413, and UTH8414) and *E.* coli WP2 uvrA/pKM101 with and without S-9 metabolic activation at concentrations up to 100 μL/plate (85,800 μg/plate[7]) (Connor et al., 1985; DeGraff, 1983; Florin et al., 1980; Gomes-Carneiro et al., 2005; Haworth et al., 1983; Heck et al., 1989; Jagannath, 1984; Müller et al., 1993; National Toxicology Program, 2010; National Toxicology Program, 2016; Rockwell and Raw, 1979). A reverse mutation assay in *S. typhimurium* strains TA98 and TA100 showed no evidence of mutagenicity of potential urinary metabolites from SD rats treated by gavage with a single dose of 0.5 mL of α-pinene (1716 mg/kg bw) (Rockwell and Raw, 1979). In this assay, 24 h urine samples (500 μL), ether extracts of urine samples, and aqueous fractions of ether extracts, diluted in phosphate buffer containing β-glucuronidase (for hydrolysis of glucuronide conjugates) were separately incubated with *S. typhimurium* strains TA98 and TA100 with S-9 activation and were all negative for mutation induction (Rockwell and Raw, 1979). Consistently negative genotoxicity results were obtained for α-pinene in the UDS assay in rat hepatocytes at concentrations up to 10,000 μg/mL (Curren, 1988; Heck et al., 1989) and for β-pinene in an SCE assay in CHO cells at concentrations between 4.5 and 136.2 μg/mL (Anderson et al., 1990; Kauderer et al., 1991; Sasaki et al., 1989). In an *in vitro* assay, V79−Cl3 cells were exposed to α-pinene at concentrations of 0, 25, 30, 35, 40, 45 and 50 μM to evaluate its potential for cytotoxicity and genomic damage. DNA damage was detected in an alkaline comet assay in which mammalian V79−C13 cells were exposed to increasing concentrations, up to 35 μM α-pinene. Morphological analyses of exposed cells indicated mitotic alterations and chromosome breaks (Catanzaro et al., 2012). This result, however, was not confirmed by an *in vivo* micronucleus assay discussed below.

As part of a 14-week inhalation study in B6C3F$_1$ mice (10/sex/group), α-pinene was administered at concentrations of 0, 25, 50, 100, 200 or 4000 ppm for 6 h/day, 5 days/week. Peripheral blood samples showed no increase in the frequencies of micronucleated erythrocytes of significant changes in the percentages of polychromatic erythrocytes, indicating an absence of bone marrow toxicity (National Toxicology Program, 2016).

[7] Based on density of 0.858 g/mL.

7.5.3.4. β-Caryophyllene. No evidence of mutagenicity has been reported for β-caryophyllene when tested in the Ames assay in several *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 and *E. coli* WP2*uvrA* with and without S-9 metabolic activation at concentrations up to 150,000 μg/plate (Di Sotto et al., 2008; Heck et al., 1989; Jagannath, 1984). In two additional *in vitro* assays, no evidence of genotoxicity was found for β-caryophyllene in the UDS assay in rat hepatocytes at concentrations up to 10,000 μg/mL or in an SCE assays in CHO K-1 hamster cells at concentrations up to 333 μM (68,000 mg/mL) (Anderson et al., 1990; Kauderer et al., 1991; Sasaki et al., 1989).

In an *in vivo* micronucleus assay, male mice (5/sex/dose) were administered a single dose of 0, 20, 200 or 2000 mg/kg bw of β-caryophyllene in corn oil by gavage. No significant increase in the induction of micronucleated polychromatic erythrocytes in sampled blood was observed in any of the treatments groups (Molina-Jasso et al., 2009). In a follow up study, groups of male mice (5/sex/dose) were administered 0, 20, 200 or 2000 mg/kg bw β-caryophyllene by corn oil gavage for three consecutive days with blood sampled and smears for analysis prepared at 24, 48, 72 and 96 h post administration. There was no significant increase in miconucleated polychromatic erythrocytes observed (Molina-Jasso et al., 2009).

7.5.3.5. p-Mentha-1,4-diene. No evidence of mutagenicity has been reported for *p*-mentha-1,4-diene when tested in the Ames assay in several *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 with and without S-9 metabolic activation at concentrations up to 50,000 μg/plate (DeGraff, 1983; Heck et al., 1989). Similarly, no evidence of genotoxicity was found for *p*-mentha-1,4-diene in the UDS assay in rat hepatocytes at concentrations up to 30 μg/mL (Heck et al., 1989).

7.5.3.6. Other hydrocarbons. No evidence of mutagenicity has been reported for *p*-mentha-1,3-diene when tested in the Ames assay in several *S. typhimurium* strains (TA 97a, TA98, TA100 and TA1535) with and without S-9 metabolic activation at concentrations up to or 5000 μg/plate, respectively (Gomes-Carneiro et al., 2005). SCE assays in CHO cells for camphene, and α-phellandrene at concentrations up to 136.2, and 136.2 μg/mL, respectively showed no evidence for genotoxicity (Sasaki et al., 1989).

Overall, *Citrus* oils and the constituents of the major chemical group of hydrocarbons are consistently negative for genotoxicity. While no *in vivo* genotoxicity tests have been reported for *Citrus* oils, *in vitro* and *in vivo* genotoxicity assays of the major hydrocarbon constituents have confirmed the lack of genotoxicity for this group.

8. Recognition of GRAS status

The *Citrus* NFCs discussed here were determined to be GRAS under conditions of intended use as flavoring ingredients by the FEMA Expert Panel in 1965 and in subsequent years. Based on the safety evaluation described in this report, the FEMA Expert Panel has affirmed the GRAS status for the materials listed in Table 6.

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

As discussed earlier in the report, several of the *Citrus* flavoring materials evaluated have been folded, i.e. concentrated by fractional distillation to remove the monoterpenes, typically *d*-limonene, to produce a more concentrated *Citrus* oil. In commerce, *Citrus* oils of various folds are used as flavoring materials. In this evaluation, the FEMA Expert Panel evaluated and determined that the continuum of folded oils in use are GRAS under the conditions of their intended use as flavoring ingredients based on an evaluation of each NFC listed below and the constituents and congeneric groups therein.

FEMA 2530: Grapefruit Oil (*Citrus paradisi* Macf.)

FEMA 2625: Lemon Oil (*Citrus limon* (L.) Burm. F.)
FEMA 2631: Lime Oil, Distilled (*Citrus aurantifolia* (Christman) Swingle)
FEMA 2657: Mandarin Oil (*Citrus reticulata* Blanco)
FEMA 2821: Orange Essence Oil (*Citrus sinensis* (L.) Osbeck)
FEMA 2823: Orange Peel Bitter Oil (*Citrus aurantium* L.)
FEMA 2825: Orange Peel Sweet Oil (*Citrus sinensis* (L.) Osbeck)
FEMA 3041: Tangerine Oil (*Citrus reticulata* Blanco)

The FEMA GRAS *Citrus* flavoring materials listed in Tables 6 and 7 were evaluated using a rigorous procedure that considers the chemical composition, *per capita* intake, metabolic fate and toxicity of the identified constituents and potential toxicity and genotoxicity of unidentified constituents. This evidence provides reassurance of the safety profiles of these NFCs and together with their long history of safe use as flavoring agents supports their GRAS status.

9. Declaration of interests

Drs. Cohen, Eisenbrand, Fukushima, Gooderham, Guengerich, Hecht, and Rietjens, are members of the Expert Panel of the Flavor and Extract Manufacturers Association. Authors Bastaki, Davidsen, Harman, McGowen and Taylor are employed by Verto Solutions which provides scientific and management support services to FEMA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.052.

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