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FEMA GRAS assessment of natural flavor complexes: Asafetida oil, garlic oil and onion oil

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ABSTRACT

The Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) applies its procedure for the safety evaluation of natural flavor complexes (NFCs) to re-evaluate the safety of Asafetida Oil (*Ferula assa-foetida* L.) FEMA 2108, Garlic Oil (*Allium sativum* L.) FEMA 2503 and Onion Oil (*Allium cepa* L.) FEMA 2817 for use as flavoring in food. This safety evaluation is part of a series of evaluations of NFCs for use as flavoring ingredients conducted by the Expert Panel that applies a scientific procedure published in 2005 and updated in 2018. Using a group approach that relies on a complete chemical characterization of the NFC intended for commerce, the constituents of each NFC are organized into well-defined congeneric groups and the estimated intake of each constituent congeneric group is evaluated using the conservative threshold of toxicological concern (TTC) concept. Data on the metabolism, genotoxic potential and toxicology for each constituent congeneric group are reviewed as well as studies on each NFC. Based on the safety evaluation, Asafetida Oil (*Ferula assa-foetida* L.), Garlic Oil (*Allium sativum* L.) and Onion Oil (*Allium cepa* L.) were affirmed as generally recognized as safe (GRASa) under their conditions of intended use as flavor ingredients.

1. Introduction

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

A key part of FEMA's GRAS program is the cyclical re-evaluation of GRAS status of flavoring ingredients. GRAS flavoring ingredients may be chemically defined or complex mixtures known as natural flavor complexes (NFCs). The FEMA Expert Panel is currently conducting a multi-year project in which a safety evaluation is conducted on more than 250 FEMA GRAS NFCs. The scientifically-based procedure used in the safety evaluation was first published in 2005 (Smith et al., 2005) and updated in 2018 (Cohen et al., 2018a). This program, initiated in 2015, began with the evaluation of 54 NFCs derived from the *Citrus* genus that included orange, lemon, lime and grapefruit-derived NFCs (Cohen et al., 2019). A series of safety evaluations of NFCs derived from genera such as *Mentha*, *Cinnamonum*, *Eugenia, Lavandula*, *Eucalyptus* and *Origanum*, as

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Abbreviations

AST	Aspartate transaminase
CA	Chromosomal aberration
CF	Correction factor
CG	Congeneric group
CHO	Chinese hamster ovary (cells)
DTC	Decision tree class
EFFA	European Flavour Association
EFSA	European Food Safety Authority
FCC	Food Chemicals Codex
ERS/USDA	Economic Research Service/United States Department of Agriculture
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
FID	Flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GRAS	Generally recognized as safe
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
ip	intraperitoneal injection
LDH	Lactate dehydrogenase
LOD	Limit of detection
MoS	Margin of safety
NFC	Natural flavoring complex
NOAEL	No-observed-adverse-effect-level
OECD	Organization for Economic Co-Operation and Development
PCI	Per capita intake
RBC	Red blood cells
SCE	Sister chromatid exchange (assay)
TD50	Dose giving a 50% tumor incidence
TTC	Threshold of toxicological concern
WBC	White blood cells
WHO	World Health Organization

well as others, has been completed (Cohen et al., 2020, 2021; Eisenbrand et al., 2021; Fukushima et al., 2020; Gooderham et al., 2020b; Rietjens et al., 2020). The procedure for the safety evaluation requires a complete constituent characterization and usage data for each NFC. The constituents are organized into congeneric groups that are similar in chemical structure and share similar pathways of metabolism and detoxication. Information is gathered on estimated intake, metabolism, toxicity, including genotoxicity, for each constituent congeneric group and the threshold of toxicological concern (TTC) approach is applied (Kroes et al., 2000). In addition, an assessment of the potential toxicity, genotoxicity and intake of the fraction of unidentified constituents is a component of the procedure. In this manuscript, eighth in the series, the safety evaluations of Asafetida Oil (FEMA 2108), Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817), whose constituent profiles consist primarily of sulfides and other sulfur derivatives, are presented.

The FEMA Expert Panel issued a call for data requesting detailed constituent data for Asafetida Oil, Garlic Oil and Onion Oil. The most recent annual volume and estimated intake for these NFCs are provided in Table 1. Members from the International Organization of the Flavor Industry (IOFI), including FEMA, the Japan Fragrance and Flavor Materials Association (JFFMA), the European Flavour Association (EFFA), and the International Federation of Essential Oils and Aroma Trades (IFEAT), provided data on these *Ferula* and *Allium* derived NFCs that are currently used for flavoring food and beverage products.

Table 1
NFCs evaluated by the FEMA Expert Panel.

Name	FEMA No.	Estimated daily intake (μg/person/day)[a]	Most recent annual volume (kg)[b]
Asafetida Oil (*Ferula assa-foetida* L.)	2108	2	24
Garlic Oil (*Allium sativum* L.)	2503	280	26,300
Onion Oil (*Allium cepa* L.)	2817	260	2530

[a] For high volume materials (greater than 22,500 kg/year), the PCI per capita is shown. For materials with a lower surveyed volume (less than 22,500 kg/year, PCI * 10 ("eaters only') calculation is shown.

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

2. History of food use

Ferula assa-foetida L. is native to Iran, Turkey and Afghanistan, and is a strongly aromatic, oleoresin gum exuded by its roots which comprised a popular condiment in Persian cuisine known as "food of the gods" (Mahendra and Bisht, 2012; Uhl, 2000). Due to its strong flavor, it is typically diluted by mixing the resin with a starch or cereal, resulting in a form known as Hing (Attokaran, 2017; Ravindran et al., 2006). Hing is used to enhance the flavor of curries, meats, spice blends, condiments, sauces, nuts and legumes, marinades and pickles of Indian, Persian, Iranian and Afghani cuisine (Mahendra and Bisht, 2012; Ravindran et al., 2006; Uhl, 2000). Reported historical uses of asafetida in the United States include its use as an alcoholic hangover cure by cowboys, pioneers and homesteaders and as a flavor enhancer of seafood, meats and sauces (Owens, 1897). It is often an ingredient in Worcestershire sauce (Arctander, 1961; Ravindran et al., 2006; Uhl, 2000). The essential oil of asafetida is also used in nonalcoholic beverages, candy, various desserts, meats, condiments and other food categories in small quantities (Khan and Abourashed, 2010; Ravindran et al., 2006).

Of the many botanicals historically used to flavor and preserve food, garlic (*Allium sativum* L.) and onion (*Allium cepium* L.) have perhaps the longest known history of use (Pandley, 2006). Garlic is native to Asia and as early as 3000 BCE, garlic was a popular ingredient in various medicinal formulations for pulmonary and respiratory health in Indian Ayurveda as well as in the ancient Chinese, Japanese, Egyptian, Roman and Israelite civilizations (Petrovska and Cekovska, 2010; Rivlin, 2001). Garlic was also used in a famous medical formulation known as the "Four Thieves Vinegar" as a method to combat the Bubonic Plague in Europe (Henaut and Mitchell, 2018; Small, 2006). It was a staple spice and vegetable ingredient in seafood, meats, broths, cheeses and cakes of the ancient Roman, Greek, European, Asian and African civilizations and was an economical alternative for asafetida (Petrovska and Cekovska, 2010; Rivlin, 2001). While garlic was commonly consumed in the lower class populations, it was not known to be an important part of the diets of the more upper class peoples in these Mediterranean and European civilizations due to its strong aroma (Faas, 2005; Rivlin, 2001; Small, 2006). New World explorers introduced garlic to North America (Rivlin, 2001). Currently, China has the highest garlic production, followed by South Korea, India, Spain, Egypt, the United States, Indonesia and Tajikistan. California is the main supplier of garlic within the United States (Small, 2006; Uhl, 2000). Today, garlic is heavily used in European, Middle Eastern, Asian and Latin American cuisines in stir-fry, pastas, soups, salads, salad dressings, pickles, vinegars, sauces, aioli,

Table 2
Estimated intakes of garlic and onion oil from the consumption as food in 2015.

	Retail *per capita* (lbs/person)	Retail *per capita* (g/person)	Yield Essential Oil (%)	Essential oil *per capita* (g/person)	Estimated intake of essential oil from food (μg/person/day)	Consumption Ratio Estimated intake from food:Estimated intake as flavoring*
Onion	17.2	7800	0.02	1.6	4300	>16
Garlic	1.9	860	0.1	0.9	2300	>8

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

curries, vegetables, meats, seasonings and marinades, casseroles, breads, salts and spice blends and condiments in its fresh, dried or powdered forms (Small, 2006; Uhl, 2000). The essential oil is used to flavor stews and soups, frozen and baked desserts, candies, gums, beverages and to flavor meat and vegetarian dishes (Khan and Abourashed, 2010; Ravindran, 2017).

As early as 4000 BCE, onions were cultivated in Asia and the Middle East for use in food, medicine or spiritual worship (Havey, 1995; McCallum, 2007; Shaath and Flores, 1998). Onion was a staple ingredient in ancient Egyptian, Greek and Roman cuisine and spiritual offerings (Shaath and Flores, 1998; Toussaint-Samat, 2009). Onions were introduced to Native Americans in North America by European trade and colonization (Goldman et al., 2000). Commercial production of onion oil in the United States dates to the 1950s and was used to flavor meats, soups and sauces (Guenther, 1952). Currently, onions are produced in Egypt, Japan, North and South America, Europe, Southeast Asia, France and Mexico. In various ethnic cuisines, onions are widely used whole, diced or dehydrated in the preparation of various sauces, curries, stir-fries, satays, tumis, pastes, garnishing, sautés, roasts, stews, soups, spice blends, fillings, sauces, salads, pickles, condiments, sandwiches as well as vegetarian, seafood and meat dishes (Uhl, 2000). The flavoring use of the oil is reported to be in beverages, candy and desserts, meats, condiments, sauces, relishes, salad dressings and oils, soups, snacks and gravies (Khan and Abourashed, 2010).

3. Current usage

Of the three NFCs listed in Table 1, Garlic Oil (FEMA 2503) has the greatest 2015 annual volume of use, 26,300 kg. The annual volumes of Onion Oil (FEMA 2817) and Asafetida Oil (FEMA 2108) are significantly smaller, 2530 kg and 24 kg, respectively. Onion, garlic and asafetida are commonly used foods, although asafetida is less popular than onion and garlic in the USA. In the USA, the Economic Research Service of the United States Department of Agriculture (ERS/USDA) has compiled data on the total production and *per capita* availability of the supply of onion and garlic in the USA (ERS/USDA, 2015). The essential oil content of onion bulbs is approximately 0.02% and the essential oil content for fresh garlic bulbs is 0.1–0.25% (Fenaroli et al., 1975; Shaath and Flores, 1998; Uhl, 2000). Based on the ERS/USDA estimation of retail *per capita* availability of onion and garlic and conservative estimates of the percentage of essential oil in onion and garlic, estimated intakes of onion oil and garlic oil from the consumption of onion and garlic as food are calculated for 2015 and shown in Table 2.

Based on this analysis, the estimated intake from the consumption of onion oil from food is 4300 μg/person/day and significantly higher than the estimated intake of Onion Oil (FEMA 2817) used as added flavoring of 260 μg/person/day. For garlic, the estimated intake from the consumption of garlic oil from food is 2300 μg/person/day which is more than eight (8) times the estimated intake of Garlic Oil (FEMA 2503), 280 μg/person/day, used as added flavoring. Finally, although asafetida is commonly used in food in some cultures, data on its *per capita* availability in the marketplace to estimate its consumption are not available.

4. Manufacturing methodology

Asafetida is a natural resin exuded from the rhizome of the female plant when the overground parts of the plant are cut off (Arctander, 1961; Ravindran et al., 2006; Vacchiano, 1992). Plant resins are sometimes referred to as "gums" in commerce, although a gum is technically a polysaccharide mixture (Langenheim, 2003). Asafetida natural resin is a milky exudate that dries to a sticky, brown resinous consistency (Mahendra and Bisht, 2012). Approximately 1 kg may be obtained from 10 to 15 cycles of cutting and collection over a three month period (Attokaran, 2017; Ravindran et al., 2006). Steam distillation of the natural resin yields 7–9% essential oil (Arctander, 1961; Fenaroli et al., 1975; Sefidkon et al., 1998).

Garlic contains 0.1–0.25% essential oil (Fenaroli et al., 1975; Uhl, 2000). The clear yellow to red-orange volatile oil is obtained by steam distillation of crushed bulbs or cloves of garlic *Allium sativum* L. (Fam. Liliaceae) and has a strong, pungent garlic flavor and odor (FCC, 2021; Shaath et al., 1995). Similarly, the clear, amber yellow to amber orange volatile oil of onion, characterized by its strong, pungent aroma and taste, is obtained by steam distillation of the crushed bulbs of *Allium cepa* L. (Fam. Liliaceae). The volatile oil content of onion ranges from 0.015 to 0.02% (Fenaroli et al., 1975; Shaath and Flores, 1998).

5. Chemical composition

The compositions of Asafetida Oil (FEMA 2108), Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817) were analyzed, and their volatile

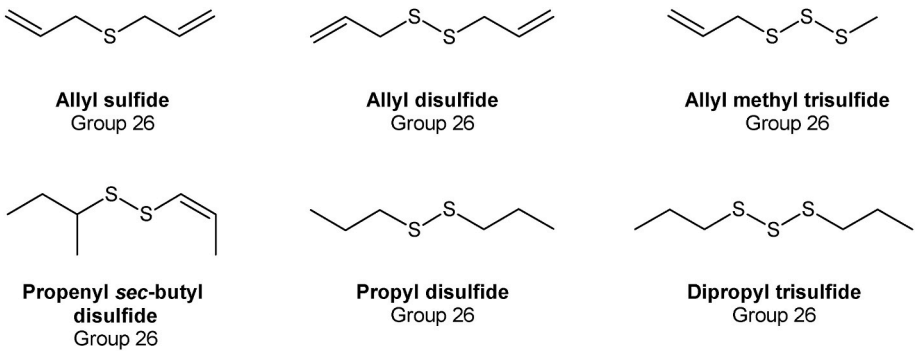


Fig. 1. Structures of commonly reported Group 26 (Aliphatic and aromatic sulfides and thiols) constituents.

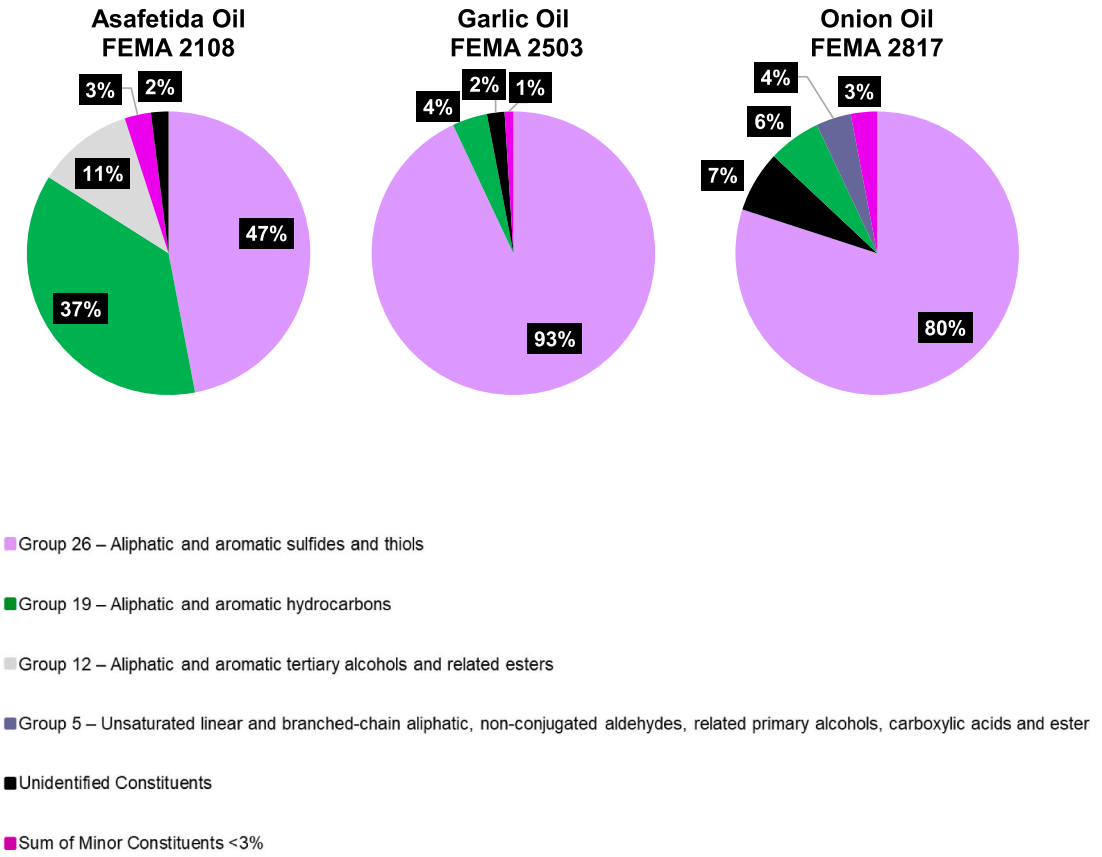
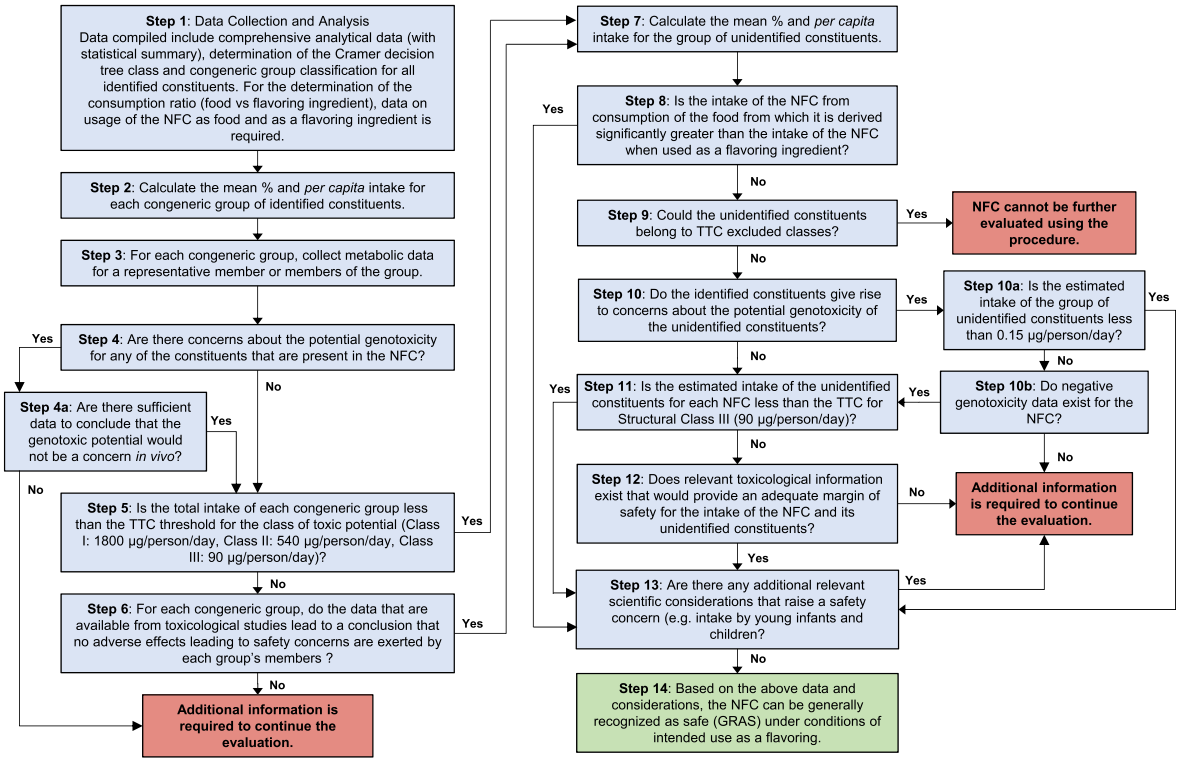


Fig. 2. Composition of the NFCs under consideration.



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

Fig. 3. Procedure for the safety evaluation of NFCs (Cohen et al., 2018a).

constituents were identified using gas chromatography-mass spectrometry (GC-MS). To quantify the chromatographic peaks, a similar gas chromatographic analysis was used employing a flame ionization detector (FID). Identified and unidentified GC peaks were reported as the percent area of the chromatogram. For each NFC, the constituent data were collected and analyzed (Appendix A). In Appendix A, the constituents present at greater or equal to 1% are listed by their respective congeneric groups. The chemical structures of some common constituents of these NFCs are shown in Fig. 1.

The constituent profile for each NFC, summarized in the pie charts shown in Fig. 2, all show a large percentage of Group 26 (Aliphatic and aromatic sulfides and thiols) constituents such as allyl sulfide, allyl disulfide, allyl methyl trisulfide, propenyl sec-butyl disulfide, propyl disulfide and dipropyl trisulfide. Group 19 (Aliphatic and aromatic hydrocarbons) is a major constituent congeneric group of Asafetida Oil (FEMA 2108). Minor constituent groups include Group 12 (Aliphatic and aromatic tertiary alcohols and related esters), Group 5 (Unsaturated linear and branched-chain aliphatic, non-conjugated aldehydes, related primary alcohols, carboxylic acids and esters) and Group 23 (aliphatic and aromatic ethers).

6. Safety Evaluation

The procedure for the safety evaluation for NFCs is guided by a set of criteria as outlined in two publications (Smith et al., 2004, 2005) with a recent update (Cohen et al., 2018a). The updated procedure with a more detailed evaluation of unidentified constituents inherent in NFCs is summarized in Fig. 3. 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, including genotoxicity, of the identified constituents by application of the threshold for toxicologic concern (TTC) approach and scientific data on metabolism and toxicity for each congeneric group; Steps 7-12 address the potential toxicity, including genotoxicity, of the unidentified constituents; in Step 13 the overall safety is evaluated along with considerations of safety for use by children, given their lower body weights; lastly in Step 14, the final determination of GRAS status is made. The safety evaluation is presented below in which each step of the procedure (Cohen et al., 2018a) (provided in italics) is considered and answered for the NFCs under consideration.

Step 1

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

a. Consumption of foods from which the NFCs are derived

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

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

PCI is calculated as follows:

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

where:

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

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

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

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

If applicable, estimate the intake resulting from consumption of the commonly consumed food from which the NFC is derived. The aspect of food use is particularly important. It determines whether intake of the NFC occurs predominantly from the food of which it is derived, or from the NFC itself when it is added as a flavoring ingredient (Stofberg and Grundschober, 1987).[1] *At this Step, if the conditions of use*[2] *for the NFC result in levels that differ from intake of the same constituents in the food source, it should be reported.*

The estimated intake for each NFC under consideration (Table 1) was calculated using the PCI × 10 method that assumes consumption of the annual usage by 10% ('eaters only') of the population. Asafetida resin and 'Hing', its powdered form, are widely used in Indian and Middle-Eastern dishes and spice blends for their garlic-onion flavor (Uhl, 2000). However, no quantitative data are available on the consumption of asafetida as a spice for the estimation of the intake of its essential oil from the consumption as food. In Table 2 above, the estimated intakes for both onion oil and garlic oil from the consumption as food was calculated based on the ERS/USDA estimation of retail *per capita* availability of onion and garlic in the USA in 2015 and conservative estimates of the percentage of essential oil in onion and garlic (ERS/USDA, 2015; Fenaroli et al., 1975; Shaath and Flores, 1998; Uhl, 2000). In Table 2, the estimated intake of Onion Oil (FEMA 2817) and Garlic Oil (FEMA 2503) from the consumption from food and added flavoring are shown. The estimated intake from the consumption of onion oil from food is 4300 μg/person/day and significantly higher than the estimated intake of Onion Oil (FEMA 2817) used as added flavoring of 260 μg/person/day. Similarly for garlic, the estimated intake from the consumption of garlic oil from food is 2300 μg/person/day which is more than eight (8) times the estimated intake of Garlic Oil (FEMA 2503), 280 μg/person/day, resulting from its use as added flavoring.

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

In this Step, the results of the complete chemical analyses for each NFC are examined, and where appropriate for each constituent the Cramer Decision Tree Class (DTC) is determined (Cramer et al., 1976).

The constituents for each NFC are organized by their respective congeneric groups in Appendix A. The congeneric groups are listed in order of decreasing mean %. Only constituents with a mean % greater or equal to 1% of the total NFC are included. Minor constituent amounts (<1% of the total NFC) are grouped together under each of the listed congeneric groups and the total mean % for each listed congeneric group is reported.

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

Proceed to Step 2.

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

Step 2

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

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

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

where:

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

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

Proceed to Step 3.

In the summary report for each NFC provided in Appendix A, the

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

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

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

Table 3
Natural occurrence and estimated intake of methyl eugenol in Asafetida Oil (FEMA 2108).

Name (FEMA No.	Constituent of Concern	Mean %	Estimated Intake (μg/person/day)
Asafetida Oil (FEMA 2108)	Methyl eugenol	0.03	0.0007

total mean % for each congeneric group is subtotaled and reported with the DTC and intake (PCI x 10 or PCI, as appropriate) for each group.

Step 3

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

Proceed to Step 4.

For each NFC reported in Appendix A, the constituent congeneric groups are listed. For each congeneric group, sufficient data on the metabolism of constituents or related compounds exist to conclude that members of the respective groups are metabolized to innocuous products. The use of metabolic data in the safety evaluation of flavoring compounds and a summary of the expected metabolism of flavoring compounds by congeneric group is described in a FEMA Expert Panel publication (Smith et al., 2018). For Group 26 (Aliphatic and aromatic sulfides and thiols) constituents, the metabolism of aliphatic and aromatic sulfides and thiols was reviewed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2000, 2008, 2011). The FEMA Expert Panel has published a safety assessment for thiophene flavoring ingredients that includes a discussion of their metabolism (Cohen et al., 2017). In addition, the FEMA Expert Panel has reviewed metabolism data for Group 19 (Aliphatic and aromatic hydrocarbons) and Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) constituents as part of their safety evaluations of flavoring ingredients of these congeneric groups (Adams et al., 2011; Marnett et al., 2014a).

Step 4

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

If Yes, proceed to Step 4a.

If No, proceed to Step 5.

Group 26 (Aliphatic and aromatic sulfides and thiols) constituents, the major constituent group for Asafetida Oil (FEMA 2108), Onion Oil (FEMA 2817) and Garlic Oil (FEMA 2503), do not present a genotoxic concern based on an analysis by JECFA (JECFA, 2000, 2008, 2011). In its review of *in vitro* and *in vivo* genotoxicity studies for Group 19 (Aliphatic and aromatic hydrocarbons) and Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) flavoring ingredients, the FEMA Expert Panel determined a lack of genotoxic potential for these and related compounds (Adams et al., 1996; Cohen et al., 2019; Fukushima et al., 2020; Marnett et al., 2013). In addition, the minor constituents have also been reviewed and raise no concern for genotoxic potential. Genotoxicity studies on the NFCs described later in the "Biochemical and Toxicological Supporting Information Relevant to the Safety Evaluation" section of this manuscript, also indicate no concern.

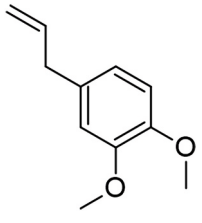


Fig. 4. Structure of methyl eugenol.

Low concentrations of naturally occurring methyl eugenol were reported in Asafetida Oil (FEMA 2108). Methyl eugenol is an allylalkoxybenzene, a structural motif that raises a concern for genotoxicity. The mean % and estimated intake for methyl eugenol is summarized in Table 3 and its occurrence in Asafetida Oil (FEMA 2108) is further evaluated in Step 4a. Similar compounds were not reported in Onion Oil (FEMA 2817) or Garlic Oil (FEMA 2503) and these NFCs proceed to Step 5.

Step 4a

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

If Yes, proceed to Step 5.

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

The structure of methyl eugenol (see Fig. 4) has a motif of a phenyl ring substituted with an alkoxy group located *para* to a 2-propenyl substituent. Allylalkoxybenzene compounds, such as methyl eugenol, are capable of forming DNA adducts upon bioactivation by cytochrome P450s catalyzing the formation of a 1′-hydroxy metabolite followed by sulfation at this site by a sulfotransferase. Elimination of sulfate from the 1′-sulfoxy metabolites creates a DNA reactive species (Daimon et al., 1997; Herrmann et al., 2012, 2014; Jeurissen et al., 2004, 2007; Phillips et al., 1984; Randerath et al., 1984; Rietjens et al., 2005, 2014; Ueng et al., 2004; Wiseman et al., 1987). Rodent studies have indicated methyl eugenol is hepatocarcinogenic at high dose levels (NTP, 2000).

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

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

Table 4
Data on Group 26 constituents for Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817) where intake of the Congeneric Group exceeds the respective TTC.

Name (FEMA No.)	DTC	Estimated intake of CG (μg/person/day)	Estimated intake of CG (mg/kg bw/day)	NOAEL (mg/kg bw/day)[a]	MoS
Garlic Oil (FEMA 2503)	III	260	0.0044	>6	>1300
Onion Oil (FEMA 2817)	III	210	0.0035	>6	>1500

[a] The NOAEL was derived from the highest dose tested in an OECD guideline 90-day repeat dose toxicity study in which methyl propyl trisulfide was administered to male and female rats by oral gavage (Koetzner, 2016).

Table 5
Estimated intake of unidentified constituents.

Name	FEMA No.	Estimated Intake of Unidentified Constituents (μg/person/day)
Asafetida Oil (*Ferula assa-foetida* L.)	2108	0.04
Garlic Oil (*Allium sativum* L.)	2503	8
Onion Oil (*Allium cepa* L.)	2817	19

flavored with flavorings and food ingredients in which these constituents occur naturally (European Commission, 2008).

The estimated intake of methyl eugenol from the use of Asafetida Oil (FEMA 2108) as a flavoring ingredient is 0.0007 μg/person/day. This value is below the TTC of 0.15 μg/person/day for compounds with structural alerts for genotoxicity, as originally stated by (Kroes et al., 2004). This value was determined based on an analysis of the dose-response data for carcinogenic compounds, provided by the Gold database of carcinogens[4] presenting the dose giving a 50% tumor incidence (TD_{50}) (Gold et al., 1984; Kroes et al., 2004). By linear extrapolation of these TD_{50} data to a dose resulting in a 1 in 10^6 tumor incidence, an exposure level or TTC at which the lifetime risk of cancer was less than 1 in 10^6 was determined to be 0.15 μg/person/day (Kroes et al., 2004). In a recent EFSA/WHO review of the TTC approach, a 0.15 μg/person/day threshold was proposed and considered sufficiently protective for compounds with structural alerts for genotoxicity with the exclusion of high potency carcinogens (the Cohort of Concern) specified by Kroes and co-workers (EFSA, 2016; Kroes et al., 2004; Nohmi, 2018). Asafetida Oil (FEMA 2108) proceeds to Step 5.

Step 5

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

If Yes, proceed to Step 7.

If No, proceed to Step 6.

The estimated intakes for the constituent congeneric groups of Asafetida Oil (FEMA 2108) are less than the TTC for their respective decision tree class. Asafetida Oil (FEMA 2108) proceeds to Step 7. For Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817), the estimated intake of Group 26 (Aliphatic and aromatic sulfides and thiols) constituents exceeds the TTC for their structural class and are further evaluated 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.

As summarized in Table 4, MoS for Group 26 (Aliphatic and aromatic sulfides and thiols) constituents of greater than 1300 and greater than 1500 were calculated for Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817), respectively. These MoS were calculated based on a no-observed-adverse-effect-level (NOAEL) of 6 mg/kg bw/day from an OECD guideline-compliant 90-day repeated dose toxicity study in male and female rats administered methyl propyl trisulfide (FEMA 3308) by oral gavage (Bastaki et al., 2018; Koetzner, 2016). In this study, the NOAEL, 6 mg/kg bw/day, was the highest dose tested. In the section 'Biochemical and Toxicological Supporting Information Relevant to the Safety Evaluation', a review of this study and the two range-finding studies conducted prior to the performance of this 90-day oral gavage study are discussed. An initial dietary range finding study determined that administration of methyl propyl trisulfide in the feed resulted in environmental issues and palatability problems that made administration in the feed impractical (Bauter, 2015b). This study was followed by a 14-day range finding oral gavage study at much lower doses (Bauter, 2015a) in which hemolytic effects were noted at the 12.5 mg/kg bw/day dose level of methyl propyl trisulfide. Based on these studies, the true NOAEL is expected to be greater than 6 but less than 12.5 mg/kg bw/day methyl propyl trisulfide. The MoS for Group 26 constituents of greater than 1300 calculated for Garlic Oil (FEMA 2503) and greater than 1500 for Onion Oil (FEMA 2817, based on a NOAEL of 6 mg/kg bw/day for methyl propyl trisulfide are therefore conservative values. A NOAEL for garlic oil of greater than 50 mg/kg bw/day was recently reported for a 28-day study in male and female ICR mice (Lin et al., 2022). Based on this NOAEL, an MoS of greater than 10,000 was calculated for Garlic Oil (FEMA 2503). 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.

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

Proceed to Step 8.

Appendix A details the mean % for the group of unidentified constituents and the *per capita* intake for each NFC. The estimated intake of the group of unidentified constituents for each NFC is summarized below in Table 5.

Step 8

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

If Yes, proceed to Step 13.

If No, proceed to Step 9.

As summarized in Step 1 and Table 2, the consumption of onion and garlic oil from food is much greater than the consumption of Onion Oil (FEMA 2817) and Garlic Oil (FEMA 2503) used as added flavoring. These NFCs, therefore, proceed to Step 13. Due to a lack of quantitative data on the use of asafetida natural resin as a spice, the consumption of asafetida is presumed to be primarily as added flavoring and as a result, the evaluation of Asafetida Oil (FEMA 2108) proceeds to Step 9.

Step 9

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

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

If No, proceed to Step 10.

No, members of the TTC excluded classes are not likely to be present in Asafetida Oil (FEMA 2108) and the evaluation of this NFC proceeds to Step 10. Based on the identified constituents, the unidentified fraction is most likely to be comprised of unidentified sulfides and terpenoids. For the materials that are prepared by distillation in which only the volatile fraction is used, the presence of the substances from the TTC excluded classes is unlikely. In addition, over the long history of use of these substances, there have not been any reports of constituents or contaminants of concern in Asafetida Oil (FEMA 2108).

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.

These NFCs are primarily constituted of Group 26 (Aliphatic and aromatic sulfides and thiols), Group 19 (Aliphatic and aromatic hydrocarbons) and Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) that are not genotoxic. In Step 4, methyl eugenol was reported to occur in small amounts in Asafetida Oil (FEMA 2108). The estimated intake of this constituent was estimated to be less than the TTC for compounds with structural alerts for genotoxicity of 0.15 μg/person/day and thus does not raise a safety concern. Allylalkoxybenzene compounds such as estragole, methyl eugenol, safrole, elemicin and myristicin are represented in the current mass spectral libraries and are readily detected and identified by GC-MS. These compounds may be part of the unidentified fraction at concentrations below the respective limit of detection (LOD). Depending on the analytical method employed to collect the data contributing to this safety evaluation, the LOD is estimated to be 0.01–0.1% of the NFC. The estimated intake of an unidentified constituent occurring at the upper end of this range, at a concentration of 0.1%, in Asafetida Oil (FEMA 2108) is 0.002 μg/person/day, which is less than the TTC of 0.15 μg/person/day for compounds with structural alerts for genotoxicity and thus does not raise a safety concern. A review of available genotoxicity and toxicological studies on the NFCs under consideration are presented later in the manuscript. These studies reported no evidence of genotoxic potential. Based on these data, it is concluded that the unidentified constituents in Asafetida Oil (FEMA 2108) do not raise a concern for genotoxicity. Asafetida Oil (FEMA 2108) proceeds to Step 11.

Step 10a

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

If Yes, proceed to Step 13.

If No, proceed to Step 10b.

Not required.

Step 10b

Do negative genotoxicity data exist for the NFC?

If Yes, proceed to Step 11.

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

Not required.

Step 11

Is the estimated intake of the unidentified constituents (calculated in Step 7) less than the TTC (Kroes et al., 2004; Munro et al., 1996) for Structural Class III (90 μg/person/day)?[7]

If Yes, proceed to Step 13.

If No, proceed to Step 12.

Yes, as shown in Table 5, the estimated intake of the unidentified constituents of Asafetida Oil (FEMA 2108) is less than the Structural Class III TTC of 90 μg/person/day and the evaluation of this NFC proceeds to Step 13.

Step 12

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

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

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

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

Table 6
Summary of genotoxicity assay data for NFCs and Group 26 constituents of NFCs.

Test Substance	Test Type (System)	Doses Tested	Results	Reference
Sulfur derivative constituents				
In vitro				
Allyl disulfide	Bacterial reverse mutation assay *Salmonella typhimurium* TA100[a]	1.5–150 μg/mL[c]	Negative[a]	Eder et al. (1980)
Allyl disulfide	*In vitro* chromosome aberration assay - Chinese Hamster Ovary (CHO) cells[a]	2–25 μg/mL	Positive[a,b]	Musk et al. (1997)
Allyl disulfide	Sister Chromatid Exchange CHO Cells[a]	2–10 μg/mL	Positive[a,b]	Musk et al. (1997)
Allyl sulfide	Bacterial reverse mutation assay *Salmonella typhimurium* TA100[a]	4–450 μg/mL[c]	Negative[a]	Eder et al. (1980)
Allyl sulfide	*In vitro* chromosome aberration assay CHO cells[a]	200–600 μg/mL	Positive[a,b]	Musk et al. (1997)
Allyl sulfide	Sister Chromatid Exchange CHO Cells[a]	200–600 μg/mL	Positive[a,b]	Musk et al. (1997)
37% Allyl propyl disulfide, 31% propyl disulfide and 32% allyl disulfide	Bacterial reverse mutation assay *S. typhimurium* TA100[a]	1.5–150 μg/ml[c]	Negative[a]	Eder et al. (1980)
Allyl propyl disulfide	Bacterial reverse mutation assay *S. typhimurium* TA97, TA98, TA100, TA1535 and TA1537[a]	0–333 μg/plate	Negative[a]	Zeiger et al. (1988)
In vivo				
Diallyl thioethers(68.1% diallyl disulfide; 19.7 diallyl sulfide; 12.2% diallyl trisulfide)	*In vivo* micronucleus assay Male ICR/C3H mice	32.8, 66.7 mmol/kg bw	Negative	Marks et al. (1992)
Natural flavor complexes				
In vitro				
Alcoholic extract of Asafetida	Bacterial reverse mutation assay – Streptomycin-dependent *S. typhimurium* TA98	5000-50,000 μg/plate	Weakly positive[d]	Shashikanth and Hosono (1986)
Asafetida Extract	Bacterial reverse mutation assay –*S. typhimurium* TA100 and TA1535	25,000, 50,000 μg/plate	Negative[d]	Soudamini et al. (1995)
Garlic oil	Bacterial reverse mutation assay *S. typhimurium* TA98, TA 100, TA1535, TA1537 and TA1538[a]	5–2000 μg/plate	Negative[a]	Hachiya et al. (1983)
Garlic oil	Bacterial reverse mutation assay - *S. typhimurium* TA100[a]	62.5–500 μg/plate	Negative[a]	Park (2002)
Garlic oil	Bacterial reverse mutation assay *S. typhimurium* TA98, TA 100, TA1535 and TA1537[a]	0.5–1000 μg/plate	Negative[a]	Lin et al. (2022)
Garlic oil	Rec assay – *B. subtilis* H17 Rec+, M45 Rec-[a]	1,3 and 5 mg/disk	Negative[a]	Ueno et al. (1983)
Garlic oil	Rec assay – *B. subtilis* H17 Rec+, M45 Rec-[a]	5 mg/disk	Negative[a]	Hachiya et al. (1983)
Garlic oil	*In vitro* chromosome aberration assay in Chinese hamster ovary cells (CHO–K1)[a]	0, 1, 1.5 2.5, 5 and 10 μg/mL	Negative[a,e]	Lin et al. (2022)
Onion oil	Bacterial reverse mutation assay – *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538[a]	1 - 50,000 μg/plate	Negative[a]	Hachiya et al. (1983)
95% ethanol extract of powdered onion	Bacterial reverse mutation assay –*S. typhimurium* TA98 and TA102[a]	10,000 μg/plate	Negative[a]	Mahmoud et al. (1992)
Aqueous and 99% ethanol extract of sliced and homogenized onion	Bacterial reverse mutation assay – *S. typhimurium* TA98 and TA100[a]	0–10 mg/ml	Negative[a]	Martínez et al. (1999)
Onion oil	Rec assay – *B. subtilis* H17 Rec+, M45 Rec-[a]	Max. 10 mg/disk	Equivocal[a]	Hachiya et al. (1983)
Onion oil	Rec assay – *B. subtilis* H17 Rec+, M45 Rec-[a]	10 mg/disk	Equivocal[a]	Ueno et al. (1984)
Onion oil	*In vitro* chromosome aberration assay in Chinese hamster fibroblasts	0.04 mg/ml	Equivocal	Ishidate et al. (1984)
In vivo				
Asafetida powder suspended in saline solution	*In vivo* Sister chromatid exchange assay – C57Bl/6 mice	0, 500 and 1000 mg/kg bw	Positive	Abraham and Kesavan (1984)
Garlic oil	*In vivo* comet assay – Male mice (5/dose)	0.25, 0.5 ml	Negative	Kaur and Singh (2007)
Garlic oil	*In vivo* micronucleus assay – male ICR mice	0, 15, 25, 50 mg/kg bw	Negative	Lin et al. (2022)
Onion oil	*In vivo* micronucleus assay – male ddY mice	0, 250, 500, 1000 mg/kg bw	Negative	Hayashi et al. (1988)

[a] With and without S9 metabolic activation system.
[b] Positive result correlated to cytoxicity.
[c] Modified suspension assay used.
[d] Without S9 metabolic activation.
[e] Same concentrations used in both 3 h and 20 h experiments.

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

If Yes, proceed to Step 13.

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

Not required.

Step 13

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

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

If No, proceed to Step 14.

The FEMA Expert Panel concurs with other food ingredient safety evaluation bodies that the TTC is applicable to the entire population (EFSA, 2012, 2016), when taking the lower body weight of children into account. An evaluation to consider possible exposure of children, given their lower body weights, and the potential for toxicokinetic and toxicodynamic differences as compared to adults, was conducted for each NFC under consideration. The NFCs under consideration would not be added to foods specifically consumed by infants (CAC, 2007; CAC, 2017; CAC, 2019), indicating that exposure of infants is not expected. For Asafetida Oil (FEMA 2108) the total estimated intake for each of the congeneric groups present in the NFC is at least 70-fold below the corresponding TTC for the group, with none close to the TTC threshold,

indicating no concern for consumption by children. Low concentrations of methyl eugenol, which has a potential genotoxicity concern, were reported in Asafetida Oil (FEMA 2108) but its estimated intake is at least 200-fold below the TTC for compounds with structural alerts for genotoxicity of 0.15 μg/person/day, indicating no safety concern for children.

For Onion Oil (FEMA 2819) and Garlic Oil (FEMA 2503), the TTC was exceeded for Group 26 constituents. In Step 6, an MoS for Group 26 constituents of greater than 1300 and greater than 1500 were calculated for Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817), respectively, based on a body weight of 60 kg and a NOAEL of 6 mg/kg bw/day, the highest dose level tested, from an OECD guideline-compliant 90-day repeat dose toxicity study in which male and female rats were administered methyl propyl trisulfide (FEMA 3308) by gavage (Bastaki et al., 2018; Koetzner, 2016). These MoS values for both Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817) are considered also adequate for children, considering their lower body weights. The estimated intakes for the other constituent congeneric groups of Onion Oil (FEMA 2819) and Garlic Oil (FEMA 2503) are at least 4-fold below the corresponding TTC for the group, with none close to the TTC threshold, indicating no safety concern for the consumption by children of these NFCs when used as flavoring in food.

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 assessment performed for Asafetida Oil (FEMA 2108), Onion Oil (FEMA 2819) and Garlic Oil (FEMA 2503), the FEMA Expert Panel affirms these NFCs as "generally recognized as safe" under intended conditions of use as flavoring ingredients.

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

The major constituent congeneric group for Asafetida Oil (FEMA 2108), Onion Oil (FEMA 2819) and Garlic Oil (FEMA 2503) is Group 26 (Aliphatic and aromatic sulfides and thiols). As compiled in Appendix A, reported Group 26 constituents of these NFCs contain a large number of mono-, di- and trisulfides, whose safe use as flavoring ingredients has been reviewed by JECFA (JECFA, 2000, 2008, 2011). The Panel has also reviewed the safety of flavoring ingredients of other congeneric groups represented in the constituent profiles of these NFCs including Group 19 (Aliphatic and aromatic hydrocarbons) and Group 12 (Aliphatic and aromatic tertiary alcohols and related esters) (Adams et al., 2011; Cohen et al., 2019; Fukushima et al., 2020; Marnett et al., 2014b). The additional information presented here includes studies on the NFCs themselves, studies on the principal constituents of these NFCs and newly available studies on constituents not considered within the reviews mentioned above. Studies concerning genotoxicity are summarized in Table 6.

7.1. Allyl disulfide (FEMA 2028)

7.1.1. Genotoxicity

No evidence of mutagenicity was observed in a reverse mutation assay using a liquid suspension test system, when allyl disulfide was tested in *Salmonella typhimurium* strain TA100 at concentrations of 1.5–150 μg/mL[8] in the absence and presence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats (Eder et al., 1980).

In an *in vitro* chromosome aberration (CA) assay, allyl disulfide was tested at concentrations ranging from 2 to 25 μg/mL in Chinese Hamster Ovary (CHO) cells in both the absence and presence of S9 metabolic activation. Increases in CAs were observed at concentrations of 10 μg/mL and higher in the absence of S9 and in a non-dose responsive manner at concentrations of 4–25 μg/mL in the presence of S9 (Musk et al., 1997). In a sister chromatid exchange (SCE) assay in CHO cells, a small dose dependent increase in the induction of SCEs was observed with allyl disulfide in the concentration range 2–10 μg/mL in both the presence and absence of S9 metabolic activation. In both assays, allyl disulfide was tested at concentrations that induced greater than 50% cytotoxicity in the CHO cells, which likely resulted in false positive assessments (Musk et al., 1997). The *in vitro* SCE study guidance has been removed from the OECD reference library due to the current lack of evidence that this test is predictive of a heritable mutagenic event (OECD, 2015).

7.2. Allyl sulfide (FEMA 2042)

7.2.1. Genotoxicity

Allyl sulfide (97%) was not mutagenic in a reverse mutation assay in *S. typhimurium* strain TA100 at concentrations ranging from 4 to 450 μg/mL[9] using a modified liquid suspension test system both with and without S9 prepared from Aroclor 1254- induced rat liver (Eder et al., 1980).

In an *in vitro* CA assay, allyl sulfide was tested at concentrations of 200–600 μg/mL in CHO cells in the absence or presence of rat liver S9 metabolic activation system. An increase in chromosome aberrations was observed at concentrations of 200 μg/mL and above in the absence of S9 metabolic activation and at concentrations of 300 μg/mL and above in the presence of S9 metabolic activation (Musk et al., 1997). In an SCE assay in CHO cells, a dose-dependent induction of SCEs was observed at concentrations of 300 μg/mL allyl sulfide and above in the presence and absence of S9 metabolic activation. In both assays, allyl sulfide was tested at concentrations that induced greater than 50% cytotoxicity in the CHO cells at all tested concentrations in the presence of S9 metabolic activation at 400 μg/mL and above in the absence of S9 (Musk et al., 1997) that may have resulted in positive results in the CA and SCE assays. This study is not considered relevant to the safety evaluation due to the high cytotoxicity in these assays and in addition, the *in vitro* SCE guidance has been removed from the OECD reference library due to the current lack of evidence that this test is predictive of a heritable mutagenic event (OECD, 2015).

7.3. Diallyl trisulfide (FEMA 3265)

7.3.1. Subchronic toxicity

In a 90-day single dose dietary study, diallyl trisulfide was provided in the diet to albino weanling FDRL rats (15/sex) at doses of 0 (control) and 4.16 mg/kg bw/day. The rats were observed daily for survival, behavior and physical appearance. Weekly measurements of body weight and food consumption were recorded. At weeks six and twelve, blood samples were drawn for clinical chemistry and hematology analysis and urine analyses were performed on 8 male and 8 female animals from each group. All test animals survived the treatment period and at the end of the study, all animals were terminated. At termination, the liver and kidney weights were recorded and histopathological examinations of the major organs and tissues were conducted. There were no signs of clinical toxicity or differences in body weight gains, food consumption, survival, hematology, clinical chemistry, urine analysis parameters or liver and kidney weights between the male and female

[8] Calculated using the average density of 1.007 g/mL (Source: Joint FAO/WHO Expert Committee on Food Additives (JECFA) http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-flav/details/en/c/145/).

[9] Calculated using the average density of 0.890 g/mL (Source: Joint FAO/WHO Expert Committee on Food Additives (JECFA) http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-flav/details/en/c/105/).

test groups and the control group. Incidental necropsy and histopathology findings included instances of intercurrent infections evidenced by perivascular or interstitial mononuclear cell infiltration of the lung, liver and kidneys in one animal in the control and test groups. Incidental necropsy and histopathology findings included instances of intercurrent infections evidenced evidenced by perivascular or interstitial mononuclear cell infiltration of the lung, liver and kidneys in one animal in the control and test groups. Macroscopic black and dark red areas of the lungs and hydronephrosis of the kidney were reported in both the control and treated rats. These effects were not related to administration of the diallyl trisulfide (Morgareidge and Oser, 1970a). However, the presence of infection with pneumonia in the animals in this study limits the usefulness for an overall risk assessment and conclusions on the test material cannot be drawn (NRC,2011; OECD, 2018).

7.4. Propyl disulfide (FEMA 3228)

7.4.1. Subchronic toxicity

In a 90-day dietary toxicity study, Sprague Dawley rats (14/sex/group) were administered propyl disulfide (FEMA 3228; 99% purity) emulsified with gum arabic in an aqueous solution and added to food at concentrations of 0.0060% (weeks 0–4), 0.0102% (weeks 5–10) and 0.0120% (weeks 11–13). The study authors calculated that the average dose amounts were 7.29 mg/kg bw/day for males and 8.12 mg/kg bw for females in the test group. Behavior, physical appearance, body weights, absolute and relative weights of the liver and kidneys, hematological parameters with exception of the blood urea levels, which were elevated, were not significantly different in the test subjects compared to the control group. Histopathological examinations revealed non-statistically significant findings of interstitial inflammatory lesions in the renal cortex and lymphohistiocytic infiltrations in the liver in control and test rats. No test-substance related adverse clinical, hematological or histopathological effects were found during the study period (Posternak et al., 1967, 1969).

7.5. Dipropyl trisulfide (FEMA 3276)

7.5.1. Subchronic toxicity

In a 90-day dietary toxicity study, dipropyl trisulfide was provided in the diet to albino weanling FDRL rats (15/sex) at doses of 0 (control) and 4.16 mg/kg bw/day. The rats were observed daily for survival, behavior and physical appearance. Weekly measurements of body weight and food consumption were recorded. At weeks six and twelve, blood samples were drawn for clinical chemistry and hematology analysis and urine analyses were performed on 8 male and 8 female animals from each group. At the end of the study, all control and treatment group rats were terminated and necropsied. The liver and kidney weights were recorded. No statistically significant differences were reported in the parameters studied. Necropsy and histopathology revealed incidental findings such as infrequent instances of intercurrent infections evidenced by perivascular or interstitial mononuclear cell infiltration of the lung, liver and kidneys, macroscopic black spots and dark red areas of the lungs and hydronephrosis of the kidneys were reported in control and treated rats. These effects were not attributed to administration of dipropyl trisulfide (Morgareidge and Oser, 1970b). Since the animals' general health for both test and control groups was compromised by an unspecified pathogen affecting the lungs and kidneys, no NOAEL could be determined for this study (NRC,2011; OECD, 2018).

7.6. Methyl propyl trisulfide (FEMA 3308)

7.6.1. Range-finding studies

In an initial dietary range-finding study, CRL Sprague-Dawley (SD) CD® IGS rats (5/sex/dietary level) were administered methyl propyl trisulfide at levels of 1800, 3600 or 7200 ppm in the feed for 14 days (Bauter, 2015b). Based on the stability data, weekly dietary refreshment and measured daily intake, the adjusted mean daily intakes were calculated to be 98, 180 and 343 mg/kg bw/day, respectively, for males and 98, 192 and 349 mg/kg bw/day, respectively, for females. This study was met with laboratory environmental concerns due to the volatility and odor of the test substance and decreased palatability of the feed. In this study, feed consumption was reduced by up to 25% at the top concentration in both sexes. At the highest dose, there was a reduction in food efficiency (52% and 60% for males and females, respectively) and body weights (60 and 70% for males and females, respectively) (Bastaki et al., 2018). The rats also had enlarged spleens at the highest dose. Based on these effects, a second range-finding study was conducted in which methyl propyl trisulfide was orally administered, by gavage, to CRL SD CD® IGS rats (5/sex/group) at doses of 0 (vehicle control), 12.5, 50 or 100 mg/kg bw/day for 14 days (Bauter, 2015a). In this second range-finding study, there were no clinical observations or changes in body weight, body weight gain, mean daily food consumption or food efficiency in the treatment groups compared to the control group. In the mid- and high dose groups, splenic enlargement and/or dark red discoloration of the spleen were observed in all animals with microscopic findings of increased spleen iron deposits and evidence of increased splenic erythropoiesis. These findings also occurred in individual low dose male and female rats. Based on these results, the maximum tolerated dose for a 90-day study was estimated to be the lowest dose tested, 12.5 mg/kg bw/day.

7.7. Subchronic toxicity study

In an OECD guideline 90-day oral gavage toxicity study, methyl propyl trisulfide (purity, 57% methyl propyl trisulfide with secondary components originating from intramolecular rearrangement identified as 32% dipropyl trisulfide, 6.4% dipropyl disulfide and 4.3% methyl isopropyl tetrasulfide) was administered to male and female CRL SD CD® IGS rats (10/sex/dose) at 0 (vehicle control), 0.5, 2 or 6 mg/kg bw/day by corn oil gavage. These doses were based on a dose range-finding study in the same strain of rats, discussed above, where the maximum tolerated dose was observed to be less than 12.5 mg/kg bw/day based on the occurrence of hemolytic events at higher doses (Bauter, 2015a).

No mortalities or significant clinical, body weight, food consumption, food efficiency or ophthalmological differences were observed between the test and control groups. Clinical findings were incidental and considered transient and not toxicologically relevant. Slight but statistically significant decreases in red blood cell counts were observed in high dose females without histological correlate in the spleen or bone marrow and therefore considered not toxicologically relevant. Minimal decreases in red blood cell count, hemoglobin and hematocrit parameters were within historical controls and were considered to be non-adverse findings. In high dose males, a decrease in mean corpuscular volume and an increase in absolute reticulocyte counts were within historical controls and were considered to be non-adverse. No significant changes in coagulation, clinical chemistry or urinalysis parameters were observed between the control and test groups, although an incidental decrease of sorbitol dehydrogenase values was observed in males in the mid- dose group and an incidental decrease in serum calcium concentration was observed in the males in the low dose group (Koetzner, 2016).

No toxicologically relevant macroscopic and microscopic findings related to the test substances were observed. The uteruses contained fluid in three test and one control group animals that corresponded microscopically to luminal dilation related to the variation in estrous cycles. There was minimal to moderate ovarian atrophy with large follicular structures and reduced or absent corpora lutea, and the appearance of secondary reproductive tissues indicated early estrus in these animals. These findings, in conjunction with persistent estrus, were indicative of reproductive senescence (Shirai et al., 2015). Macroscopic ear lesions in male and female control, mid- dose and high dose rats corresponded with microscopic findings of auricular

chondropathy, a common age-related occurrence in Sprague Dawley rats (Chiu and Lee, 1984; Koetzner, 2016). There was no difference in organ weights, organ-to-body or organ-to-brain weight ratios in female rats. Absolute and brain-relative thymus weights were decreased in high dose males but there was no microscopic correlate and were therefore considered to be non-adverse. Significant increases in epididymis-to-body weights in high dose males were considered to be of no toxicological importance due to a lack of correlating adverse histopathology findings. Based on these observations, a NOAEL of 6 mg/kg bw/day, the highest dose level tested, was determined for both male and female SD rats (Bastaki et al., 2018; Koetzner, 2016). Based on this NOAEL, MoS of greater than 1300 and greater than 1500 were calculated for Group 26 (Aliphatic and aromatic sulfides and thiols) constituents of Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817), respectively, in Step 6 of the safety evaluation.

7.8. Allyl propyl disulfide (FEMA 4073)

7.8.1. Genotoxicity

Allyl propyl disulfide did not induce an increase in reverse mutations in an Ames assay conducted in *S. typhimurium* strains TA97, TA98, TA100, TA1535 and TA1537 at concentrations up to 333 μg/plate in the presence and absence of 10 and 30% S9 fraction isolated from Aroclor 1254-induced male SD rat and male Syrian hamster livers (Zeiger et al., 1988).

7.9. Sulfide mixtures

7.9.1. Genotoxicity

7.9.1.1. Genotoxicity studies of sulfide mixtures. A mixture of 37% allyl propyl disulfide, 31% propyl disulfide and 32% allyl disulfide did not induce reverse mutations in *S. typhimurium* strain TA100 at concentrations ranging from 1.5 to 150 μg/mL using a modified liquid suspension test system in both the absence and presence of S9 metabolic activation derived from the livers of Aroclor 1254-induced rats (Eder et al., 1980).

In a non-OECD guideline-compliant study, male ICR and C3H mice were administered a mixture of 0.33 mmol/kg bw or 0.67 mmol/kg bw allyl disulfide (68.1%), allyl sulfide (19.7%) and diallyl trisulfide (12.2%) twice in 48-h intervals by oral gavage using corn oil as the vehicle. These doses were estimated by the authors to correspond to 32.8 and 66.7 mg/kg bw of allyl disulfide as well as 7.2 and 14.5 mg/kg bw each of allyl sulfide and diallyl trisulfide, respectively. Twenty-four hours after the last dose, mice were terminated and bone marrow cells were collected. The ratio of micronucleated polychromatic erythrocytes and monochromatic erythrocytes was calculated. No induction of micronucleated polychromatic erythrocytes in ICR mouse bone marrow cells was observed (Marks et al., 1992).

7.10. Natural flavor complexes

7.10.1. Asafetida oil

7.10.1.1. Subchronic toxicity. In a short-term toxicity study, male Wistar albino rats (4/group) were orally administered 0, 25, 50, 100 or 200 mg/kg bw/day aqueous suspension of dried powder of crude asafetida oleoresin gum (composition not specified) for 6 weeks (Bagheri et al., 2015). The control group was exposed to an equal volume of physiological saline. Blood was collected at termination for limited biochemical and hematological analyses. The livers and kidneys were preserved for histological examination. No other toxicological parameters were reported. Significantly higher serum aspartate transaminase (AST) and lactate dehydrogenase (LDH) levels were reported in all test groups compared to the control group but were not dose-related and not greater than two times the concurrent control levels. Significant non-dose-related decreases in white and red blood cell and platelet counts occurred in all treated groups when compared to the control group. Significant, non-dose-related decreases in hematocrit values were also reported in the 50, 100 and 200 mg/kg bw/day treatment groups. The biochemical and hematological changes were not dose-dependent. Although the structure of the lobules of the livers of treated rats were normal, histopathological examination revealed that in the 50 mg/kg bw/day and higher dose groups, the hepatocytes were larger in size with a prominent nucleus when compared to the controls. In the 50, 100 and 200 mg/kg bw/day dose groups, hypertrophied Kupffer cells, dilated blood vessels and sinusoids were increased in a dose-dependent manner. Renal tubular necrosis and mild infiltration of inflammatory cells around the blood vessels and interstitial spaces were present in the kidneys of treated rats compared to the control group. The kidneys of high dose male rats had enlarged glomeruli or inflammation of glomeruli and slight signs of tubular degeneration, but the authors concluded that the changes were not significant. Furthermore, there was no increase in serum BUN or creatinine. The study authors concluded that the test substance caused adverse effects in the liver and hematological effects. The FEMA Expert Panel could not draw a definitive conclusion on this study given the lack of a dose response, the mild changes observed, lack of statistical significance, and the possibility that the animals were infected, along with considerable uncertainty that the composition of the administered substance corresponded to the NFC in commerce.

7.10.1.2. Genotoxicity. A crude alcoholic extract of asafetida (specification not provided) induced significant increases in revertant colonies compared to an unspecified control at concentrations of 5000 to 20,000 μg/plate in TA98 streptomycin-dependent *S. typhimurium* strains #510 and #4 (Shashikanth and Hosono, 1986). Mutagenicity was reported as a ratio of spontaneous revertants of the test plate to the control plates and a ratio greater than 5 was considered a significant response. However, the biological relevance of these results is questionable due to the lack of characterization of the test material, lack of a dose response in both strains, the high test concentrations applied (greater than 5000 μg/plate) and the use of strains that are not validated by the OECD (OECD, 1997).

When a crude 70% ethanol asafetida extract (specifications undefined) was tested in *S. typhimurium* strains TA100 and TA1535 at concentrations of 25,000 and 50,000 μg/plate, no mutagenicity was observed compared to untreated plates in the absence of metabolic activation (Soudamini et al., 1995). It should be noted that these concentrations are in excess of the recommended top test substance limit of 5000 μg/plate in the OECD guideline (OECD, 1997).

In an *in vivo* SCE assay, C57Bl/6 Ffm mice were orally administered a single dose of 0, 500 or 1000 mg/kg bw of asafetida powder in saline suspensions following a 24-h subcutaneous bromodeoxyuridine infusion (Abraham and Kesavan, 1984). The test substance was prepared by finely grinding the spice and suspending it in saline. The spermatogenia cells were harvested for examination 40 h after dose administration. Weak induction of SCEs was observed at 1000 mg/kg bw/day in the spermatogenia of test subjects compared to the negative control. Due to a lack of understanding of the underlying mechanism(s) of action of the SCE assay, the assay was removed from the OECD library of standardized assays in 2014 (OECD, 2015) and its relevance to the safety evaluation cannot be assessed (Gooderham et al., 2020a).

7.10.1.3. Summary on the genotoxicity of asafedtia oil. In summary, data on the genotoxicity assays for asafedtia extracts are mixed and data for asafedtia oil are not available. In addition, the *in vitro* studies conducted for extracts of asafedtia were performed under non-standard conditions. The *in vivo* SCE assay reported weak induction of SCEs but only at a relatively high concentration, and this assay is no longer supported by OECD guidance for genotoxicity testing (OECD, 2015). This study did

not report the determination of a maximum tolerable dose. These studies are not useful for risk assessment. The composition of asafedtia oil is 47% Group 26 constituents (Aliphatic and aromatic sulfides and thiols) primarily propenyl sec-butyl disulfide, 37% Group 19 constituents (Aliphatic and aromatic hydrocarbons), 11% Group 12 constituents (Aliphatic and aromatic tertiary alcohols and related esters) with other minor constituents. A review of genotoxicity studies on Group 26 constituents indicates no concern for genotoxicity. In addition, there is no genotoxic concern for Group 12 and Group 19 constituents (Cohen et al., 2019; Fukushima et al., 2020). Based on the lack of genotoxicity of the constituents of asafedtia oil, the Panel concluded that there is no genotoxic concern for this NFC.

7.11. Garlic oil

7.11.1. Short-term toxicity

In an OECD guideline subacute toxicity study, garlic oil obtained by steam distillation of fresh garlic bulbs containing 40.7% allyl disulfide, 21.6% diallyl trisulfide and 6.7% allyl sulfide DAS consistent with the constituent profile of FEMA 2503 Garlic Oil, was administered to ICR mice (10/sex/dose) at doses of 0 (olive oil), 15, 25 or 50 mg/kg bw/day for 28 days (Lin et al., 2022). At the end of the study, the mice underwent blood collection and laparotomy under anesthesia and the major organs, the brain, heart, liver, spleen, lung, kidney, adrenal, thymus, testis (male), epididymis (male), ovary (female), and uterus (female), were isolated, weighed and preserved.

There were no deaths or ophthalmological abnormalities observed during the study. In addition, there were no significant differences in total food intake or body weights between the treatment and control groups. There were no significant changes in the measured hematological parameters. Also, there were no significant differences in relative organ weights in the treatment versus the control groups and no histopathological findings were found in the brain, heart, liver, spleen kidney, stomach or intestines of the treatment and control groups. Based on the lack of adverse effects, the study authors determined a NOAEL for garlic oil of greater than 50 mg/kg bw/day for male and female ICR mice (Lin et al., 2022). Based on this NOAEL, an MoS of greater than 10,000 was calculated for Garlic Oil (FEMA 2503).

Groups of male SD rats were administered 0 (corn oil), 30, 80 or 200 mg/kg bw of garlic oil (composition not provided) by gavage three times per week for 6 weeks as part of a study examining the effect of garlic oil in rats administered low and high corn oil or a fish oil diet (Chen et al., 2003). For the groups administered garlic oil, there were no changes in body weights or food consumption compared to the control group for each of the three diets. Relative jugular lymph node, liver, heart and kidney weights in the garlic oil treatment groups were similar to the control group but there was a dose dependent increase in relative spleen weights in each dietary group. The increase in relative spleen weights is not considered adverse in the absence of extramedullary hematopoiesis or other specific hematological effect. Garlic oil had no effect on the total lipid content in the liver tissues but decreased glutathione (GSH) peroxidase activity, increased GSH reductase and glutathione-*S*-transferase activities in the liver. Based on the absence of adverse effects at the highest dose of garlic oil in this study, the FEMA Expert Panel determined a NOAEL of 200 mg/kg bw/day for garlic oil in male SD rats. Based on this NOAEL, an MoS of greater than 42,000 was calculated for Garlic Oil (FEMA 2503).

As part of a short term study on the effect of garlic oil on the metabolism of rats on high versus low fat diets, 200 mg/kg bw/day of garlic oil (containing 38.6% diallyl disulfide, 5% diallyl sulfide, 30.8% diallyl trisulfide and minor constituents) was administered to two groups of male SD rats by corn oil gavage for 7 weeks (Sheen et al., 1999). One of the groups was fed a low-fat diet and the other a high-fat diet. At the end of the treatment period the rats were euthanized, blood was collected and the livers and spleens were harvested for further analysis. Body weight gains at the end of the 7-week treatment period were significantly reduced for both garlic oil groups. Absolute and relative spleen weights were significantly increased for the garlic oil groups compared to controls. The increase in relative spleen weights is not considered adverse in the absence of extramedullary hematopoiesis or other specific hematological effect. Garlic oil did not have an effect on absolute or relative liver weights in either diet.

In a 6 week study, groups of six male SD rats were administered 200 mg/kg bw of garlic oil (described as 10% diallyl sulfide, 40% diallyl disulfide, 35% diallyl trisulfide and other minor components) by corn oil gavage 3 times per week (Wu et al., 2001). Body weight gain over the course of the study was similar between controls and the group administered garlic oil. Absolute and relative liver weights and absolute spleen weights were comparable to controls but relative spleen weights were significantly increased. The increase in relative spleen weights is not considered adverse in the absence of extramedullary hematopoiesis or other specific hematological effect.

7.11.2. Genotoxicity

In an OECD-guideline Ames assay, no mutagenicity was reported when garlic oil was incubated with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 at concentrations of 0.5–1000 μg/plate in the absence and presence of an S9 metabolic activation system derived from the livers of 3-methylcholanthrene-treated male rats (Lin et al., 2022). The garlic oil was obtained by steam distillation of fresh garlic bulbs and was determined to contain 40.7% allyl disulfide, 21.6% diallyl trisulfide and 6.7% allyl sulfide by GC analysis which is consistent with the preparation and composition of FEMA 2503 Garlic Oil. No reverse mutations were reported when garlic oil was incubated with *S. typhimurium* strains TA98, TA100, TA1537, TA1535 and TA1538 at concentrations of 5–2000 μg/plate in the absence and presence of an S9 metabolic activation system (Hachiya et al., 1983). In a single strain Ames assay, garlic oil, obtained by steam distillation of the bulb of the plant was not mutagenic in *S. typhimurium* strain TA100 at concentrations ranging from 62.5 to 250 μg/plate in the presence and absence of an S9 metabolic activation (Park, 2002). In a spore rec assay conducted in strains *B. subtilis* strains H17 Rec^+, M45 Rec^-, garlic oil was not mutagenic at concentrations up to 3 mg/disk in the absence of an S9 metabolic activation system and was not mutagenic at concentrations up to 5 mg/disk in the presence of S9 metabolic activation (Ueno et al., 1983). Additionally, no mutagenicity was observed when garlic oil was incubated with *B. subtilis* strains H17 Rec^+, M45 Rec^- at concentrations up to 5000 μg/disk in the absence or presence of S9 metabolic activation (Hachiya et al., 1983). The rec assay has not been standardized in an OECD guideline for genotoxicity testing, and OECD has noted that indicator tests such as the rec assay should be correlated to the results of other assays that measure DNA damage or mutagenicity that can be passed on to subsequent generations (OECD, 2015).

In an OECD-guideline *in vitro* chromosomal aberration study, CHO cells (CHO–K1) were incubated with garlic oil (composition consistent with FEMA 2503) in 3 h experiments in the presence and absence of S9 metabolic activation at concentrations of 1, 1.5, 2.5, 5 and 10 μg/mL (Lin et al., 2022). An additional 20 h incubation with garlic oil at these concentrations was also done in the absence of S9. The S9 metabolic activation system was derived from the livers of 3-methylcholanthrene--treated male rats. Under the conditions tested, garlic oil did not induce the formation of chromosomal aberrations in mammalian CHO–K1 cells.

In an *in vivo* comet assay, male mice (LACA strain) were orally administered 0 (olive oil), 0.25 or 0.50 mL garlic oil (specification not reported) for six consecutive days. Mice were terminated 24, 48 and 72 h after the last dose and the liver, kidney, lung, spleen and testes were harvested and prepared for comet analysis. Results of the comet analysis in each organ were reported as averages with standard deviations from 50 replications per test subject. No significant differences were observed in the percentage of cells with the appearance of comets or in average tail length in all four tissue examined (Kaur and Singh, 2007).In an OECD guideline *in vivo* micronucleus induction study, a single dose

garlic oil (composition consistent with FEMA 2503) was administered to male ICR mice (10/dose) at levels of 0 (olive oil), 15, 25 or 50 mg/kg bw by oral gavage (Lin et al., 2022). At 24 h and 48 h post-administration, blood samples were drawn for analysis of micronucleated reticulocytes. Garlic oil did not induce the formation of micronuclei in the peripheral blood of mice under the conditions tested.

7.11.3. Summary on the genotoxicity of garlic oil

Rec and Ames assays of garlic oil, including an OECD guideline Ames study, were negative for mutagenicity. OECD guideline *in vitro* chromosomal aberration and *in vivo* micronucleus assays of garlic oil with a composition and preparation similar to FEMA 2503) were negative for genotoxicity. Finally, garlic oil was non-genotoxic in an *in vivo* comet assay in the kidney, lung, spleen and testes in mice. Based on the available OECD guideline Ames, *in vitro* chromosomal aberration and *in vivo* micronucleus induction assay studies as well as an *in vivo* comet assay for garlic oil indicating a lack of genotoxicity, the FEMA Expert Panel concludes that the weight of evidence indicates that garlic oil is not genotoxic.

7.12. Onion oil

7.12.1. Genotoxicity

No evidence of mutagenicity was reported in an Ames assay when concentrations of 1–50,000 μg/plate of onion oil were tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 in the presence and absence of S9 using the preincubation method (Hachiya et al., 1983). Onions (*Allium cepa*) were obtained in the local (Jordan) market, dried, ground and a 95% ethanol cold extract of 0.05 kg of finely powdered onion was prepared and concentrated in vacuo. This extract was tested for mutagenicity in triplicate in *S. typhimurium* strains TA98 and TA102 using the plate incorporation method at 10,000 μg/plate in the presence and absence of S9. The onion extract showed no induction of reverse mutations in either strain in the presence and absence of S9 (Mahmoud et al., 1992). Aqueous and 99% ethanol extracts of sliced and homogenized onion (*Allium cepa*) were tested for mutagenicity in *S. typhimurium* strains TA98 and TA100 in the presence and absence of S9 metabolic activation system at concentrations up to 10,000 μg/mL. Under the conditions tested, onion extract did not induce increases in reverse mutations in either strain in the presence and absence of S9 (Martínez et al., 1999). The FEMA Expert Panel noted that the concentrations tested in this study exceeded the maximum concentration of no more than 5 mg/plate recommended in OECD guideline 471 (OECD, 2020) and that the composition of the onion extracts used in the Martinez et al., and Mahmoud et al. studies are not representative of Onion Oil (FEMA 2817).

In two spore rec-assay in *B. subtilis* strains H17 Rec^+ and M45 Rec^-, onion oil, at a concentration of 10 mg/disk resulted in an equivocal or weakly positive result in the absence of S9 metabolic activation system and negative results in the presence of metabolic activation. Cytotoxicity was noted for onion oil in the experiment with S9 metabolic activation (Hachiya et al., 1983; Ueno et al., 1984). The rec assay has not been standardized in an OECD guideline for genotoxicity testing, and OECD has noted that indicator tests such as the rec assay should be correlated to the results of other assays that measure DNA damage or mutagenicity that can be passed on to subsequent generations (OECD, 2015).

In an *in vitro* chromosome aberration assay conducted in Chinese hamster fibroblasts, equivocal results were reported for onion oil, 48 h following treatment, tested at concentrations up to 40 μg/ml in the absence of metabolic activation (Ishidate et al., 1984). Because fewer than 300 cells were scored for each test, this study does not meet the current OECD guideline (OECD, 2016).

No mutagenicity was reported for 0, 250, 500 and 1000 mg/kg bw of onion oil in an *in vivo* micronucleus assay conducted in male ddy mice provided *ad libitum* access to water and food. The maximum dose used was based on the maximum tolerated dose (Hachiya et al., 1983). Single intraperitoneally administered doses of onion oil in an olive oil vehicle resulted in no mortality after 24 h and no significant increases in polychromatic erythrocytes and micronucleic polychromatic erythrocytes in the bone marrow cells obtained from test mice compared to the negative control. A 4-day repeat dose intraperitoneal administration of 200 mg/kg bw of onion oil resulted in no mortalities and no significant changes in the frequency of micronucleic polychromatic erythrocytes and polychromatic erythrocytes compared to the negative controls (Hayashi et al., 1988).

7.12.2. Summary on the genotoxicity of onion oil

In summary, rec and Ames assays on onion extracts and onion oil were negative for mutagenicity. The OECD notes that indicator tests such as the rec assay should be considered with the results of other assays that measure DNA damage or mutagenicity that can be passed on to subsequent generations (OECD, 2015). Although equivocal results were reported for an *in vitro* chromosome aberration assay conducted in Chinese hamster fibroblasts, onion oil was negative in an *in vivo* micronucleus assay conducted in male ddy mice. Based on the weight of evidence, there is no genotoxic concern for onion oil.

Recognition of GRAS status

Asafetida Oil (FEMA 2108), Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817) were determined to be GRAS under conditions of intended use by the FEMA Expert Panel in 1965 and have been used as flavoring ingredients for over fifty years. Studies gathered for the safety evaluation presented here indicated adequate margins of safety between conservative estimates of exposure and the NOAELs in animal short and long-term toxicity studies in addition to a lack of genotoxic potential. The safety of Asafetida, Garlic and Onion NFCs is further supported by their self-limiting properties as flavoring ingredients resulting in use levels that do not saturate pathways of metabolism or elimination. By application of the safety procedure, the FEMA Expert Panel affirms the GRAS status of Asafetida Oil (FEMA 2108), Garlic Oil (FEMA 2503) and Onion Oil (FEMA 2817) as flavoring ingredients in food.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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