Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food and Chemical Toxicology 43 (2005) 1141–1177
www.elsevier.com/locate/foodchemtox

Review

Criteria for the safety evaluation of flavoring substances The Expert Panel of the Flavor and Extract Manufacturers Association

Robert L. Smith [a], Samuel M. Cohen [b], John Doull [c], Victor J. Feron [d], Jay I. Goodman [e], Lawrence J. Marnett [f], Ian C. Munro [g], Philip S. Portoghese [h], William J. Waddell [i], Bernard M. Wagner [j], Timothy B. Adams [k,*]

[a] *Division of Biomedical Sciences Section of Molecular Toxicology, Imperial College School of Medicine, South Kensington, London SW7 2AZ, United Kingdom*
[b] *Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198, USA*
[c] *Department of Pharmacology and Toxicology, University of Kansas Medical Center, Kansas City, KS 66103, USA*
[d] *TNO Nutrition, Toxicology, Utrechtseweg 48, The Netherlands*
[e] *Department of Pharmacology and Toxicology, Michigan State University, B440 Life Science Building, East Lansing, MI 48824, USA*
[f] *Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, USA*
[g] *CanTox Health Sciences International, Mississauga, Canada, ON L5N 2X7*
[h] *Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, USA*
[i] *Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, USA*
[j] *New York University, School of Medicine, New York, New York Bernard M. Wagner, Associates, Millburn, NJ 07078, USA*
[k] *Scientific Secretary to the FEMA Expert Panel, Flavor and Extract Manufacturers Association, 1620 I Street, NW Suite 925, Washington, DC 20006, USA*

Received 8 September 2004; accepted 26 November 2004

Abstract

The current status of the GRAS evaluation program of flavoring substances operated by the Expert Panel of FEMA is discussed. The Panel maintains a rigorous rotating 10-year program of continuous review of scientific data related to the safety evaluation of flavoring substances. The Panel concluded a comprehensive review of the GRAS (GRASa) status of flavors in 1985 and began a second comprehensive review of the same substances and any recently GRAS materials in 1994. This second re-evaluation program

Abbreviations: ABS, chromosomal aberration; ADH, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; AE, anethole epoxide; CYP450, Cytochrome P450; CHO, Chinese hamster ovary; CoA, coenzyme A; CoE, Council of Europe; CCK, cholecystokinin; DDA, detailed dietary analysis; DNA, deoxyribonucleic acid; DSB, double strand breaks; EC, European Commission; ECETOC, European Centre for Ecotoxicology and Toxicology of Chemicals; EFSA, European Food Safety Authority; EH, epoxide hydrolase; *E. coli*, *Escherichia coli*; EPA, Environmental Protection Agency; F, female; FAA, Food Additives Amendment; FDA, United States Food and Drug Administration; FEMA, The Flavor and Extract Manufacturers Association; FSM, robust stochastic model; GSH, glutathione; GST, glutathione-*S*-transferase; GRAS, Generally Recognized as Safe; GRASa, GRAS affirmed; GRASr, GRAS reaffirmed; IARC, International Agency for Research on Cancer; IOFI, International Organization of the Flavor Industry; i.p., intraperitoneal; LD50, median lethal dose; M, male; MLA, mouse lymphoma cell assay; MRCA, Market Research Corporation of America; NAS, National Academy of Science; NCI, National Cancer Institute; NFC, natural flavor complex; NOAEL, no observable adverse effect level; NOEL, no observed effect level; NR, not reported; NTP, National Toxicology Program; MW, molecular weight; OECD, Organisation for Economic Cooperation and Development; PADI, possible average daily intake; PCI, per capita intake; PPARα, peroxisome proliferator-activated receptor; PE, polychromatic erythrocytes; ppm, parts per million; PTS, Primary Toxicity Screen; *S. typhimurium*, *Salmonella typhimurium*; SCE, sister chromatid exchanges; SCF, European Scientific Committee for Food; SLR, Scientific Literature Review; TAMDI, theoretical added maximum daily intake.

* Corresponding author. Tel.: +1 202 293 5800; fax: +1 202 463 8998.
E-mail address: tadams@therobertsgroup.net (T.B. Adams).

0278-6915/$ - see front matter
doi:10.1016/j.fct.2004.11.012

of chemical groups of flavor ingredients, recognized as the GRAS reaffirmation (GRASr) program, is scheduled to be completed in 2005.

The evaluation criteria used by the Panel during the GRASr program reflects the significant impact of advances in biochemistry, molecular biology and toxicology that have allowed for a more complete understanding of the molecular events associated with toxicity. The interpretation of novel data on the relationship of dose to metabolic fate, formation of protein and DNA adducts, enzyme induction, and the cascade of cellular events leading to toxicity provides a more comprehensive basis upon which to evaluate the safety of the intake of flavor ingredients under conditions of intended use. The interpretation of genotoxicity data is evaluated in the context of other data such as in vivo animal metabolism and lifetime animal feeding studies that are more closely related to actual human experience. Data are not viewed in isolation, but comprise one component that is factored into the Panel's overall safety assessment.

The convergence of different methodologies that assess intake of flavoring substances provides a greater degree of confidence in the estimated intake of flavor ingredients. When these intakes are compared to dose levels that in some cases result in related chemical and biological effects and the subsequent toxicity, it is clear that exposure to these substances through flavor use presents no significant human health risk.

Contents

1. Introduction

For more than four decades, the Expert Panel of FEMA [Flavor and Extract Manufacturers Association (of the United States)] has maintained a program to evaluate the safety evaluation of flavor ingredients for their intended use in food. During this period, the Panel has evaluated and regularly re-evaluated the "safety-in-use" of more than 1700 chemically identified substances used as flavor ingredients in foods. The Panel's determination of whether a flavor ingredient is "generally recognized as safe" (GRAS), is always made with the distinct limitation that the substance is concluded to be safe "as a flavoring substance under conditions of intended use in food".

Flavoring substances are defined by their action on the human senses. The flavoring effect of a substance is determined by its impact on membrane receptors of the human gustatory and olfactory systems. The partial or complete saturation of these receptors provides the physiologic response to a flavoring agent. Optimum response frequently occurs at extremely low levels of exposure. Concentrations of individual flavoring ingredients added to food are, almost without exception, <0.1% with the majority at <0.001%. These levels simulate concentrations of flavoring substances as they occur naturally in traditional foods. Therefore, the intentional addition of flavoring substances to food is, in almost all cases, associated with the property of self-limitation. Within the context of low exposure, the Panel performs

a rigorous evaluation of the available scientific data and evaluates the safe use of substances as flavor ingredients. The principal objective of the safety evaluation of flavoring substances (i.e., GRAS program) by the Panel is "to protect the consumer's health in the context of flavor use and to provide the scientific basis for helping the flavor industry maintain a pattern of self-regulation" (FEMA Expert Panel, 1986[1]). To realize this objective the multi-disciplinary Panel relies on expert judgment achieved through decades of experience evaluating flavoring substances. All decisions of the Panel concerning the safety of flavoring substances must be unanimous.

1.1. Historical background to the safety evaluation of flavor materials and the formation of the FEMA expert panel

Historically, the safety evaluation of flavor substances has not been seen as a high priority issue. The reasons for this are fairly obvious. Firstly, the safety assessment of other groups of socially used chemicals such as drugs, pesticides, major food additives and environmental substances were seen as a more pressing priority and would inevitably consume restricted resources. Secondly, there were widespread perceptions that food flavors posed no significant safety issues as exposure levels were very low, bordering in many cases on the trivial, and quite frequently there was a long history of human exposure to these materials as naturally occurring components of traditional foods.

The first move to regulate the safety of food flavors appears to be that of the US Food and Drug Administration in 1958. This Governmental Agency issued the first so called GRAS List ("Generally Recognized as Safe") of flavor substances in 1958. This list accommodated some 188 traditional herbs and spices and was compiled in response to the then recently passed Food Additives Amendment (FAA) of the Federal Food and Drug and Cosmetic Act.

A second GRAS list was published by the FDA in 1960 and this comprised some 27 single chemical entities, mainly synthetic in nature. There was little scientific information concerning the safety of these materials. The statutory basis for those flavors in use before 1958 is that they had a long history of "common use in food" as defined in the FAA.

The passage of the 1958 Amendment and the publication of the two FDA GRAS Lists of flavor materials elicited within the US Flavor Industry a perception that future developments would inevitably require the gathering and systematic evaluation of information relating to safety aspects such as chemical identity and specifications, conditions of use and scientific data on metabolism and toxicology.

Through the combined initiatives of Dr. Bernard L. Oser and Dr. Richard Hall it was proposed that a scientifically independent group of Experts (The Expert Panel) should be formed and they should be given the remit of: (a) attempting to extract what judgments they could from the data available for flavor materials; (b) specify what additional studies would be needed; and (c) make GRAS decisions.

The legal basis for the existence and operation of the FEMA Expert Panel is embedded in the phraseology of the Food Additives Amendment of 1958 which defines a "food additive" as "any substance the intended use of which results or may reasonably be expected to result in its becoming a component of food if such substance is not generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been shown to be safe under the conditions of its intended use". The three conditions specified in the Amendment which underpin and define the operation of the Expert Panel are: "general recognition", "qualified experts" and "conditions of use". When this program began, it was without precedent. The flavor industry, a small segment of the entire food industry, not only originated this program, but has continuously and strongly supported its existence and activities (Hallagan and Hall, 1995).

The FEMA Expert Panel first met in 1960 and since then has regularly met 3–4 times a year for the purpose of assessing the safety of flavor materials. The GRAS determinations made by the Panel are published in Food Technology and to date, there are 21 GRAS publications covering over 2000 substances. Membership of the Panel reflects the wide knowledge and experience that is judged to be necessary for the evaluation of these materials via experts in biochemistry, metabolism and pharmacokinetics, natural product chemistry, clinical medicine, pathology and toxicology.

In other safety evaluation programs, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has regularly evaluated individual flavoring agents beginning in 1967. During the next 30 years approximately one substance was evaluated annually by JECFA and an acceptable daily intake was assigned. In 1995, JECFA adopted a novel safety evaluation program for flavoring substances. Using a group assessment approach, JECFA has approved the use of more than 1300 substances for use as flavoring agents under current conditions of intake. In the case of Europe, the Council of Europe (CoE) published its so-called "Blue Book" in 1973 which provides a list of "permitted flavor substances" the majority of which appear to have been adopted from the FEMA GRAS Lists. From 1993 to 1995, the European Scientific Committee for Food

[1] FEMA Expert Panel (1986) Private communication.

(SCF) convened a Flavor Working Group to evaluate the safety of chemically-defined flavoring substances. After a short hiatus the Flavor Working Group was reconvened in 2000 to continue the evaluation process. Since then the SCF and its Working Group has been succeeded by a new Scientific Panel, under the auspices of the European Food Safety Authority (EFSA), which will assess the safety of flavors as part of its ongoing responsibilities. Under EFSA, a database (FLAVIS) has been developed to collect and organize scientific data on "European only" flavors. These data are to be further evaluated by the EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food.

What has emerged over the years with the evaluation of so many materials is the recognition, by Governmental and non-Governmental Agencies that they cannot, for many reasons, be evaluated by traditional toxicology and that novel approaches are required. In recent years there has developed an encouraging convergence of approaches adopted by the various Agencies and these place emphasis on exposure, structural group approach, metabolic fate and toxicology. In particular these approaches take advantage of the fact that the vast majority of single chemical entity flavors (≈1700) belong to relatively few well-defined structural groups (≈40) where there exists a reasonable homology in terms of likely toxicity and metabolic fate.

1.2. Scope of flavoring substances reviewed by the expert panel

Two types of flavor ingredients exist, chemically identified (e.g., ethyl butyrate and *l*-carvone) and naturally-occurring flavor mixtures (e.g., essential oils, oleoresins). The vast majority of chemically identified flavoring substances (≈1700) exists naturally in foods or are formed during the preparation of foods via heating or mixing. However, with few exceptions, the naturally-occurring individual substances used in commerce are not isolated from nature, but are synthesized chemically in order to ensure the purity and identity of the product intended for commerce. The availability of technologically advanced methods of synthesis, separation and identification of flavoring substances has provided the opportunity to produce and maintain a quality of product not available by isolation of the same substance from natural sources. The remaining chemically identified flavoring substances (≈350) that are produced by synthesis but not found in nature are, almost without exception, structurally related to naturally-occurring flavoring substances. For example, cinnamyl acetate and butyrate are reported to occur in nature while cinnamyl propionate and valerate are not (Nijssen et al., 2003). Regardless of their origin, essentially all chemically identified flavoring substances belong to well-defined chemical groups (e.g., pyrazines, aliphatic lactones, cinnamyl derivatives). The ability to organize the large number of substances into a limited number of chemical groups provides an efficient and effective approach to evaluate chemically identified flavor ingredients. In this framework, each flavor ingredient can be evaluated individually and within the context of its chemical group.

The second type of flavor ingredient is the naturally-occurring flavor mixtures isolated primarily from plants. Historically, they have been recognized as essential oils, extracts, oleoresins, tinctures, and in some cases foods (e.g., cardamom and black pepper). Approximately 300 mixtures are now collectively recognized as natural flavor complexes (NFC). The volatile components of most NFCs are mixtures containing as many as 250 chemically identified constituents with likely more to be discovered. Advances in analytical technology will doubtless yield more constituents. A limited number of these constituents are responsible for the technical flavor effect of the NFC itself. As the flavor industry has developed over time, most of these important flavoring constituents were separated, identified, and synthesized. They now constitute many of the important chemically identified flavor ingredients (e.g., citral and limonene) used by the flavor industry worldwide.

Being of plant origin, NFCs form their chemical constituents by well-recognized biochemical pathways (e.g., isoprene and shikimic acid pathways). Constituents formed by these pathways exhibit similar skeletal structures and possess a limited number of functional groups. In fact, NFCs of different plant origin contain many of the same constituents. The vast majority of constituents fall into one of a few well-defined chemical groups (e.g., terpene hydrocarbons and alicyclic terpene secondary alcohols and ketones). Although the safety evaluation of chemical mixtures involves significant complexity, the safety evaluation of NFC mixtures can be reduced to an analysis of relatively few chemical groups each containing one or a number of constituents. As is evident from the above discussion the use of the chemical group approach provides a powerful tool for the safety evaluation of both chemically identified and NFC flavor ingredients.

Although these two groups constitute what is commonly known as flavor ingredients, flavors added to food are a mixture of flavor ingredients. Commercial flavors added to food normally contain 50 or more substances, both chemically identified and NFCs, and accompany other ingredients that perform non-flavor functions such as preservatives (BHA), solvents (ethyl alcohol), modifiers (neohespiridin dihydrochalcone), and emulsifiers (guar gum). Substances that do not impart flavor may serve several functions and have significant uses in the food supply. In these instances, the

Panel evaluates the substance for GRAS status based only on its intended use as a component of a food flavor. This criterion is entirely consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (21 CFR 172.515 a and b) which states that in order for a substance to be considered GRAS it must be "safe under conditions of intended use." It is also consistent with past Panel decisions in which substances having non-flavor functions (solvents, modifiers, antioxidants, etc.) have been recognized as GRAS for their intended use in a compounded flavor.

2. GRAS, GRASa, and GRASr evaluation

2.1. GRAS process

The GRAS assessment performed by the Expert Panel includes a thorough evaluation of all the available data on the candidate flavor ingredient and on structurally related substances in the same chemical group. The analyses include a comprehensive evaluation of the potential exposure to the flavor ingredient through food, compared with the available toxicologic, metabolic, and pharmacokinetic characteristics of the substance and its structural relatives. Available information on the absorption, distribution, excretion, and metabolic options on the flavoring ingredient and structurally related substances provide the basis for understanding the biochemical fate of the substance. Data from acute, subchronic and long-term oral studies provide the fundamental basis to understand the toxic potential of the substance. In addition, in vitro and in vivo genotoxicity data for the substances, principal metabolites, and members of the respective chemical group are evaluated to screen for potential genotoxic effects. Behavioral, neurotoxicity, immunotoxicity, and reproductive effects are evaluated, if available. The combined expert judgment of the Panel is then applied to the above data and any other data deemed to be relevant to the safety evaluation. In order to reach a GRAS decision, there must be (1) an understanding of the known or anticipated biochemical fate of the substance and its potential to produce toxicity, and (2) establishment of the safety of low levels of use of the substances in the context of its toxic potential. The criteria for the final decision of the Panel are described below. For the vast majority of flavoring substances, very low levels are required to create the intended flavor effect and low volumes are added to the food supply annually. For many of these substances, little or no toxicology data is available. This, however, does not preclude evaluation of the substance for its intended use as a flavoring substance. If the structure of the substance (e.g., benzyl butyrate) is such that it can be assigned to a well defined chemical group (e.g., benzyl alcohol, benzaldehyde, benzoic acid and related esters) for which extensive safety data exists the safety evaluation can proceed. For instance, benzyl butyrate participates in the same biochemical pathways and exhibits similar toxicologic potential as the well-studied substances, benzyl acetate and benzyl propionate. Provided the substance is (1) metabolized by well-recognized detoxication pathways, (2) anticipated exposure is orders of magnitude less than no effect levels reported in studies on structurally related substances, (3) The group of structurally related substances exhibit no potential for genotoxicity effects at anticipated levels of use as flavoring substances and (4) the substance presents no unique structural features that warrant special considerations, the substance is considered safe under intended conditions of use.

Beginning with the publication of GRAS 3 in 1965 the Expert Panel has published nineteen (19) GRAS articles identifying more than 2000 substances as GRAS for their intended use as flavor ingredients. GRAS 20 (Smith et al., 2001) represented a landmark in that it contained the 2000th substance (FEMA No. 4000) to be recognized as "GRAS" by the FEMA Expert Panel. In addition to a complete listing of new GRAS flavor ingredients, the Expert Panel regularly presents critical interpretations of recent scientific studies on flavor ingredients with the goal to ensure that the results of these studies are consistent with their current GRAS status. Flavor issues, for example, the evaluation of methods for the calculation of human dietary exposure to flavoring substances and a novel method for the safety evaluation of NFC's are also discussed in GRAS articles.

The application for GRAS recognition is submitted by FEMA member companies. This Association represents flavor companies accounting for at least 90% of all flavor business conducted in the US. The GRAS application contains physical and chemical data to identify the structure of the candidate flavoring substance and verify its purity. Results of literature searches and company generated safety data on the substance and structurally related substances are included in the application to support the safety for the substance under conditions of use. Information on the anticipated pattern of use as a flavor (use levels in food categories), technical flavor function and the results of sensory panel evaluations are also provided. Upon completion of the evaluation of these data and other information the Panel deems appropriate, the Panel has three options. They may conclude that the substance is GRAS for its intended use as a flavoring substance; they may place the substance on HOLD requesting that the applicant provide additional data; or notify the applicant that the substance in not GRAS, in which case the applicant usually withdraws the application. Over the last four decades, more than 150 applications have been withdrawn. Far more applications have been placed on

HOLD and additional data is later provided to complete the GRAS evaluation. Normally, the applicant performs a cost benefit analysis prior to submitting a GRAS application. However, in some cases, the request for additional studies alters the results of the analysis such that completing GRAS submission is economically unfeasible. Under these circumstances, the application is withdrawn.

During its review of the scientific data related to flavors, the Panel identifies new data that may affect prior GRAS decisions. In these cases, the Panel reevaluates the safety of the flavor in the context all relevant safety data. Upon completion of the review they may conclude the substance is no longer GRAS and remove it from the GRAS list of flavors (deGRASed). In some instances, they request additional studies be performed by industry members or the industry as a whole. If sufficient interest exists in the continued use of the substance as a flavor, the studies are performed and the reevaluation of the GRAS status of the substance is completed. In the absence of industry interest and data, the Panel completes the revaluation based on the data available. Over the last four decades, this review process has lead to the deGRASing of 10 substances (e.g. o-vinylanisole, 2-methyl 5-vinylpyrazine,), mainly due to the lack of interest in the continued use of the substance as a flavor.

2.2. GRASa and GRASr

As advancements are made in science, new information becomes available on existing FEMA GRAS flavoring substances. The dynamic FEMA GRAS assessment program incorporates this new information into the program by way of systematic reviews of all GRAS flavor ingredients.

Between 1965 and 1985, the first comprehensive and systematic scientific literature reviews (SLRs) of flavoring substances were completed by FEMA under contract by the US Food and Drug Administration. These SLRs served as the basis for a comprehensive review of substances already designated as FEMA GRAS. This GRAS status reassessment program was known as "GRAS affirmation" or "GRASa" and was completed in 1985.

In 1994, the Expert Panel initiated a second comprehensive reassessment program known as "GRAS reaffirmation" or "GRASr". It is anticipated that this reaffirmation program will be completed in 2005. As part of the GRASr program, the Expert Panel regularly publishes interpretations of key scientific data on structurally related groups of flavoring substances on which GRAS decisions are based. Since 1995, FEMA GRAS assessments of alicyclic substances, pyrazines, cinnamyl derivatives, furfural, lactones, and *trans*-anethole, allylalkoxybenzene derivatives (methyl eugenol and estragole) have been published as part of the GRASr program (Adams et al., 1996; Adams et al., 1997; Adams et al., 1998; Adams et al., 2004; Newberne et al., 1999; Smith et al., 2002a; Smith et al., 2002b). Regular publication of at least 20 other structurally related groups of flavoring substances are scheduled.

2.3. GRASr group assessments

In its initial years of operation, the Panel used its combined background of expertise and judgment to deal with the often limited amount of safety data available on a flavoring substance. As the effort progressed, the Panel was exposed to a broad array of simple chemical substances, most of which could be assigned to specific chemical classes (e.g., benzyl alcohol, benzaldehyde, benzoic acid and related esters and acetals) based on their close structural relationship, common pathways of metabolism and consistent pattern of toxicologic potential. The Panel came to use these well-established patterns, not as a substitute for data-driven judgments, but to assure that those judgments were applied consistently across increasingly larger amount of data for growing numbers of structurally related substances. These patterns in the form of group assessments are regularly published in the peer-reviewed literature (Adams et al., 1996; Adams et al., 1997; Adams et al., 1998; Adams et al., 2004; Newberne et al., 1999; Smith et al., 2002a; Smith et al., 2002b).

During its 40 year-plus history, the Expert Panel has evaluated and reevaluated chemically identified flavor ingredients within the context of their respective chemical group (e.g., benzyl derivatives). This experience has provided the basis for understanding the relationship between the biochemical fate of members of a chemical group and their toxicologic potential. Within this framework, the Panel regularly assesses new scientific data for GRAS candidates, GRAS substances, and structurally related non-GRAS materials. The objective is to continuously build a more complete understanding of the absorption, distribution, metabolism and excretion of members of the chemical group and their potential to cause systemic or target-organ toxicity. Against this background, exposure to each chemically identified flavor ingredient is evaluated for safety (see example of geranyl butyrate, Fig. 1).

One of the principal objectives of the GRASr program is to publish the Panel's chemical group safety assessments in the peer-reviewed scientific literature (see above for listing). These assessments contain all the key scientific data relevant to the safety of chemical groups of flavoring substances. Upon completion of the GRASr program, approximately 35 chemical group assessments covering approximately 1600 flavor ingredients will be published. The chemical groups of flavor ingredients currently being evaluated by the Expert Panel are listed in Table 1.

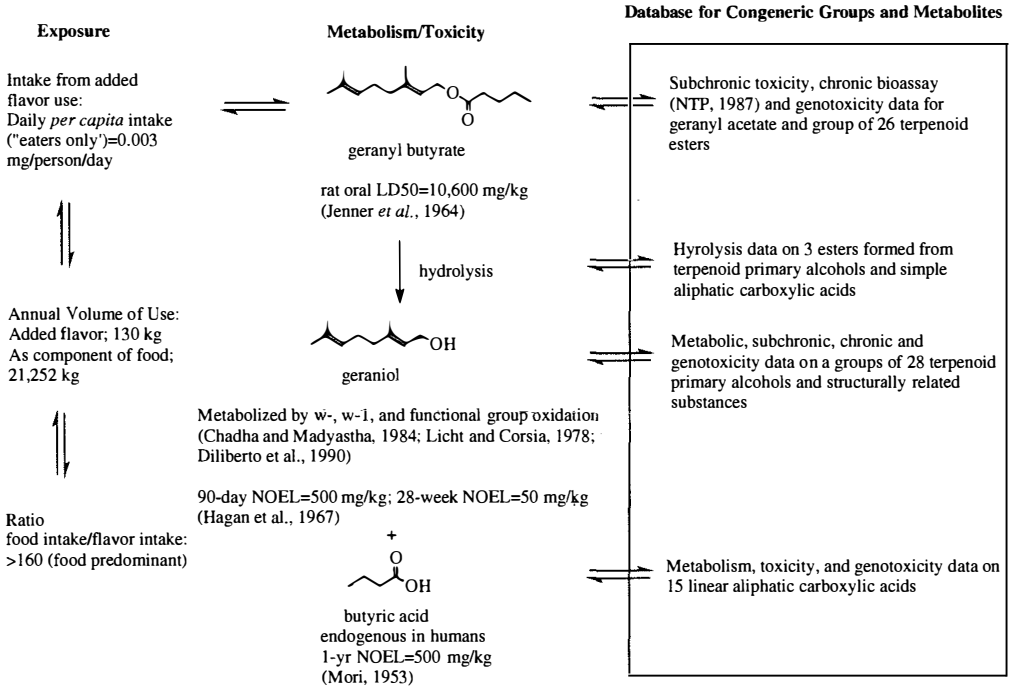


Fig. 1. Safety evaluation of a flavor ingredient-geranyl butyrate.

During the last three years, the Panel has expanded the application of the group assessment approach to the safety evaluation of NFCs. As noted above, NFCs are mixtures of constituents, most of which have been chemically identified, that belong to a limited number of chemical groups. For instance, greater than 98% of the composition of coriander oil is accounted for by approximately 30 constituents that belong to three distinct chemical groups, aliphatic terpene hydrocarbons, terpene tertiary alcohols and esters, and alicyclic terpene secondary alcohols and ketones (Lawrence and Shu, 1993; FEMA, 2003,[2] Heck et al., 1989). Studies on the biochemical fate and toxicologic potential along with intake data for members of each of these chemical groups provide the scientific basis to perform a comprehensive safety evaluation of the NFC. Because of the complexities involved in the evaluation of a mixture, the Panel has developed a scheme for the systematic evaluation of the chemical composition of an NFC. The principles, details and examples of this novel approach to safety evaluation are the subject of recent publications (Newberne et al., 1998; Smith et al., 2004; Smith et al., 2005).

The types and numbers of chemical groups in the safety evaluation program are, by no means, static. As new scientific data and information become available, some chemical groups are combined while others are subdivided. This has been the case for the group of alicyclic secondary alcohols and ketones that were the subject of a comprehensive scientific literature review (SLR) in 1978 (FEMA, 1978). Over the last two decades, experimental data has become available indicating that a few members of this group exhibit biochemical fate and toxicologic potential inconsistent with that for other members of the same group. These inconsistencies, almost without exception, arise at high dose levels that are irrelevant to the safety evaluation of low levels of exposure to flavor use of the substance. However, given the importance of the chemical group in the safety assessment program, it is critical to resolve these inconsistencies. Often the Expert Panel has requested additional metabolic and toxicologic studies to distinguish the factors that determine these differences. In many cases, the effect of dose and a unique structural feature results in utilization of another metabolic pathway not utilized by other members of a chemical class.

For instance, within the class of alicyclic ketones mentioned above, (+)-isomenthone and (+)-pulegone differ only in that the former contains an isopropyl substituent adjacent to the ketone function whereas the latter contains an isopropylidene substituent at the same position. The presence of an exocyclic isopropylidene group induces increased allylic hydroxylation (Moorthy et al., 1989a,b; Thomassen et al., 1988, 1990, 1991; Madyastha and Raj, 1990, 1991, 1992, 1993) eventually leading to a reactive cis-2-ene-1,4-dicarbonyl metabolite that readily forms protein adducts in the liver (McClanahan et al., 1989; Thomassen et al., 1992) and induces hepatotoxicity (Moorthy and Madyastha, 1989; Madyastha and Raj, 1993) at dose levels >75 mg/kg per day in mice

[2] FEMA (2003) Private communication.

Table 1
Chemical groups of flavor materials

1	Saturated aliphatic, acyclic, linear primary alcohols, aldehydes, carboxylic acids and related esters
2	Saturated aliphatic, acyclic, branched-chain primary alcohols, aldehydes, carboxylic acids and related esters
3	Aliphatic linear and branched-chain α,β-unsaturated aldehydes and related alcohols, acids and esters
4	Aliphatic allyl esters
5	Unsaturated linear and branched-chain aliphatic, non-conjugated aldehydes, related primary alcohols, carboxylic acids and esters
6	Aliphatic primary alcohols, aldehydes, carboxylic acids, acetals and esters containing additional oxygenated functional groups
7	Saturated alicyclic primary alcohols, aldehydes, acids, and related esters
8	Saturated and unsaturated aliphatic acyclic secondary alcohols, ketones and related esters
9	Aliphatic acyclic and alicyclic α-diketones and related α-hydroxyketones
10	Alicyclic ketones, secondary alcohols and related esters
11	Pulegone and structurally- and metabollically-related substances
12	Aliphatic and aromatic tertiary alcohols and related esters
13	Aliphatic, alicyclic, alicyclic-fused and aromatic-fused ring lactones
13	Aliphatic and aromatic hydrocarbons
14	Benzyl derivatives
15	Hydroxy- and alkoxy-substituted benzyl derivatives
16	Cinnamyl alcohol, cinnamaldehyde, cinnamic acid, and related esters
17	Phenyl-substituted primary alcohols, aldehydes, carboxylic acids, and related esters
18	Phenyl-substituted secondary alcohols, ketones, and related esters
20	Phenol derivatives
21	Hydroxyallylbenzene and hydroxypropenylbenzenes derivatives
22	Phenethyl alcohol, phenylacetaldehyde and related acetals and esters
23	Aliphatic and aromatic ethers
24	Furfuryl alcohol, furfural, and related substances
25	Furan derivatives
26	Aliphatic and aromatic sulfides and thiols
27	Sulfur-substituted furan derivatives
28	Sulfur-containing heterocyclic and heteroaromatic derivatives
30	Aliphatic and aromatic amines and related amides
31	Nitrogen-containing heterocyclic and heteroaromatic substances
32	Pyrazine derivatives
33	Anthranilate derivatives
34	Amino acids
35	Maltol derivatives
36	Epoxide derivatives

(NTP, 2002a) and >10 mg/kg per day in rats (NTP, 2002b). Conversely, the menthone derivative cannot undergo exocyclic allylic hydroxylation because it contains no allyl group. As expected, the menthone derivative and other structurally related alicyclic ketones, do not exhibit hepatoxicity at dose levels at or above those causing hepatotoxicity with pulegone (Adams et al., 1996). Thus, the pulegone derivatives are now considered as a unique chemical group separate from other alicyclic ketones.

2.4. Formation, function, and composition of the FEMA expert panel

GRAS decisions rely on a broad range of knowledge, experience, and judgment. Biological events are the result of a network of chemical reactions involving macromolecules (proteins, enzymes, nucleic acids) with micromolecules (e.g., flavoring substances) that may, given sufficient concentration, produce physiologic and/or toxicologic effects. The interpretation of these effects in animals, including humans, requires expert scientific judgment that is dependent upon the training, experience, and expertise of the individual. Given the number of different disciplines involved in understanding these biological events, a multidisciplinary approach provides the comprehensive analysis required to address issues regarding human health. Since its inception, the composition of the Panel has been determined by the scientific expertise required to perform an exhaustive assessment of the safety of flavor ingredients added to food. Over time, the Panel's expertise has broadened as new disciplines have developed (i.e. molecular toxicology and genetic toxicology). Currently the areas of expertise among the members of the Panel include toxicology, molecular toxicology, metabolism and disposition, biochemistry, pathology, nutrition, organic chemistry, medicinal chemistry and some related disciplines. Ad hoc consultants provide special expertise not available through regular members of the Panel.

The Panel acts as an independent body of experts and has set its own agenda for more than 40 years. Although Panel decisions affect the flavor industry marketplace, a clear separation exists between the safety activities of the Panel and the commercial interests of the flavor industry. The industry has long recognized the critical part

the Panel plays in self-regulatory aspects of GRAS. It is continuously aware of the need to maintain the unquestioned credibility of the Panel to perform it safety evaluations in a completely independent manner.

3. Criteria for the GRAS evaluation of flavoring substances

The criteria used by the Panel for the determination of GRAS for a flavor ingredient are (1) intake of the flavor ingredient intentionally added to food based on (a) recommended levels of use in specific food categories (e.g., baked goods, chewing gum) and (b) the total annual amount of flavor ingredient sold into the marketplace by the flavor manufacturers of the USA; (2) natural occurrence, purity and specifications; (3) chemical structure and interaction with biologically important macromolecules; (4) metabolic and pharmacokinetic characteristics; and (5) toxicity testing including that for general toxicology, carcinogenicity, genotoxicity screening, and developmental and reproductive toxicology, immunotoxicity, and neurotoxicity. The Panel applies the above criteria simultaneously in the evaluation of individual defined chemical substances and NFCs for their intended use as flavor ingredients. All criteria must be mutually supportive and the data being reviewed must be consistent with similar data for the chemical group or groups to which those substances or mixture of substance belong. All criteria must be unanimously agreed upon by the Panel for it to conclude that a substance can be used safely as a flavor ingredient. The Panel decision is a subjective scientific judgment based on a consideration of all relevant factors, which of course, reflects the scientific training, experience, and expertise of its individual members.

The criteria discussed below provide the basis for the Panel's safety evaluation program for flavoring substances. In some cases, the discussion of individual criteria is supplemented with examples to illustrate how the criteria are applied to individual flavoring substances.

3.1. Estimate of intake of flavor ingredients

The intake of flavor ingredient is defined as the amount of substance ingested and is essential to assessing the safety of food ingredients. Quantifying intake of flavor ingredients is an intimidating task and challenged by many technical and economic difficulties. There are greater than 20,000 different food products and more than 2000 flavor ingredients available for consumption in the western diet (FMI, 1998; Woods and Doull, 1991). Food products are occasionally consumed by a large heterogeneous population, which makes it difficult to determine any one individual's intake of a food constituent. Added to this is the difficulty and expense of obtaining accurate intake data from a detailed dietary analysis of a large enough population to obtain statistically significant results for the diverse group of eaters.

Over its 40 year-plus history, the Expert Panel has used various methods of estimating exposure to flavor ingredients in food. In the absence of any accurate intake data, the Panel in the 1960's estimated daily intake using a method called "the possible average daily intake" (PADI) which is based on the level of flavor ingredient added to various food categories and the amounts of those foods consumed (see below). In Europe, an approach similar to PADI is currently used. The theoretical added maximum daily intake (TAMDI) paradigm is based on the same assumptions as the PADI estimate.

However, as actual intake data became available, it became apparent that the assumptions inherent in the PADI approach produced vastly exaggerated overestimates of intake. The PADI method estimates intake by (1) multiplying usual use levels of the substance in each of 33 food categories (e.g., baked goods and meat products) times the average amount of that food category consumed daily, and (2) summing the intake over all 33 food categories (USDA, 1972). The PADI calculation assumes that all foods in a food category always contain that substance and that the food category is consumed daily (Oser and Hall, 1977). An example of how these assumptions can be problematic is illustrated with the flavoring ingredient, ethyl methylphenylglycidate, which is added to impart strawberry flavor to hard candy. The PADI method assumes all hard candy, including peppermints, cherry-flavored lollipops, and butterscotch, contains ethyl methylphenylglycidate. In addition, the approach assumes that the amount added to the food is actually the amount ingested. This is often not the case in that it fails to account for significant quantities of flavor ingredient lost during food processing (heat processing, baking, etc.). For example, the majority of allyl disulfide, a volatile disulfide, added to garlic breads is lost during the baking process. Since more than 98% of the chemically identified flavor ingredients are low molecular weight substances (MW < 300 D_a), processing (heating) will lead to substantial loss and concomitant lower levels of intake. Based on results of numerous industry surveys (NAS, 1970, 1975, 1982, 1987; Lucas et al., 1999; IOFI, 1995[3]) of the annual volumes of use of flavor ingredients, it is evident that, for the vast majority of flavor ingredients that have low reported annual volumes of use, the PADI method, as currently determined, provides a gross exaggeration of the average daily intake. However, the PADI method could be vastly improved if more accurate data were available on (1) the concentration, not added levels, of flavoring

[3] International Organization of the Flavor Industry (IOFI) (1995) European inquiry on volume use. Private communication to the Flavor and Extract Manufacturers Association (FEMA).

substances in specific foods and (2) the pattern of intake (e.g., number of servings consumed per week) of the foods. The PADI calculation would then provide a meaningful estimate of intake of flavoring substances.

Over the last three decades, two comprehensive studies of flavor intake have been undertaken. One involved a detailed dietary analysis (DDA) of a panel of 12,000 consumers. The other is based on a robust, stochastic model (FSM). The results of the data intensive DDA method and the model-based FSM support the use of annual volume of use data in the determination of daily intake.

The DDA method is based on the detailed reporting of dietary intake of all foods by a panel of consumers over the period of 14 days spread over a year to accommodate seasonal variations in diet (Hall, 1976; Hall and Ford, 1999). The Market Research Corporation of America (MRCA) enlisted a diverse panel of 12,000 consumers that came from urban, suburban and rural communities and ranged in age from infant to over 65 years. The results were then statistically analyzed and sorted by age groups and consumption patterns. The participants had more than 4000 descriptors to choose from over all food eaten. Food categories were narrow and highly specific. For example, the baked goods category was divided into 500 subcategories allowing for garlic bread to not be combined with cinnamon-flavored coffee cake.

This study assessed the amount of each specific food eaten, the frequency of consumption of each food, the amount of flavoring agent in each food and the classification of consumer by age, weight or other pertinent characteristics. The amount of flavoring agent in foods was difficult to determine since flavor formulas were proprietary. These levels were assured by a panel of food chemists and flavorists familiar with the flavor substance levels in particular food products. Once all of the data were compiled, it was taken through 8 steps of analysis to produce average intake levels for both eaters-only groups and non-eaters. Eaters can be defined as participants who consumed foods containing specific flavoring substances; and non-eaters are consumers with zero reported intake for a particular flavoring substance.

Although this data intense method is accurate and reliable, it is expensive and time consuming. Conservative estimates derived from this data are obtained from using the 99th percentile intake levels. In the vast majority of cases, estimates of intake are orders of magnitude less than those obtained from PADI calculations. Based on the results of the DDA study, it was determined that intake could be reasonably predicted by using data from the industry-wide annual poundage surveys. The method is known as the per capita intake (PCI) $\times$ 10 method (Rulis et al., 1984; Woods and Doull, 1991). The method assumes that only 10% of the population consumes the total annual reported volume of use of a flavor ingredient. This approximation provides a practical and cost effective approach to the estimation of intake for flavoring substances. The annual volumes of flavoring agents are relatively easy to obtain by industry-wide surveys, which can be performed on a regular basis to account for changes in food trends and flavor consumption. The 1995 poundage survey of US flavor producers was published by FEMA in 1999 (Lucas et al., 1999).

This method can be evaluated by comparison to the data obtained by the DDA method discussed above. Since the dietary analyses were completed in 1970, it is necessary to use poundage information from that time (NAS, 1970). To correct for possible incompleteness in the 1970 poundage survey, these data are assumed to be 60% of total annual volume of the flavoring agents (0.6 correction factor in the equation below) actually used. The per capita daily intakes (PCI) are then calculated from the annual volume, in kg, for the US population in 1970 (i.e., 210,000,000) by the following equation:

$$\mathrm{PCI(mg/day)} = \mathrm{kg} \times 10^6/210,000,000 \times 0.6 \times 365. \quad (1)$$

The calculated PCI is then multiplied by ten to obtain a reasonably conservative estimate for intake by the eaters of the ingredient. The data obtained from PCI $\times$ 10 is more conservative than that obtained from the DDA method.

For 10 of the substances studied in the panel survey, the PADI is a gross over-estimation of the DDA intake. For two high volume substances, β-ionone and methyl salycilate, PADI is comparable to PCI $\times$ 10. This demonstrates that PADI is a reasonable model to follow for intake estimation of high volume substances that are used in many food categories. However, for low volume substances (i.e., allyl disulfide), it gives an estimation three or four (4) orders of magnitude higher than DDA and three (3) orders of magnitude higher than PCI $\times$ 10.

A second method that has been developed to improve upon our understanding and estimation of exposure to flavoring agents is based upon the theoretical full stochastic model (FSM) (Lambe et al., 2002). It was developed to assist the European Union in their goal to evaluate flavor ingredients. Previously, European intake estimates were based on a TAMDI approach. TAMDI estimates intake based on maximum levels of incorporation to 31 different categories of food or beverages. Dietary intake data were collected from British males aged 16–24 years in the 1988 Dietary and Nutritional Survey of British Adults. This data provided the maximum concentrations, distributions of concentrations and the maximum probability of encountering each substance in a flavored food or beverage in any one of the 31 different categories. TAMDI produces intake estimates,

which are on the same order of magnitude as PADI. If the model is refined and full stochastic treatment of the data is performed, the FSM data are lower than TAMDI estimates by three orders of magnitude for the twelve substances studied.

The FSM allows for the complete randomization of conventional intake data and assumes that only a small portion of the population consumes a given flavoring substance at its maximum level on a daily basis. This application of probability to dietary intake provides a more realistic estimation of intake in that it eliminates the exaggeration that the maximum level of added flavoring is consumed daily in each food category. To some extent even the FSM method overestimates intake in that it does not account for loss due to processing (cooking) or manufacturing waste and the market share of flavored foods in that food category, which has the potential to skew the data depending on the concentration of substance reported in that food. An example of this would be a soft drink containing a maximum concentration of isoamyl acetate which has market dominance at the time of data collection. Therefore, the concentration of isoamyl acetate assumed for all similar soft drinks will be higher in all cases, even though only one brand contains the maximum concentration. If that brand dominance fades, then the familiar scenario of higher estimated intake than manufacture of flavoring agent would occur.

Upon comparison of the FSM and TAMDI methods to the PCI × 10 method it is revealed again that TAMDI, like PADI, is an over estimation of exposure to flavoring ingredients through food consumption. The PCI × 10 method is a reasonably conservative estimation for safety analysis when compared to the levels of exposure calculated by the FSM. The FSM estimates are comparable to those obtained for 10 different substances by the DDA method with respect to order of magnitude.

The FSM study points out that the PCI × 10 estimates are a close match to the FSM data, which is lower by at least one order of magnitude than TAMDI estimates, in most cases. The probability (pFSM>) of FSM methods overestimating intake determined by either TAMDI or PCI × 10 is very small This analysis affirms that FSM estimates are in good agreement with PCI × 10 estimates.

An advantage of using PCI × 10 estimations is that the common problem shared by other methods of a decreasing supply of flavoring substance being eclipsed and surpassed by intake estimates based on food categories cannot occur. The exposure to flavoring agents is strictly limited to the volume distributed for the use in food. Industry poundage surveys are regularly updated as are estimations of the population through census. Improved intake determinations can be obtained provided accurate concentrations of flavoring agents in food and comprehensive food intake data become available. Certainly for particular types of flavoring substances, such as "niche" flavoring substances, it may be useful to collect concentration and intake data. Flavorings used in niche food products consumed by a limited population in a specific geographic region would cause significantly higher exposure even for flavorings that have a low annual volume of reported use. Similar to the DDA study, it seems reasonable to collect appropriate data for selected low volume flavoring substances (Arcella and Leclerq, 2004).

It can be concluded that the DDA and FSM approaches offer a more realistic assessment of intake of flavoring substances through consumption of food. The drawback to the DDA method is the cost and time needed to evaluate the data on a fairly regular basis. The FSM approach requires a fairly extensive food intake survey as well, which is economically challenging. The PCI × 10 method offers a simple calculation based on easily obtained data and its results are consistent with those provided by the DDA and FSM methods. Therefore PCI × 10 offers conservative intake estimates and would be easy to implement on a national and global basis. The Expert Panel uses the PCI × 10 method as a satisfactory means of assessing exposure to flavoring substances.

3.2. The relationship of intake to threshold of toxicologic concern

Based on the results of the most recent annual poundage survey (Lucas et al., 1999) and application of the PCI x10 method, the estimated daily per capita intake ("eaters only") of approximately 85% of all flavoring substances is less than 1 μg/kg/day in the USA. Less than 5 chemically identified substances have a daily per capita intake ("eaters only") greater than 1 mg/kg/day. However, exposure to these few substances occurs principally from their consumption as naturally occurring components of food and not as intentionally added flavoring substances. At these low levels of exposure, it is reasonable to expect that substances used as flavors would not exhibit any significant toxic effects. Compared to structures of other chemicals used as pesticides, herbicides, pharmaceuticals, and industrial chemicals, flavoring agents present no significant structural features that would raise safety concerns, especially at such low levels of use. Based primarily on these two factors, the concept of threshold for toxicologic concern was born, that is, the quantitative levels of exposure below which there is no significant concern for toxic effects.

Early on, the ability to associate structure and exposure to toxic potential was limited to the effect of an atom, functional group (e.g., sulfur atom, aldehyde, amino, carbamoyl, carboxyl, cyano, ester, hydroxyl, nitro, nitrile, and phosphoryl groups), or carbon skeletal

Table 2
Structural class definitions and their human intake thresholds

Class	Description	Fifth percentile noel (mg/kg/day)	Human exposure threshold[a] (μg/day)
I	Structure and related data suggest a low order of toxicity If combined with low human exposure, they should enjoy an extremely low priority for investigation. The criteria for adequate evidence of safety would also be minimal. Greater exposures would require proportionately higher priority for more exhaustive study	3.0	1800
II	Intermediate substances. They are less clearly innocuous than those of Class I, but do not offer the basis either of the positive indication of toxicity or of the lack of knowledge characteristic of those in Class III	0.91	540
III	Permit no strong initial presumptions of safety, or that may even suggest significant toxicity. They thus deserve the highest priority for investigation. Particularly when per capita intake is high of a significant subsection of the population has a high intake, the implied hazard would then require the most extensive evidence for safety-in-use	0.15	90

[a] The human exposure threshold was calculated by multiplying the fifth percentile NOEL by 60 (assuming an individual weighs 60 kg) and dividing by a safety factor of 100.

structure (aliphatic or aromatic compounds). The first attempt to formalize the relationship of chemical structure and the level of use (and importance) to the estimation of toxic hazard was the Cramer et al. decision tree (Cramer et al., 1978). The "decision tree" assigns substances into three classes, *Class I* being those chemicals with simple structures and related data suggesting a low order of oral toxicity. *Class II* substances were those not clearly as harmless as the first class but which did not show positive indications of toxicity or the structural complexity of *Class III*. The last class comprised "those substances that permit no strong initial presumption of safety, or that may even suggest significant toxicity". Class I contains several chemical groups of substances consisting of more than 80% of chemically identified flavor ingredients that are predictably substrates in high capacity detoxication pathways and that are without any adverse biochemical or pharmacologic effect. The chemicals in Class III (i.e., approximately 200 flavor ingredients) that exhibit the highest toxic potential at the lowest levels of exposure are of the greatest interest. In hindsight, it is now apparent that the decision tree was a conservative, but valid approach to screening a large number of chemicals for their toxic potential. However, when evaluating individual chemicals, the structural requirements for assignment to Class III are, in most cases, too generalized and result in the inclusion of many relatively innocuous substances.

The toxic potential of each of the three structural classes has been quantified (Munro et al., 1996). An extensive toxicity database has been compiled for substances in each structural class. The database covers a wide range of chemical structures, including food additives, naturally-occurring substances, pesticides, drugs, antioxidants, industrial chemicals, flavors and fragrances. Conservative no observable effect levels (fifth percentile NOELs) have been determined for each class. These 5th percentile NOELs in each structural class are converted to human exposure threshold levels in μg/day by applying a 100-fold safety factor and correcting for mean bodyweight (60/100). The 5th percentile NOELs and human exposure thresholds are recorded for each structural class (see Table 2). With regards to flavoring substances, these thresholds are even more conservative, given that the vast majority of NOELs for flavoring substances are above the 90th percentile. These conservative exposure thresholds have since been adopted by JECFA (1997) and Commission of the European Communities (EC, 2000) for use in the evaluation of chemically identified flavoring agents.

The Panel has recently adopted these thresholds for their application to the safety evaluation of natural flavor complexes. With the caveat that expert judgment is used at every step in the procedure, these thresholds provide an efficient method to organize and prioritize the significant amount of data on a relatively large number of chemical constituents and chemical groups in an NFC.

In a series of recent papers, Waddell (2002, 2003a,b,c,d) has proposed that the high dose carcinogenicity studies in rodents show evidence of a threshold dose for carcinogenicity. This conclusion was based on fundamental principles of chemistry and the observation that the animal studies show a linear increase in the incidence of tumors in the animals over the range of 2–99% tumors. The analysis of data is based on a dose-response relationship in which dose is plotted as the logarithm of dose in units of molecules/kg bw/day with the scale continuing down to one molecule/kg/day. The dose is typically plotted as the incidence of tumors at each dose level. This scale was first proposed by Rozman et al.

(1996) and the log-linear plot is consistent with the traditional analysis of quantal responses in pharmacology and toxicology. The Panel continues to monitor the progress of the research into carcinogenic thresholds.

3.3. Natural occurrence

The objective of a flavor chemist is not to replace nature, but to duplicate it. Therefore, as analytical methodology has become increasingly sophisticated, many additional flavors and other ingredients have been found in raw and processed food. Literally thousands of flavor ingredients are present in food from natural occurrence alone before and/or after processing for final consumption. Many of the major and minor constituents of natural and/or processed foods, flavors, and otherwise have been identified as discrete chemical structures. If that naturally-occurring chemical is then synthesized it is considered to be identical to the "natural" product. Such substances are recognized as natural-identical. For the evaluation of any synthetic flavor ingredient, the Panel requires documentation of identity, purity, and the method of chemical synthesis. The latter information allows the Panel to evaluate potential toxic intermediates or by-products.

3.4. Chemical structure and interaction with biologically important macromolecules

Key to the safety evaluation process is understanding the relationships between structure and inherent toxicity, and between dose and the onset of toxicity. Without exception, every chemical will become toxic provided a threshold is reached. Although the current state of the science does not permit the exact prediction of the toxic effect of a new chemical, recent studies of organic molecules possessing a range of heteroatoms, functional groups, skeletal structures, and substituents have provided new insight on the interaction of these molecules with bio-functional macromolecules such as protein, glycoprotein, lipoprotein, and nucleic acids. Reaction of these low molecular weight molecules with macromolecules that serve as enzymes, receptors, genes, or permeability barriers is the primary event that eventually determines the biological response to a substance. A reciprocal relationship exists between the macromolecule and the low molecular weight substance. The beneficial or toxic biological response to any substance, a drug, flavoring agent, nutrient or pesticide, is determined by the specific conformation of the target macromolecule. On the other hand, the three-dimensional structure of the substance determines the action on the macromolecule (receptor, enzyme, etc.). Flavor substances are selective for the olfactory and gustatory receptors. Although these functional effects are not of direct concern to the Panel, flavoring substances must be evaluated for their potential to interact with other macromolecules.

The presence of specific atoms, functional groups and substituents greatly influence the nature and extent of interaction of a chemical substance with a macromolecule. The interaction may be direct or indirect, in that the substance may be metabolized prior to interaction with the macromolecule. For chemically identified flavor ingredients used at low levels, the substance undergoes short-lived binding with an enzyme and in the process is converted to an innocuous product that is less reactive with macromolecules and is easily eliminated from the animal. Alternately, the substance may be a substrate for high capacity enzymes involved in catabolism resulting in the complete breakdown of the substance to carbon dioxide and water. Provided levels of intake are low, these macromolecular interactions are either beneficial in that the resulting product is of lower toxicity and, in most cases, more easily excreted that the parent substance. However, as levels of exposure increase, primary enzymatic pathways may become saturated creating conditions for the involvement of other pathways, one or more of which may produce reactive metabolites of greater toxic potential than the parent substance. Reactive metabolites may bind covalently to proteins, membrane components and even nucleic acids.

In the last two decades, research in flavor safety has focused on those few flavor ingredients possessing structural features and concomitant reactivity with macromolecules producing toxic effects at low dose levels in animal studies. It should be emphasized that, even in these cases, the dose levels causing deleterious reactions with macromolecules and subsequent toxicity are orders of magnitude greater than the average daily intake of these substances used as flavor ingredients. The following discussion is intended to demonstrate the type of data on the interaction of flavoring substances with macromolecules currently being considered by the Expert Panel during the safety evaluation of important flavoring substances.

Formation of protein and DNA adduct of p-allylalkoxybenzene derivatives

Based on the metabolic and biochemical evidence, the hepatotoxicity and hepatocarcinogenicity of allylalkoxybenzene derivatives (i.e., estragole and methyl eugenol) in rodents have been related to the dose-dependent formation of the corresponding 1′-hydroxy metabolite (Miller et al., 1985). In repeated oral dose studies in rats, low doses (10 or 30 mg/kg/d for 5 days) of methyl eugenol have been shown to produce a single 44 kDa microsomal protein adduct which is likely formed from the reaction of the electrophilic 1′-hydroxylation metabolite (carbonium

ion) with a peripheral membrane protein (Gardner et al., 1996). It is also the major adduct at higher hepatotoxic dose levels (100 and 300 mg/kg/d) which have been shown to produce as many as 20 other protein adducts. The formation of protein adducts has been directly related to the formation of the 1′-hydroxy metabolite (Borchert et al., 1973; Gardner et al., 1995) in a dose-related manner (Gardner et al., 1995). A similar pattern of adduct formation occurs in vitro when the 1′-hydroxy metabolite is incubated with rat hepatocytes (Gardner et al., 1995, 1996).

In studies beginning two decades ago, methyl eugenol and estragole, the 1′-hydroxy metabolites of methyl eugenol and estragole, and the corresponding sulfate esters of the 1′-hydroxy metabolites were shown to form DNA adducts in vivo and in vitro. Adult female CD-1 mice (mean weight 35 g) were given 12 umol/mouse (58 mg/kg) of [2′,3′-^{3}H]-1′-hydroxyestragole by intraperitoneal injection in trioctanoin, and DNA adduct formation was monitored over 20 days post exposure. Similarly, 9-day old male or female B6C3F1 mice (mean weight, 6g) were given intraperitoneal injections of 0.5 μmol (14 mg/kg) of labeled estragole and sacrificed after 23 h. Three adducts were formed by the reaction of 1′ or 3′ positions (*cis* or *trans* isomers) of estragole with the exocyclic amino group (N^2) of deoxyguanosine. An additional adduct was formed by the reaction of the 3′ position of estragole and the N^6 position of deoxyadenosine. Unlike adducts of aromatic amines (e.g., N-acetyl-2-aminofluorene) which persist at near maximum levels of binding for several weeks, the three adducts of estragole-deoxyribonucleoside were removed rapidly from mouse liver DNA. Timed measurement of DNA adducts revealed a biphasic loss indicated by a sharp decline in one of the two major 1′-hydroxyestragole adducts followed by relatively constant levels of liver DNA adducts from days 3 to 20. This suggests that at least one of the adducts undergoes excision repair. Dose levels of the 1′-hydroxyestragole in the adult female and pre-weanling male and female mice were approximately 58 mg/kg bw and 14 mg/kg bw, respectively (Phillips et al., 1981).

In ^{32}P-post-labelling experiments with adult female CD-1 mice (mean weight, 25 g) (Randerath et al., 1984), a 2 or 10 mg dose of estragole, methyl eugenol, safrole or other alkenylbenzene derivative was given by intraperitoneal injection and liver DNA samples were collected 24 h later. The dose levels in this study were equivalent to 100 or 500 mg/kg bw of each test substances. Safrole, methyl eugenol, and estragole show binding activities higher than allylbenzene, anethole, and other allyl substituted benzene derivatives. Similar to the previous experiment, a rapid drop in total adduct formation occurred within 7 days after dosing and was followed by a relatively constant level over the next 140 days. The authors noted that the significant decrease in DNA adduct levels was probably related to DNA repair processes.

In a related ^{32}P-post-labelling experiment (Phillips et al., 1984), newborn male B6C3F1 mice were given 0.25, 0.5, 1.0, and 3.0 umol of the same series of allylbenzene derivatives by intraperitoneal injection on day 1, 8, 15, and 22, respectively, after birth. Dose levels on days 1 and 22 were estimated to be approximately 27 and 35 mg/kg bw, 1′-hydroxyestragole and 1′-hydroxysafrole, respectively. Mice were terminated on days 23, 29, and 43 and their liver DNA was isolated and analysed. Highest DNA adduct levels were measured for methyl eugenol, estragole, and safrole. A significant ($p < 0.05$) amount of adduct was detected at 43 days. Based on the results of a study of carcinogenic activity of these substances in the same species and strain (Miller et al., 1983), the authors concluded that threshold adduct levels of at least 15 pmoles/mg of DNA at 23 days were required for statistically significant tumour formation (Phillips et al., 1984). The authors also noted that, compared to adults, newborn mice showed greater sensitivity to alkenylbenzene carcinogenicity.

Based on the protein and DNA adduct studies the Expert Panel concludes that methyl eugenol and estragole can form covalently-bound protein and DNA adducts. It is anticipated that the 1′-hydroxy derivative is the reactive intermediary metabolite that forms protein and DNA adducts, since the 1′-hydroxy metabolite forms these same adducts but at lower dose levels than for the parent substance. Studies performed at relatively high dose levels (15 mg/kg bw) demonstrate that the 1′-hydroxy metabolite reacts with an exocyclic amine function to form a single covalent bond to the deoxyribonucleoside. Clearly, at high dose levels, DNA adduct formation has been directly related to administration of the parent allylalkoxybenzene derivative or its principal hepatotoxic metabolite. Currently there is no information on the formation of DNA adducts at dose levels <10 mg/kg bw; there is some evidence that liver tumors are not induced at detectable levels at a low dose level (1.5 mg/kg bw) of the 1′-hydroxy metabolite (Wiseman et al., 1987).

3.5. Metabolism and disposition

Upon completion of the Scientific Literature Reviews (1974–1979), the Expert Panel concluded that data on

dose-dependent metabolism was critical to understanding the quantitative link between the biochemical fate of a substance and the dose resulting in toxicity. Beginning in 1980, a series of dose-dependent metabolism studies were undertaken on key flavor ingredients (benzyl acetate, cinnamaldehyde, furfural, anethole, estragole, 2-ethyl-1-hexanol) for which extensive toxicity and carcinogenicity data were available. The objective of the studies was to evaluate the effect of dose on the metabolic options available to a substance and correlate those data with dose levels required for the onset of toxicity.

Fundamentally, the metabolic options available to a substance depend on the chemical structure, that is, the functional group or groups, carbon skeleton, position and type of substituents, and the electronic and steric forces present. Ordinarily, the molecule is enzymatically altered to increase its hydrophilic properties to facilitate excretion via the kidney or entero-hepatic system. In this case, the toxicity of the substance is decreased by metabolism (detoxication). Often there are multiple metabolic options available in an animal for the detoxication of a substance. This is not unexpected, given redundancy in the evolutionary process. However, in many cases, as dose increases, other metabolic pathways may emerge due to saturation of high affinity metabolic pathways (e.g., sulfation) or xenobiotic induction of expression of other metabolic pathways (e.g., cytochromes P450). Detoxication and metabolic activation pathways compete and the predominance of a particular pathway is governed by a threshold dose of substance required to induce a particular pathway and the capacity of each pathway to react to a given quantity of substance.

Typically, as one or more detoxication pathways become saturated due mainly to an increase in dose, metabolic activation may emerge. This is particularly true for conjugating enzymes that scavenge reactive intermediates. Since toxicity studies are routinely performed at high dose levels (i.e., those required to induce toxicity), it is critical to evaluate the metabolic events occurring at anticipated low levels of exposure from use as a flavor ingredient. The change in metabolic disposition that occurs with increasing dose provides a basis for evaluating the safe use of a flavor ingredient at low levels compared to animal toxicity observed at high dose levels.

In addition to dose, the threshold and the capacity of a metabolic pathway are dependent on the species and sex of the animal studied. Since animal models (e.g. rats, mice, and dogs) must be relied on to evaluate the toxic potential of a substance in man, a properly designed study of metabolism should be performed over a broad range of dose levels (including toxic dose levels) in different species.

The evaluation of dose-, sex-, and species-dependent metabolic studies has become an integral part of the Panel's safety evaluation program. The metabolic fate of anethole described below demonstrates the Panel's integration of metabolic data with observed toxicological effects.

Metabolism of anethole

trans-Anethole (FEMA No. 2086) is 4-methoxypropenylbenzene. It is the major volatile component in sweet and bitter fennel, and anise. Based on the reported annual volume in 1987 of 17,100 kg (NAS, 1987), the estimated daily per capita intake ("eaters only") is 0.054 mg/kg bw/d from use of anethole as an added flavor ingredient. It is calculated as follows: $\mu g/kg\ bw = (\text{Annual volume, kg})\ (1 \times 10^9\ \mu g/kg) \times (1/260 \times 10^6\ \text{people in the US}) \times (1/365\ \text{days}) \times (1/60\ \text{kg bw}) \times (1/0.6) \times 10$ (eaters only) and is based on the assumptions that (1) only 10% of the population, the "eaters only", consumed the entire reported annual volume of a flavoring substance (NAS, 1987) and, (2) only 60% of the flavor volume was reported by flavor manufacturers in the 1987 annual survey (NAS, 1987).

In 1989, it was reported that chronic intake of high dose levels of *trans*-anethole in the female rat was associated with hepatotoxicity and a low incidence of liver tumors (Truhau et al., 1989; Newberne et al., 1989). Subsequent studies on the toxicity, pharmacokinetics, and metabolism of *trans*-anethole in laboratory animals and humans have been performed and were used in a mechanism-based safety evaluation to interpret the observed hepatotoxicity and related tumorigenic effects (Newberne et al., 1999). Changes reported in the liver of laboratory rodents exposed to *trans*-anethole in the diet for periods more than 2 years (i.e., 833 days) may be further understood in terms of the hepatic intoxication pathway in which *trans*-anethole is metabolized to the ultimate hepatotoxic agent, anethole epoxide.

In a 2-year study, Sprague–Dawley rats exhibited evidence of hepatotoxicity at dietary levels of 200 and 400 mg/kg bw/d for males and 250 and 550 mg/kg bw/d for females based on a statistically significant increased incidence of focal and nodular hyperplasia, sinusoidal dilatation, and distended bile ducts (Truhau et al., 1989). An independent histopathological evaluation confirmed the presence of hepatic injury at these dose levels (Newberne et al., 1989). Additionally, a statistically significant increase in hepatocellular carcinoma was reported in female rats at the highest dose level (i.e., 550 mg/kg bw/d) (Truhau et al., 1989; Newberne et al., 1989). The neoplasms occurred primarily in livers with significant non-neoplastic lesions indicative of hepatotoxicity and

necrosis (Newberne et al., 1989). They were found to be of late onset, after week 98, and had no effect on the longevity of the animals. Based on the evidence of hepatotoxicity in males at ⩾200 mg/kg bw and females at ⩾250 mg/kg bw, the NOAEL for the study was concluded to be 100 mg/kg bw/d for males and 120 mg/kg/bw/d for females (Truhau et al., 1989; Newberne et al., 1989). In a more recent 90-day dietary study, no significant hepatotoxicity was observed in male or female rats at dose levels up to 300 mg *trans*-anethole/kg bw/d (≈55–60 mg AE/kg bw/d) for 90 days (Minnema, 1997).

Subsequent to the 2-year study, biochemical and metabolic studies were performed that demonstrate that the hepatotoxicity observed in rats exposed to *trans*-anethole is associated with the dose-dependent metabolic formation of anethole epoxide (AE) (Sangster et al., 1984a,b; Bounds and Caldwell, 1996). At high dose levels (⩾100 mg *trans*-anethole/kg bw), a metabolic shift to greater epoxidation in rats leads to increased hepatocellular concentrations of AE. Epoxidation is more pronounced in rats than mice (Sangster et al., 1984a,b; Bounds, 1996) and significantly greater than in humans (Sangster et al., 1987; Caldwell and Sutton, 1988) at low dose levels (i.e., <12 mg *trans*-anethole/kg bw/d). Therefore, the rat is the more sensitive rodent species for evaluating the potential for AE-related hepatotoxicity in humans exposed to *trans*-anethole from use as a flavoring substance.

At low doses of *trans*-anethole in rats, AE is readily detoxified by enzymes such as epoxide hydrolase (EH; fast-acting enzyme) and glutathione-*S*-transferase (GST; slower detoxication enzyme) (Marshall and Caldwell, 1992). With increasing dose levels, hepatic levels of AE increase and these enzymes (especially EH) approach saturation leading to cytotoxicity. The female rat is probably more sensitive than the male, since it exhibits lower EH activity that would result in higher hepatocellular levels of AE (Meijer et al., 1987). Saturation of these detoxication enzymes (i.e., EH and GST) is directly related to an increase in the cytotoxic effects of *trans*-anethole. AE is approximately 10 times more cytotoxic than *trans*-anethole in the hepatocytes of rats (Marshall and Caldwell, 1992, 1996; Caldwell, 1991; Caldwell et al., 1991; Caldwell et al., 1992). This difference corresponds approximately to the proportion (12–18%) of *trans*-anethole that is metabolized to AE in rats at dose levels (⩾200 mg *trans*-anethole/kg bw/d) required to observe hepatotoxicity. Taken together, these data indicate that cytotoxicity and hepatotoxicity are linked metabolically to the formation of AE from *trans*-anethole in the liver.

Based on repeated dose metabolic studies, daily production of AE is higher in female rats as compared to male rats. At higher dose levels more *trans*-anethole is converted to AE that saturates the "fast-acting" epoxide hydrolase detoxication pathway in the liver. This leads to increased hepatocellular AE concentrations that may react with glutathione or cellular components. At the highest dose level of anethole (i.e., 400 mg/kg/d in males and 550 mg/kg bw/d in females) daily hepatic production of AE in females (120 mg/kg bw) was at least twice that of males (≈50 mg/kg bw). The NOAEL of 120 mg *trans*-anethole/kg bw/d for female rats in the 2-year study corresponds to production of approximately 22 mg AE/kg bw/d, which is >10,000 times the level of 0.002 mg AE/kg bw/d produced by humans from intake of *trans*-anethole as a flavoring substance.

The panel concludes that daily exposure to significant levels of AE must continue over a long duration in order to observe the onset of hepatotoxicity in rats. Cumulative exposure to AE may be directly related to the incidence and severity of the observed dose-dependent hepatotoxicity. Hepatotoxicity reported in female rats at dose levels ⩾ 250 *trans*-anethole/kg bw/d in the 2-year study corresponds to cumulative AE production of at least 20,000 mg AE/kg bw. It is postulated that continuous exposure to high dose levels of *trans*-anethole leads to a continuum of biochemical and toxicological events: (1) hepatic metabolism to form AE; (2) saturation of rapid (EH) detoxication pathways; (3) concomitant increase in hepatocellular concentrations of AE; (4) cytotoxicity; (5) cell death (necrosis); (6) hepatocyte proliferation; and ultimately (7) liver tumors in a few female rats as reported in the 2-year study (Truhau et al., 1989).

The low incidence (6/52) of carcinomas observed in the severely compromised livers of female rats given 550 mg *trans*-anethole/kg bw/d in the 2-year study occurred following an estimated total lifetime exposure to AE exceeding 100,000 mg/kg bw. The fact that hepatocellular carcinomas occurred only in the female rat is a reflection of a higher daily dose of *trans*-anethole for the females, increased conversion to AE compared to the male, and decreased detoxication of AE by the female which exhibits a lower activity of the AE-detoxication enzyme EH compared to that of the male (Meijer et al., 1987).

3.6. Toxicology

As previously described, the Panel considers flavoring substances in the context of homologous chemical

groups. If sufficient biochemical and metabolic information on prototype members of the chemical group are available and if the substances belong to a well-studied and toxicologically innocuous group of substances used at low levels in food, toxicological data on each member of the group may not be required to reach a GRAS decision. However, under some circumstances, even though information on safety and metabolic fate is available for other members of the chemical group, the Panel may conclude that toxicological data should be obtained for a specific substance.

In the past when additional data have been needed, a simple minimal test, referred to as a "Primary Toxicity Screen" (PTS), developed by the Panel for such needs, served to replace the standard LD50 test and to provide a minimal amount of biological data. If the results from the PTS so indicated, more definitive tests were then required, on a case-by-case basis, usually sufficient to establish a no observed adverse effect level (NOAEL). The 14-day PTS protocol provided important data in alleviating the scientific and regulatory concerns of the times. However, advances in molecular biology now reveal potential methods for enhancement of histological observations.

After carefully considering the OECD's "*Guidelines for Testing Chemicals. No. 407 Repeated Dose Oral Toxicity—Rodent: 28 day study or 14 day study*" (OECD, 1981), the Panel decided to modify the toxicity testing guidelines, now referred to as the 4-Week Toxicity Study for Flavoring Substances, to meet the following objectives: (1) provide a test that meets the special needs of flavor materials; (2) maintain a flexible test protocol that is compatible with the ones currently recommended by national and international regulatory agencies; and (3) expand the toxicity testing to characterize adverse effects to the nervous, reproductive and immune systems, if necessary.

3.6.1. Carcinogenicity studies on flavoring ingredients

For more than two decades, the Panel has critically reviewed the results of carcinogenicity studies of more than 20 flavoring substances or their principal metabolites, the majority of which were sponsored by the National Toxicology Program (NTP). Given that the studies were hazard determinations, they were normally performed at dose levels orders of magnitude greater than the daily intakes as added flavor ingredients or as naturally occurring constituents of food. Even at these high intake levels, the majority of flavoring ingredients show no carcinogenic potential (Table 3). In addition to dose, the carcinogenic potential of remaining flavor ingredients are related to several factors including mode of administration, species and sex of animal model, and target organ specificity. In the vast majority of studies, the carcinogenic effect is secondary to pre-existing chronic organ toxicity. Selected subgroups of structurally related flavor ingredients (e.g., aldehydes, terpene hydrocarbons) are associated with a single target organ and tumor type in a specific species and sex using a single mode of administration. Common features among these substances provide valuable information for the mechanism of carcinogenic activity.

Carcinogenic effects related to the mode of administration

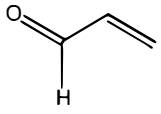
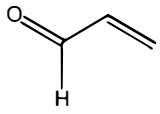
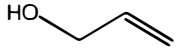
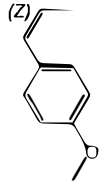
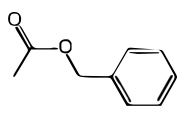
Forestomach neoplasms in rodents chronically administered irritating reactive substances by gavage. The appearance of forestomach hyperplasia and squamous cell papillomas in rodents is a regular occurrence in bioassay gavage studies in which high concentrations of an irritant material in corn oil is delivered daily by dosing tube into the forestomach. These phenomena are consistently associated with administration of high concentrations of aldehydes, e.g., malonaldehyde, furfural, benzaldehyde, and *trans,trans*-2,4-hexadienal (NTP, 1988, 1990c,b, 2003b) and other irritating substances, e.g., ethyl acrylate, benzyl acetate, dihydrocoumarin and coumarin (NTP, 1986a, 1993a,b,c) in corn oil by gavage. The lack of any evidence of carcinogenicity in bioassays in which aldehydes (e.g. citral and cinnamaldehyde) (NTP, 2003a; NTP, 2004) or reactive substances (e.g., benzyl acetate) (NTP, 1986b) were provided in microencapsulated form administered in the diet illustrates the impact of the mode of administration on the toxicological sequelae in the rodent forestomach. Future design of 2-year bioassay studies with low molecular weight, irritant substances should avoid the use of gavage as a mode of administration.

Carcinogenic effects specific to rodents

Kidney neoplasms in male rats chronically exposed mainly to hydrocarbons, ketones and other substances.

Administration of high dose levels of hydrocarbons (limonene, camphene, decalin, and JP-4 jet fuel) and other substances (e.g., isophorone, methyl isobutyl ketone, and α-methylbenzyl alcohol) in short-(Hoechst, 1991; Wolf et al., 1956; Webb et al., 1989; Spindler and Madsen, 1992) and long-term studies (EPA, 1991; or NTP, 1990) exhibit renal lesions resulting from the accumulation of aggregates of α2u-globulin (a low molecular-weight protein synthesized in the male rat liver) and the test substances or a metabolite in the P2 segment of the renal proximal tubule. These aggregates prevent lysosomal degradation which leads to accumulation

Table 3
Summary of carcinogenicity studies on FEMA GRAS substances

Substance (FEMA No.)	Daily per capita Intake ("eaters only"), μg/kg bw/day	Route of administration	Dose levels, mg/kg bw/day	Statistically significant ($P < 0.05$) dose-related neoplastic effects in rats (% tumors at each dose level)	Statistically significant ($P < 0.05$) dose-related neoplastic effects in mice (% tumors at each dose level)	Reference (date)	Evaluation of positive results
Negative results:							
Acrolein 	Not applicable	Oral, drinking water	Groups of 20m and 20f F344 rats were given drinking water containing 100, 250 or 625 mg/L 5 days/week for 124, 124 or 104 weeks, respectively	None	Not applicable	Lijinsky and Reuber (1987)	Not applicable
Acrolein 	Not applicable	Gavage, water	Groups of 70m and 70f Sprague–Dawley rats were administered 0, 0.05, 0.5 or 2.5 mg/kg bw 7 days/week for 102 weeks	None	Not applicable	Parent et al. (1992)	Not applicable
Allyl alcohol 	Not applicable	Oral, drinking water	A group of 20m and 20f F344 rats was given drinking water containing 300 mg/L allyl alcohol 5 days/week for 106 weeks	None	Not applicable	Lijinsky and Reuber (1987)	Not applicable
Anethole (2086) *(Z)* 	100.57	Oral, feeding study	CD-1 mice (f only) 0% or 0.46% for 12 months	Not applicable	None	Miller et al. (1983)	Not applicable
Benzyl acetate (2135) 	14.24	Oral, feeding study	0, 3000, 6000 or 12,000 ppm (R); 0, 330, 1000, 3000 ppm (M) 60 animals/sex/group	None	None	NTP (1993a)	Not applicable

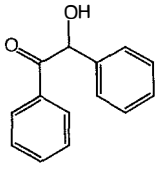
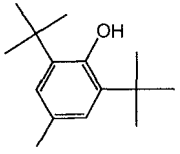
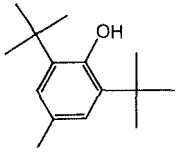
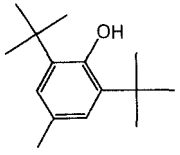
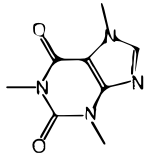
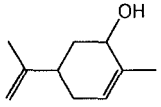
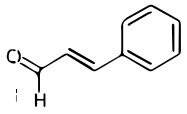
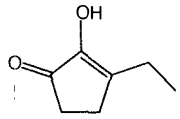
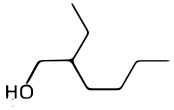
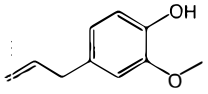
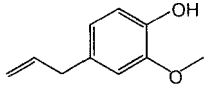
Benzoin (2132) 	0.35	Oral, feeding study	0, 125 or 250 ppm (mR) 0, 250 or 500 ppm (fR) 0, 2500 or 5000 ppm (M) 104 weeks	None	None	NCI (1980b)	Not applicable
Butylated hydroxytoluene (BHT) (2184) 	61.24	Oral, feeding study	0, 3000 or 6000 ppm 105 weeks (R) 107–108 weeks (M)	None	None	NTP (1979)	Not applicable
Butylated hydroxytoluene (BHT) (2184) 	61.24	Oral, feeding study	0%, 0.25% or 1% WistarR 57m and 57f/group 104 weeks	None	Not applicable	Hirose et al. (1981)	Not applicable
Butylated hydroxytoluene (BHT) (2184) 	61.24	Oral, feeding study	0, 200, 1000 or 5000 ppm (M) 96 weeks	Not applicable	None	Shirai et al. (1982)	Not applicable
Caffeine (2224) 	4022.90	Oral, drinking water	0%, 0.1% or 0.2% WistarR 78 weeks	None	Not applicable	Takayama and Kuwabara (1982)	Not applicable
d-Carvone (2247) 	0.01	Gavage, corn oil vehicle	0, 375 or 750 (mice only)	Not applicable	None	NTP (1990b)	Not applicable

Table 3 (*continued*)

Substance (FEMA No.)	Daily per capita Intake ("eaters only"), μg/kg bw/day	Route of administration	Dose levels, mg/kg bw/day	Statistically significant ($P < 0.05$) dose-related neoplastic effects in rats (% tumors at each dose level)	Statistically significant ($P < 0.05$) dose-related neoplastic effects in mice (% tumors at each dose level)	Reference (date)	Evaluation of positive results
trans-Cinnamaldehyde (2286) 	990.00	Diet (microencaps)	0, 50, 100 or 200 mg/kg bw in male and female F344/N rats and 0, 125, 270 or 550 mg/kg bw in male and female B6C3F1 mice	None	None	NTP (2002)	Not applicable
3-Ethyl-2-hydroxy-2-cyclopenten-1-one (3152) 	0.19	Oral, feeding study	0, 30, 80 and 200 to Charles River CD-COBS rats through 3 generations and to F1 generation for 2 years	None	Not applicable	King et al. (1979)	Not applicable
2-Ethyl-1-hexanol (3151) 	0.40	Gavage, distilled water with 5 mg Polyoxyl 35 Caster Oil (CremophorÒ EL) per 100 mL vehicle	0, 50, 200, or 750 (M) 78 weeks 0, 50, 150, or 500 (R) 104 weeks	None	None	Astill et al. (1996)	Not applicable
Eugenol (2467) 	56.06	Oral, feeding study	CD-1 mice (f only) 0% or 0.50% for 12 months	Not applicable	None	Miller et al. (1983)	Not applicable
Eugenol (2467) 	56.06	Feeding study	0, 6000 or 12,500 ppm (fR) 0, 3000 or 6000 (mR, M)	None	None	NTP (1983b)	Not applicable

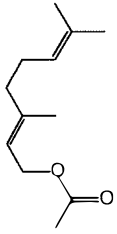
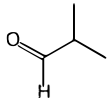
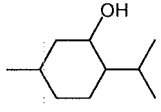
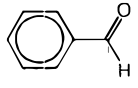
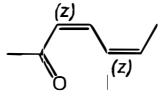
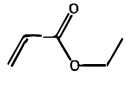
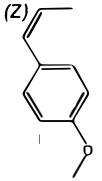
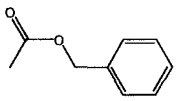
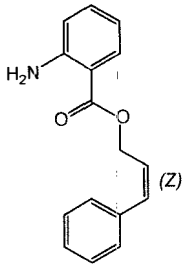
Geranyl acetate (71%), Citronellyl acetate (29%) (2509) 	8.16	Gavage, corn oil vehicle	0, 1000, or 2000 (R) 0, 500 or 1000 (M)	None	None	NTP (1987)	Not applicable
Isobutyraldehyde (2220) 	1.10	Inhalation	0, 500, 1000 or 2000 ppm	None	None	NTP (1999)	Not applicable
Menthol (2665) 	527.76	Feeding study	0, 3750, 7500 (R) 0, 2000, 4000 ppm (M)	None	None	NCI (1978)	Not applicable
Forestomach effects:							
Benzaldehyde (2127) 	600.45	Gavage, corn oil vehicle	0, 200 or 400 mg/kg bw in male and female F344/N rats and B6C3F1 mice 0, 300 or 600 mg/kg bw in female B6C3F1 mice for 104 wks	None	Forestomach: Squamous cell papillomas: (females at 300 mg/kg bw, 10%; 600 mg/kg bw, 12%) lower incidence in males	NTP (1990c)	Forestomach tumors associated with chronic irritant effects (Smith and Ford, 1993)
2,4-Hexadienal (3429) 	0.003	Diet (microencaps)	0, 22.5, 45, 90 mg/kg bw in rats and 30, 60, and 120 mg/kg bw in B6C3F1mice for 104 weeks	Forestomach: Squamous cell carcinoma and papillomas (mf); (males at 22.5 mg/kg bw, 7%; 45 mg/kg bw, 25%, 90 mg/kg bw, 67%) lower incidence in females	Forestomach: Squamous cell carcinoma and papillomas (mf); (females at 30 mg/kg bw, 4.3%; 60 mg/kg bw, 23%, 120 mg/kg bw, 39%) lower incidence in males	NTP (2003a)	Forestomach tumors associated with chronic irritant effects (Smith and Ford, 1993)
Ethyl acrylate (2418) 	0.01	Gavage, corn oil vehicle	0, 100 or 200 mg/kg bw in Fischer 344 rats and B6C3F1 mice for 104 weeks	Forestomach: Squamous cell carcinoma and papillomas: (males at 100 mg/kg bw, 36%; 200 mg/kg, 72%); lower incidence in females	Forestomach: Squamous cell carcinoma and papillomas: (males at 100 mg/kg bw, 11%; 200 mg/kg bw, 24%) lower incidence in females	NTP (1986a)	Forestomach tumors associated with chronic irritant effects (NTP, 1986a)

Table 3 (*continued*)

Substance (FEMA No.)	Daily per capita Intake ("eaters only"), μg/kg bw/day	Route of administration	Dose levels, mg/kg bw/day	Statistically significant ($P < 0.05$) dose-related neoplastic effects in rats (% tumors at each dose level)	Statistically significant ($P < 0.05$) dose-related neoplastic effects in mice (% tumors at each dose level)	Reference (date)	Evaluation of positive results
Liver effects:							
Anethole (2086) 	100.57	Oral, feeding study	0%, 0.25%, 0.5% and 1% (105–1000 mg/kg bw/day) groups of 312m and 312f Sprague–Dawley rats 117–121 weeks	Liver: Hepatocellular neoplasms (females at 1000 mg/kg bw, 23%)	Not applicable	Truhaut et al. (1989)	Although statistically significant, the increase hepatocellular carcinoma remained within the historical control range for the strain of rat and laboratory
Benzyl acetate (2135) 	14.24	Gavage, corn oil vehicle	0, 250 or 500 mg/kg bw day in groups of 50 male and 50 female F344/N rats 0, 500 or 1000 mg/kg bw day in groups of 50 male and female B6C3F1 mice for 103 weeks	Pancreas: Acinar-cell adenoma (males at 250 mg/kg bw, 54%; 500 mg/kg bw, 76%); testis mentioned but data not shown	Liver: Adenomas or carcinomas (males at 500 mg/kg bw, 37%; 1000 mg/kg bw, 46%; females-lower incidence at high dose only); Forestomach: Squamous cell papilloma or carcinoma (males at 500 mg/kg bw, 8%; 1000 mg/kg bw, 22%; females- lower incidence at high dose only)	NTP (1986b)	Neoplasms in mice associated with the gavage route of administration and high spontaneous incidence of this type of neoplasm in this mouse. No tumors were observed in a subsequent feeding study of benzyl acetate (NTP, 1993a)
Cinnamyl anthranilate (2295) 	0	Feeding study	Groups of 50 F344/N rats and 50 B6C3F1 mice were fed 0, 15,000 or 30,000 ppm (0, 750, 1500 mg/kg bw) for 103 weeks	None	Liver: Hepatocellular carcinomas or adenomas (combined) (males at 750 mg/kg bw, 60%; 1500 mg/kg bw, 79%) lower incidence in females	NCI (1980b)	Neoplasms in mice associated with high spontaneous incidence of this type of neoplasm in this strain of mouse; the MTD was exceeded and may have been a confounding effect (Adams et al., 2004)

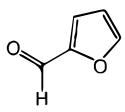
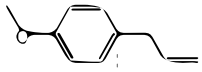
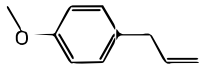
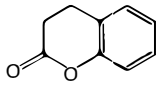
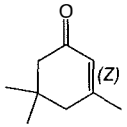
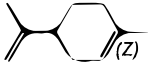
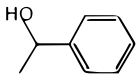
Furfural (2489) 	7.68	Gavage, corn oil vehicle	0, 30 or 60 mg/kg bw in F344/N rats and 0, 50, 100, or 175 mg/kg bw in B6C3F1 mice for 103 weeks	Liver: Cholangiocarcinomas (males at 60 mg/kg bw, 2%) and bile duct dysplasia with fibrosis	Liver: Hepatocellular carcinomas and adenomas (males at 50 mg/kg bw, 44%; 100 mg/kg bw, 35%; 175 mg/kg bw, 64%) lower incidence in females	NTP (1990c)	Rat and mouse liver tumors were either insignificant at $P < 0.01$ (Haseman et al., 1986), or secondary to chronic hepatotoxicity (Adams et al., 1997)
Estragole (2411) 	1.22	Oral, diet	CD-1 mice (f only) 0, 2300 ppm, or 4600 ppm for 12 months	Not applicable	Hepatomas (females at 2300 ppm, 58%; 4600 ppm, 71%)	Miller et al. (1983)	
Estragole (2411) 	1.22	Gavage	Weanling CD-1 mice 370 mg/kg, twice weekly for 10 times. Animals examined after 14 months	Not applicable	Hepatomas: (males at 0 mg/kg bw, 24%; 370 mg/kg bw, 73%) lower incidence in females	Miller et al. (1983)	
Pyridine (2966) 	0.07	Drinking water	8, 17, 36 mg/kg in male F344/N rats for 104 weeks and 35, 65, or 110 in B6C3F1 mice for 104 (female) or 105 (male) weeks	None	Hepatocellular carcinomas or blastomas in mice: (males at 250 mg/kg bw, 94%; 500 mg/kg bw, 94%, 1000 mg/kg bw, 94%) lower incidence in females	NTP (1997c)	
Liver and kidney effects: 3,4-Dihydrocoumarin (2381) 	18.52	Gavage, corn oil vehicle	0, 150, 300 or 600 mg/kg bw in groups of 60 male and 60 female F344/N rats and 0, 200, 400 or 800 in 70 male and 70 female B6C3F1 mice (M) for 105 weeks	Kidney: Renal tubule hyperplasia (males at 150 mg/kg bw, 5%; 300 mg/kg bw, 6%; 600 mg/kg bw, 8%) and adenomas (males at 150 mg/kg bw, 1%; 300 mg/kg bw, 3%; 600 mg/kg bw, 6%) lower incidence for both in females	Liver: Hepatocellular adenomas, carcinoma, or hepatoblastoma (combined): (males at 200 mg/kg bw, 59%; 400 mg/kg bw, 78%; 800 mg/kg bw, 68%) lower incidence in females	NTP (1993b)	Rat kidney lesions species/sex specific (Smith et al., 1996). Mouse lesions associated with high incidence of spontaneous lesions an an inconsistent dose response (Smith et al., 1996)

Table 3 (*continued*)

Substance (FEMA No.)	Daily per capita Intake ("eaters only"), μg/kg bw/day	Route of administration	Dose levels, mg/kg bw/day	Statistically significant ($P < 0.05$) dose-related neoplastic effects in rats (% tumors at each dose level)	Statistically significant ($P < 0.05$) dose-related neoplastic effects in mice (% tumors at each dose level)	Reference (date)	Evaluation of positive results
Isophorone (3553) 	0.002	Gavage, corn oil vehicle	0, 250 or 500 mg/kg bw to groups of 50 F344/N rats and 50 B6C3 F_1 mice (M) for 103 weeks	Kidney: Renal tubular cell adenomas (males at 500 mg/kg bw, 4%) or adenocarcinomas (males at 250 mg/kg bw, 6%); carcinomas of the preputial gland (males at 250 mg/kg bw, 10%; 500 mg/kg bw, 10%)	Liver: Hepatocellular adenomas or carcinomas (combined) (males at 250 mg/kg bw, 36%; 500 mg/kg bw, 58%) and mesenchymal tumors in the integumentary system (males at 250 mg/kg bw, 16%; 500 mg/kg bw, 28%)	NTP (1986c)	Kidney neoplasms, $\alpha_{2\mu}$-globulin phenomenon; preputial neoplasms, high spontaneous incidence in this rat; Liver neoplasms, a species-specific effect; neoplasms of integumentary system, high spontaneous incidence in this rat (Adams et al., 1996)
Kidney effects:							
d-Limonene (2633) 	212.10	Gavage, corn oil vehicle	0, 75 or 150 mg/kg bw to groups of 50 F344/N male rats; 0, 300, 600 mg/kg bw to groups of 50 female F344/N rats; 0, 250 or 500 mg/kg bw to groups of 50 male (mM) 0, 500 or 1000 (fM) for 103 weeks	Kidney: Renal tubular cell hyperplasia (males at 75 mg/kg bw, 8%; 150 mg/kg bw, 14%); adenomas (males at 75 mg/kg bw, 8%; 150 mg/kg bw, 16%); and adenocarcinomas (males at 75 mg/kg bw, 8%; 150 mg/kg bw, 6%) no lesions observed in females	None	NTP (1990d)	Kidney neoplasms associated with $\alpha_{2\mu}$-globulin, specific to the male rat (Burdock et al., 1990; EPA, 1991)
α-Methylbenzyl alcohol (2685) 	1.21	Gavage, corn oil vehicle	0, 375 or 750 mg/kg bw in F344/N rats and B6C3F1 mice for 105 weeks	Kidney: Renal tubular cell adenomas and adenomas or adenocarcinomas (combined) (males at 375 mg/kg bw, 4%; 750 mg/kg bw, 10%)	None	NTP (1990e)	Inadequate study (Smith and Ford, 1993)

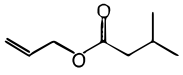
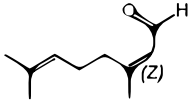
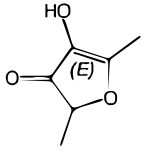
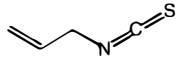
Other effects:							
Allyl isovalerate (2045) 	0.01	Gavage, corn oil vehicle	0, 31, or 62 mg/kg bw to groups of 50 male and 50 female F344/N rats and B6C3F1 mice for 105 weeks	Hematopoietic system: Mononuclear cell leukemia (females at 31 mg/kg bw, 12%; 62 mg/kg bw, 18%) lower incidence in males	Hematopoietic system: Lymphoma (females at 31 mg/kg bw, 22%; 62 mg/kg bw, 36%) lower incidence in males	NTP (1983c)	Rat and mouse lesions not statistically significant ($P < 0.01$) (Haseman et al., 1986; Smith et al., 1996); blood effects previously observed in controls at this lab, associated with *Klebsiella* infection
Citral (2303) 	116.50	Feed, microencaps	1000, 2000, or 4000 ppm (50, 100, or 210 mg/kg bw) to groups of 50 male and 50 female F344/N rats for 105 weeks 500, 1000, or 2000 ppm (60, 120, or 260 mg/kg bw) to groups of 50 male and 50 female B6C3F1 mice for 105 weeks	None	Malignant lymphoma (all types): (females at 60 mg/kg bw, 10%; 120 mg/kg bw, 18%; 260 mg/kg bw, 24%)	NTP (2003b)	High incidence in historical controls maintained on NIH diet-07 (NTP, 2001a)
4-Hydroxy-2,5-dimethyl-3(2H)-furanone 	87.00	Diet	0 (control), 100, 200 and 400 mg/kg bw/day for 24 months to Sprague–Dawley rats (60/sex/group)	Pituitary adenomas of the pars distalis in 400 mg/kg group of males after 18 months on test. Within historical control data ranges and within reported ranges in the literature for the age and strain of rat (McComb et al., 1984)	Not applicable	Kelly and Bolte (2003)	Pituitary adenomas common in aging and aged rats- appear at between 13 and 24 months of age. Peto analysis, which compares tumour rate and time to tumour formation, revealed no statistical difference between control and treated males and females in this study. Adenomas were concluded to be common, spontaneous tumours unrelated to the administration of DMHF
Allyl isothiocyanate (2034) 	2.21	Gavage, corn oil vehicle	0, 12 or 25 mg/kg bw fed to groups of 50 male and 50 female F344/N rats and B6C3F1 mice for 103 weeks	Urinary bladder: Transitional cell papillomas (males at 12 mg/kg bw, 4%; 25 mg/kg bw, 8%)	None	NTP (1982)	

Table 3 (*continued*)

Substance (FEMA No.)	Daily per capita Intake ("eaters only"), μg/kg bw/day	Route of administration	Dose levels, mg/kg bw/day	Statistically significant ($P < 0.05$) dose-related neoplastic effects in rats (% tumors at each dose level)	Statistically significant ($P < 0.05$) dose-related neoplastic effects in mice (% tumors at each dose level)	Reference (date)	Evaluation of positive results
g-Butyrolactone (3291)	2.21	Gavage, corn oil vehicle	0, 112 or 225 mg/kg bw to groups of 50 males and 50 female F344/N rats for 105 weeks. 0, 225 or 450 mg/kg bw to groups of 50 male and 50 female B6C3F1 mice for 103 weeks	None	Adrenal medulla: hyperplasia (males at 225 mg/kg bw, 18%; 450 mg/kg bw, 8%) and pheochromo-cytoma (males at 225 mg/kg bw, 12%; 450 mg/kg bw, 2%)	NTP (1992a)	Hyperplasia associated with dietary factors and not considered an adverse effect related to the test material; this type of neoplasm not relevant to humans (Adams et al., 1998)

In all studies summarized, the following protocol was used, unless otherwise indicated: Groups of 50 male and 50 female F344/N rats and B6C3F1 mice were administered the test material for 103 weeks. In gavage studies, the test material was administered 5 days/week. In inhalation studies, the test material was administered 6 h/day, 5 days/week.
M = mice; R = rats; m = male; f = female.

in the cytoplasm of the protein or the protein-chemical complex which leads to single cell necrosis, regeneration and eventually, renal neoplasia in male rats (Lehman-McKeeman et al., 1990; Hildebrand et al., 1997; Strasser et al., 1988; Borghoff et al., 1990). The accumulated evidence indicates that it is the unique interaction of the chemical or metabolites with $\alpha_{2\mu}$-globulin in the male rat kidney, especially the proximal convoluted tubule, which allows *d*-limonene to interfere with renal processing of the sex-specific $\alpha_{2\mu}$-globulin. Since humans and other species do not possess a protein comparable to $\alpha_{2\mu}$-globulin that bonds these substances and accumulates in the renal tubules, this process is not predictive of human carcinogenicity. In a comprehensive review of $\alpha_{2\mu}$-globulin nephropathy and associated renal tubule tumors produced in the male rat exposed to *d*-limonene and other simple chemical substances (e.g. isophorone, decalin and methyl isobutyl ketone), it was concluded that this mode of action in the male rat is not an appropriate model for assessing human renal carcinogenic risk (EPA, 1991; IARC, 1989). After careful review, the Panel concludes that this mode of action leading to the renal carcinogenic findings in the male rat are largely known and strongly indicate that the nephropathy associated with *d*- limonene has no significance for human risk assessment (Burdock et al., 1990).

Peroxisome proliferation-liver tumors in mice and pancreatic acinar cell tumors in rats

Hepatocellular neoplasms in mice.

Neoplastic and non-neoplastic lesions associated with administration of cinnamyl anthranilate to mice developed principally in the liver. Treated groups of male and female mice showed evidence of lipoidosis, hemosiderosis, hyperplasia of hepatocytes and a statistically significant increase in the incidence of combined hepatocellular adenomas and carcinomas {control, 14/48; 15,000 ppm or 2250 mg/kg bw, 30/50 ($p = 0.003$); 30,000 ppm or 4500 mg/kg bw, 37/47 ($p < 0.001$)} in male mice compared to that of the control group. There was a statistically significant increase in the incidence of hepatocellular carcinomas {control, 1/50; 15,000 ppm or 2250 mg/kg bw, 8/49 ($p = 0.014$); 30,000 ppm or 4500 mg/kg bw, 14/49 ($p < 0.001$)} and combined adenomas and carcinomas {control, 3/50; 15,000 ppm or 2250 mg/kg bw, 20/49 ($p < 0.001$); 30,000 ppm or 4500 mg/kg bw, 33/49 ($p < 0.001$)} in dosed groups of female mice.

Four high-dose and two low-dose females were diagnosed as having both adenomas and carcinomas.

The NTP report concluded: "*Based on increased incidences of hepatocellular adenomas, and hepatocellular adenomas and carcinomas, cinnamyl anthranilate was considered carcinogenic for male and female B6C3F1 mice receiving 15,000 or 30,000 ppm cinnamyl anthranilate in the diet*" (NCI, 1980).

Since performance of the original bioassay (NCI, 1980), additional studies on over 70 substances have established a direct correlation between the increased incidence of hepatocarcinogenicity and the induction of peroxisome proliferation in rodent livers (Ashby et al., 1994). Studies performed by the European Centre for Ecotoxicity and Toxicology of Chemicals (ECETOC) (ECETOC, 1992) show that peroxisome proliferators form a discrete category of rodent liver carcinogens, the carcinogenicity of which does not involve direct genotoxic mechanisms.

Histological evidence of peroxisome proliferation in rodents is reflected by an increased peroxisome/mitochondrial ratio which is correlated with increases in target organ weights, total cytochrome P-450 content, and activities in microsomal lauric acid hydroxylation, carnitine acetyl transferase, and cyanide (CN^-) insensitive palmitoyl-CoA (Reddy et al., 1980, 1986; Reddy and Lalwai, 1983; Barber et al., 1987). Peroxisome proliferation is a transcription-mediated process involving the peroxisome proliferator-activated receptor (PPARα) in the hepatocyte nucleus. The role of PPARα in the induction of hepatocarcinogenicity in the mouse has been clearly established (Peters et al., 1997). Carcinogenicity studies with mice genetically modified to remove PPAR show no evidence of either peroxisome proliferation or carcinogenicity. Given that levels of expression of PPARα in humans is 1–10% of levels found in the rat or mouse (Palmer et al., 1994, 1998) and that the associated response element does not appear for several of the genes in humans, it is not unexpected that humans are refractory to peroxisome proliferation following chronic exposure to potent rodent peroxisome proliferators. No significant evidence of peroxisome proliferation has been observed in human studies with several potent hypolipidemic drugs that are rodent peroxisome proliferators (reviewed in Doull et al., 1999; Ashby et al., 1994). Based on these observations, it is concluded that the hepatocarcinogenic response in rodents is not relevant to the human health assessment of cinnamyl anthranilate.

When the above information is combined with data on metabolism and enzyme induction, the panel concludes that hepatic peroxisome proliferation is both a rodent-specific and dose-dependent phenomenon induced by the intact ester cinnamyl anthranilate (Viswalingam et al., 1988; Keyhanfar and Caldwell, 1996; Caldwell, 1992). Specifically, repeated-dose metabolism studies have shown that above a threshold dose greater than 500 mg/kg bw/d, intact cinnamyl anthranilate given i.p. or in the diet to mice shows a dose-dependent increase in liver weight, total cytochrome P-450, microsomal lauric acid hydroxylation and cyanide (CN^-) insensitive palmitoyl-CoA activity, and peroxisome/mitochondria ratio in hepatic cells (Caldwell, 1992; Viswalingam et al., 1988). These markers for peroxisome proliferation correspond to dose levels at which saturation of the hydrolysis pathway leads to the presence of the intact ester in vivo. Therefore, peroxisome proliferation caused by cinnamyl anthranilate is a dose-dependent effect. In addition, the results of chronic studies on the hydrolysis product, anthranilic acid, a normal metabolite of the amino acid tryptophan, and on the intermediary metabolite cinnamyl alcohol, provide additional evidence for this mechanism of action.

Pancreatic Acinar-cell neoplasms in male rats

In an NTP bioassay using cinnamyl anthranilate (NCI, 1980), an increased incidence of pancreatic acinar-cell adenomas (2/45) and carcinomas (1/45) was reported in the high-dose males (3/45; 7%) compared to controls (0/42). According to the NTP, although the difference was not statistically significant, the incidence of this type of neoplasm in aging F344 control rats is extremely low {historical incidence for controls in participating NTP laboratories (6/1538; 0.28%)}. Therefore, the NTP considered occurrence of these neoplasms to be related to administration of the test material.

Since completion of the 2-year bioassay with cinnamyl anthranilate, other carcinogenicity studies have established a relationship between peroxisome proliferation and the appearance of pancreatic acinar-cell neoplasms in the male F344 rat (Critical Reviews in Toxicology, 11/03). The sex-specific phenomenon has been observed when F344 male rats were exposed to high dose levels of other peroxisome proliferators (*e.g.* butyl benzyl phthalate and hypolipidemic drugs, clofibrate and nafenopin) (Malley et al., 1995; NTP, 1997a; Reddy and Qureshi, 1979; Svoboda and Azarnoff, 1979). It appears that the effect on the rat pancreas is secondary to the effect of these substances on the liver.

The sequence of rat pancreatic acinar cell hypertrophy, hyperplasia, and adenomas in male rats is affected by several factors including steroids, growth factors such as cholecystokinin (CCK), growth factor receptors, and diet. Studies show that testosterone stimulates, and estrogen inhibits, the growth of

pancreatic acinar-cell neoplasms in rats (Lhoste et al., 1987a,b; Sumi et al., 1989; Longnecke, 1987; Longnecker and Sumi, 1990). Cholecystokinin has been shown to stimulate adaptive and neoplastic changes of pancreatic acinar cells (Longnecke, 1987). The impact of diet on stimulation of CCK and the subsequent appearance of acinar cell neoplasms in male rats has also been reported (Longnecke, 1987; NTP, 1997b). In rat bioassays, the corn oil vehicle has been shown to increase the incidence of pancreatic acinar call neoplasms (Longnecke, 1987). Also, the incidence of pancreatic acinar-cell neoplasms induced by benzyl phthalate was 10/50 for male rats fed *ad libitum*, but 0/10 for rats placed on a restricted feed protocol for 2 years. The latter study clearly demonstrated the effect of excess caloric intake on the incidence of pancreatic acinar cell neoplasms. In summary, the appearance of these neoplasms is sex, species, dose, and even diet specific. In addition, relevance of this tumor type to humans is unlikely since acinar cell proliferations and tumors in humans are rare.

Apparently, prolonged peroxisome proliferation in rats inhibits bile flow leading to cholestasis (Lu et al., 2000; Marrapodi and Chiang, 2000). The cholestasis, in turn, leads to a decrease in trypsin activity and an increase in monitor protein in the gut lumen which stimulates cholecystokinin (CCK) (Obourn et al., 1997a,b). CCK then acts on CCK receptors on pancreatic acinar cells leading to hyperplasia and eventually adenomas. This is a high dose phenomenon in rats and is unlikely to occur in humans. Several human studies of hypolipidemic drugs that are recognized peroxisome proliferators in rodents have failed to show any significant difference in cancer deaths between treated patients and placebo-treated group (IARC, 1996). Also, acinar cell neoplasms are extremely rare in humans. These results are expected, since humans and rodents show quantitative differences in their response to peroxisome proliferators. Furthermore, increased CCK levels in humans do not stimulate acinar cell proliferation, because humans possess a relatively small number of CCK receptors compared to the rat.

Given these more recent data and the lack of any correspondence between bioassay results and human studies with peroxisome proliferators, the Panel believes that the increased incidence of acinar-cell neoplasms in the F344 male rat are associated with peroxisomal proliferation induced by high dose levels of cinnamyl anthranilate. This effect is specific to the male F344 rat and, therefore, is not relevant to the human health assessment of cinnamyl anthranilate.

In summary, the FEMA Expert Panel concludes that the report of pancreatic neoplasms in the male F344/N rat is related to use of corn oil as a gavage vehicle and is a secondary response to peroxisome proliferation. Likewise, hepatic neoplasms in the B6C3F1 mouse in the NTP bioassay are secondary responses to peroxisome proliferation. Since peroxisome proliferation is a dose-dependent effect specific to rodents, the results of the bioassay are not relevant to the safety of cinnamyl anthranilate in humans at low levels of intake from its use as a flavoring substance.

High incidence of background spontaneous neoplasms

Liver neoplasms in male and female B6C3F1 mice.

High dose levels of groups of flavor ingredients {i.e., benzyl acetate, furfural dihydrocoumarin, isophorone, pyridine} have been associated with an increased incidence of liver neoplasms in primarily male, but also female mice in NTP gavage studies. In parallel studies, no liver neoplasms were reported in male or female rats. The high incidence of hepatocellular adenomas and carcinomas that occurs in control male and female mice demonstrates the sensitivity of the $B6C3F_1$ mouse liver to neoplastic changes. A recent analysis of the historical spontaneous incidence of liver neoplasms in control male and female $B6C3F_1$ mice has revealed background incidences of liver adenoma/carcinomas of 42.2% (range 10–68%) for males and 23.6% (6–56%) for female B6C3F1 mice (Haseman et al., 1998). Increasing liver tumor rates are strongly correlated with increasing mean body weights in control and test mice achieved when NTP altered its standard protocol from group to individual housing (Haseman et al., 1994). This change led to increased body weights in control and test animals. Analysis of body weight on the incidence of liver neoplasms for control and test mice in the NTP database revealed that males exhibiting mean body weights >49 g showed a liver tumour incidence of 54% (test) and 62% (control), respectively. Test females exhibiting mean body weights >49 g showed a liver tumour incidence of 35%. In addition, other factors (Haseman et al., 1998) such as administration of corn oil gavage vehicle and contamination with *Heliobacter hepaticus* contribute to the high background incidence of this type of neoplasm in both sexes of mice. The profile of neoplastic responses in these studies are consistent with the historically high levels of background hepatocellular neoplasms in male and female $B6C3F_1$ mice (Maron-

pot et al., 1987). Therefore, the appearance of hepatic neoplasms in NTP mouse bioassays is generally not relevant to the safety of these flavor ingredients in humans at low levels of intake from use as flavor ingredients. This conclusion is based on: the high incidence of spontaneous hepatocellular neoplasms (adenomas and carcinomas) in the $B6C3F_1$ mice related to body weight changes, the use of corn oil gavage, the absence of consistent dose-response data, the lack of hepatocellular neoplastic effects in the parallel rat studies, low incidences of liver tumors in other strains of mice that do not have a high spontaneous incidence, and the relatively high dose levels.

Lymphoma in B6C3F1 mice

An increased incidence of malignant lymphoma was reported in B6C3F1 female mice administered (1) diets containing 0, 500, 1000 or 2000 ppm of citral (a mixture of the cis and trans isomers of 3,7-dimethyl-2,6-octadienal) (NTP, 2003a), or (2) 31 or 62 mg/kg bw of allyl isovalerate by gavage daily, five days per week, for 2 years (NTP, 1983b). A dose-related increase in the incidence of lymphoma was reported that was statistically significant in the high dose in B6C3F1 female mice given citral ($P = 0.011$ by Fisher exact test; 12/50 (24%) at 2000 ppm versus 3/50 (6%) in controls) or allyl isovalerate (22% at 31 mg/kg bw and 36% at 62 mg/kg bw). There was no evidence of increased incidence of malignant lymphoma in either sex of F344/N rats or in male B6C3F1 mice.

The background incidence of malignant lymphoma in control female B6C3F1 mice maintained on a NTP-2000 diet is high (98/659), with a historical incidence of 14.0% (standard deviation ± 7.1%) and a range of 6–30% (NTP, 2003a). The incidence of spontaneous malignant lymphoma in female B6C3F1 mice in all 2-year rodent carcinogenicity studies carried out by NTP is also high (20.9%) (Haseman et al., 1998). The historical incidence in controls maintained on the NIH-07 diet at the same contract laboratory performing the citral study was high (167/953) with a historical incidence of 17.5% (standard deviation, 7.7%) and a range of 6–30%.

Therefore, these tumors occur at a high and variable rate in control animals. It is recommended (Haseman et al., 1986) that a compound is anticipated to exhibit a carcinogenic potential if the highest dose is associated with an increased incidence of a common tumor that is significant at the 1% ($p < 0.01$) level, or an increased incidence in a rare tumor at the 5% ($p < 0.05$) level. Therefore, applying a significance level of 1% ($p < 0.01$) and based on pairwise comparisons of the incidence of malignant lymphoma in the citral study by a Fisher exact test, the incidence of this commonly observed neoplasm is not considered to be statistically significant ($p = 0.011$) for female mice at the 1% level. Similar conclusions can be made for the allyl isovalerate gavage study.

Based upon the high frequency of this neoplastic response in historical controls in NTP studies (Haseman et al., 1998), the fact that toxicity was observed in female B6C3F1 mice at dose levels producing lymphoma in female mice given citral or allyl isovalerate, and the observation that the incidences of lymphoma reported in both NTP studies were not significant at the 1% level ($p < 0.01$) (Haseman et al., 1986), the FEMA Expert Panel concluded that the results of the bioassays do not provide evidence that these substances present a carcinogenic risk to humans exposed to low levels of citral or allyl isovalerate used as flavor ingredients added to food.

Carcinogenic effects in multiple species

Liver neoplasms in mice and rats.

High dose levels of allylalkoxybenzene derivatives (i.e., estragole, methyl eugenol, and safrole) have been associated with a carcinogenic potential in multiple species and sexes via different routes of administration. Both the qualitative and quantitative aspects of the molecular disposition of methyl eugenol and estragole and their associated toxicological sequelae have been relatively well defined from mammalian studies. Several studies have clearly established that the profiles of metabolism, metabolic activation, and covalent binding are dose-dependent and that their relative importance diminishes markedly at low levels of exposure (i.e., these events are not linear with respect to dose). In particular, rodent studies show that these events are minimal probably in the dose range of 1–10 mg/kg bw which is approximately 100–1000 times the anticipated human exposure to these substances. For these reasons the panel concludes that present exposure to methyl eugenol and estragole resulting from consumption of food, mainly spices, and added as such, does not pose a significant cancer risk. Nevertheless, current toxicologic and biochemical studies are being performed to confirm and more thoroughly explore both the nature and implications of the dose-response curve in rodents at low levels of exposure to methyl eugenol and estragole.

3.6.2. Conclusion

3.6.2.1. Conclusions on carcinogenicity. In summary, the majority of 2-year bioassays on flavor ingredients either show no evidence of carcinogenicity in rodents at

any dose level or exhibit carcinogenic responses that are not considered relevant to the assessment of risk to humans. These latter studies show a dose- and toxicity-dependent increase in the incidence of neoplasms in a specific rodent species and sex (i.e., α-2u-globulin in male rats and spontaneous liver neoplasms or lymphoma in B6C3F1 male and female mice, respectively), or use a mode of administration (forestomach tumors by gavage) irrelevant to the intake of flavor ingredients by humans, or the neoplasms are of a spontaneous background nature specific to a sex, strain, and species of rodent. For those substances that currently are not the subject of ongoing research (i.e., allylalkoxybenzene derivatives), it is clear that flavor ingredients at current levels of intake provide no significant carcinogenic risk to humans. However, taken together, these assays reveal the importance of chronic dose-related organ toxicity in the onset of carcinogenic effects in 2-year rodent studies. In this regard, it is relevant to discuss presence of toxicity thresholds for the appearance of carcinogenicity in these studies.

3.7. Genotoxicity

The Panel uses data from genotoxicity studies as a screening tool to evaluate the genotoxic potential of individual flavoring compounds and groups of structurally related substances. In this respect the Panel has evaluated the results of thousands of genotoxicity assays. The Panel critically evaluates the results of both in vitro and in vivo tests for genotoxicity, taking into consideration metabolic and related toxicity data in coming to a conclusion as to the biological significance and relevance of genotoxicity studies in the context of the use of flavoring substances.

The Panel is often faced with a mix of positive and negative in vitro studies. In such cases results from well conducted in vivo studies would typically outweigh positive results obtained in vitro. It is the Panel's practice to provide a clear rationale for its conclusions in resolving divergent data sets.

The original design of standardized in vitro assays was to provide a basis for understanding the potential of chemical substances to cause molecular and genetic alterations leading to carcinogenicity. However, the disparity of positive and negative results observed in the four widely used in vitro genotoxicity tests (SAL, MLA, ABS, and SCE) has caused a thorough reevaluation of the utility of these assays for predicting the carcinogenic potential of substances in long-term rodent assays. Based, in part, on the following conclusions by researchers affiliated with the NTP (Haseman et al., 1990; and Zeiger et al., 1990), the use of ABS, SCE, and MLA assays to prioritize substances for carcinogenicity testing has been curtailed within the NTP: (1) there appears to be no evidence of complementariness among the four genotoxicity assays; (2) the results of a battery of tests has not been shown to be more conclusive than the use of the SAL assay alone (Smart, 1994); and (3) the disparity of positive and negative results in selected assays precludes the formation of conclusions on the potential carcinogenicity of a substance.

The evaluation of genotoxicity assays is also often complicated by the fact that the results are inconsistent with other metabolic and toxicologic data on the substance. Thus, the panel considers the results of genotoxicity assays in the broader context of available information for a given flavoring substance. An example of such an analysis is provided by consideration of the panel's analysis of the data set available for furanones. Furanones are flavor ingredients that exhibit uniform positive responses in both in vitro and in vivo genotoxicity assays. The genotoxic potential of 3-(2H)-furanone derivatives is consistently demonstrated by the results of standardized bacterial (Gilroy et al., 1978; Xing et al., 1988; Hiramoto et al., 1996a,b; Li et al., 1998) and mammalian assays (Xing et al., 1988; Tian et al., 1992; Hiramoto et al., 1996b). Yet consideration of metabolic and toxicological studies along with comparison to data sets available for chemically-related substances suggests that furanones added as flavoring agents do not pose a significant genotoxic risk to humans (see box).

Putative mechanism of genotoxicity of furanone derivatives

Furanones such as 2,5-dimethyl-2(3H)-furanone (DMHF) induce DNA damage in vitro by generating free radicals that induce strand scission. In the presence of metals (e.g., Fe^{3+}) and dissolved oxygen, the enolic OH of the furanone is oxidized by single electron transfer to yield the corresponding carbon-centered radical and a reduced metal ion (e.g., Fe^{2+}). The carbon-centered radical can couple to molecular oxygen to produce a peroxyl radical. Alternately, the reduced metal ion can autoxidize to form superoxide radical anion. Superoxide radical then dismutates into hydrogen peroxide (H_2O_2). It is well recognized that reduced metals react with H_2O_2 to form hydroxyl radical which is a powerful oxidizing agent (see Fig. 2). Hydrogen peroxide also oxidizes glutathione leading to decreased GSH/GSSG and an increase in cellular oxidative stress.

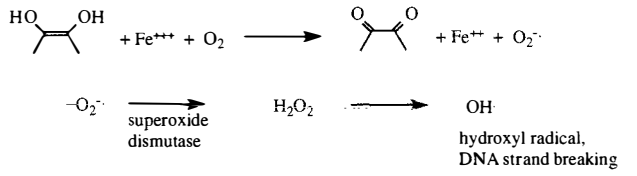


Fig. 2. Mechanism of in vitro furanone derivative oxidation.

The ability of DMHF to induce oxygen radical formation and DNA strand breaks is reminiscent of similar activities of Vitamin C, a structurally related furanone. Vitamin C (ascorbic acid) contains an enediol that is superficially related to the enol of DMHF. Being both an enol ether and α,β-unsaturated ketone, DMHF is anticipated to undergo hydrolytic ring opening to yield an enediol. Like DMHF, Vitamin C also reduces metal ions and produces superoxide anion to generate hydroxyl radicals that cleave DNA. As anticipated, vitamin C exhibits genotoxicity in test systems similar to those in which furanones test positive. In standard Ames assays, ascorbic acid (vitamin C) induces reverse mutations in *S. typhimurium* strains TA104, TA102, TA100, and TA98 at concentrations 352–1,761 μg/plate (D'Agostini et al., 2000; Ichinotsubo et al., 1981). In the *E.coli* Mutoxitest, positive results were obtained when 200, 300, or 400 μg/plate of ascorbic acid in the presence of Cu^{++} was incubated with *E. coli* strain IC203. *E.coli* IC203 carries a Δ *oxy* R30 mutation effectively removing its ability to turn on the biosynthesis of hydrogen peroxide protective proteins and making the strain sensitive to DNA damage under conditions of oxidative stress (Blanco et al., 1998; Martinez et al., 2000).

Increased levels of micronuclei were observed when ascorbic acid (400, 500, or 600 μg/ml) was incubated with Chinese hamster cells (Miller et al., 1995). An increase in SCE was observed in Chinese hamster ovary (CHO) cells in the presence of 500 μg/ml of ascorbic acid without metabolic activation (Tennant et al., 1987). Ascorbic acid (1 or 2 mg/kg bw) did not increase the incidence of replicative DNA synthesis in F344 rats or B6C3F1 mice (Miyagawa et al., 1995). But in a standard mouse micronucleus assay, 1500 mg/kg bw ascorbic acid induced a significant increase in micronuclei in B6C3F1 mice (Shelby et al., 1993).

Furanones are a class of substances present in food naturally that are also added as flavoring agents. The principal furanone used as a flavoring agent is DMHF. In humans, DMHF is rapidly absorbed in the gastrointestinal tract and conjugated with glucuronic acid in the liver. Free DMHF is not detected in the blood of human volunteers to whom it is administered as a constituent of strawberries; its glucuronic acid conjugate is the principal urinary metabolite (Roscher et al., 1997). Thus, the potential for chemical reaction of DMHF with important cellular macromolecules, especially DNA, appears low.

Despite the fact that DMHF is genotoxic, it does not produce carcinogenic effects in rodents. Two studies, one on DMHF and the other on a structurally furanone show no evidence of carcinogenicity at intake levels orders of magnitude greater than the intake of furanones added as flavoring agents (Kelly and Bolte, 2003; Munday and Kirkby, 1973). Furthermore, Vitamin C, a furanone with a genotoxicity test profile similar to that of DMHF, does not demonstrate carcinogenicity (NRC, 1996). As a result of the 2-year bioassay of DMHF, a NOAEL of 200 mg/kg bw per day was established in rodents. This intake level is approximately 2000 times the daily per capita intake ("eaters only") of 0.088 mg/kg bw per day form use of DMHF as a flavoring agent. Based on the available data, it is highly unlikely that DMHF or other furanones pose any significant genotoxic risk to humans under conditions of use as flavoring agents.

4. Conclusions

The Panel will continue to evaluate genotoxicity studies for their relevance to the human health assessment of flavor ingredients. Certainly as a screening tool, the results have been consistent with the metabolic and toxicologic data available for the vast majority of chemical groups reviewed by the Panel. However, at this time, the relevance of genotoxicity data must be evaluated in the context of other data more closely related to actual human experience, i.e., the results of genotoxicity testing are not viewed in isolation, these data comprise one component that is factored into the Panel's overall safety assessment.

5. Summary

This review describes the current status of the GRAS evaluation of flavoring substances by the Expert Panel of FEMA. The Panel currently maintains a rotating 10-year program of continuous review of the safety data related to chemical groups of flavor ingredients. As scientific methodology has evolved, new data relevant to the safety of individual and groups of structurally related flavoring substances have become available. Studies in dose-dependent metabolism, enzyme induction, two year bioassays, and in vitro and in vivo genotoxicity have become a routine part of flavor safety evaluation. Maintaining its multidisciplinary approach to safety evaluation, the Panel's objective is to integrate relevant information from these different scientific disciplines to achieve a truly comprehensive safety evaluation program for flavoring substances. Major emphasis has been placed on understanding biochemical and biological events leading to toxicity, the relationship of toxicity to dose, and comparison of dose to intake of the

flavoring substance. The mechanistic approach continues to evolve.

The Panel has now concluded a second comprehensive review of all available GRAS chemically identified flavoring substances. This second re-evaluation of chemical groups of flavor ingredients is recognized as the GRAS reaffirmation (GRASr) program The Expert Panel is alert to new methodology that conceivably could be applied to the evaluation of the safety-in-use of flavor ingredients. However, the application of such tests would need to be evaluated within the context of the very low exposure resulting from the use of flavor ingredients in order to be validated.

References

Adams, T.B., Hallagan, J.B., Putman, J.M., Gierke, T.L., Doull, J., Munro, I.C., Newberne, P.M., Portoghese, P.S., Smith, R.L., Wagner, B.M., Weil, C.S., Woods, L.A., Ford, R.A., 1996. The FEMA GRAS assessment of alicyclic substances used as flavor ingredients. Food and Chemical Toxicology 34, 763–828.

Adams, T.B., Doull, J., Goodman, J.I., Munro, I.C., Newberne, P.M., Portoghese, P.S., Smith, R.L., Wagner, B.M., Weil, C.S., Woods, L.A., Ford, R.A., 1997. The FEMA GRAS assessment of furfural used as a flavor ingredient. Food and Chemical Toxicology 35, 739–751.

Adams, T.B., Greer, D.B., Doull, J., Munro, I.C., Newberne, P.M., Portoghese, P.S., Smith, R.L., Wagner, B.M., Weil, C.S., Woods, L.A., Ford, R.A., 1998. The FEMA GRAS assessment of lactones used as flavor ingredients. Food and Chemical Toxicology 36, 249–278.

Adams, T.B., Cohen, S., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Munro, I.C., Portoghese, P.S., Smith, R.L., Waddell, W.J., Wagner, B.M., 2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. Food and Chemical Toxicology 42, 157–185.

Arcella, D., Leclerq, C., 2004. Assessment of dietary intake of flavouring substances within the procedure for their safety evaluation: advantages and limitations of estimates obtained by means of a per capita method. Food and Chemical Toxicology 43, 99–104.

Ashby, J., Brady, A., Elcombe, C.R., Elliot, B.M., Ismael, J., Odum, J., Tugwood, J.D., Kettle, S., Purchase, I.F.H., 1994. Mechanistically-based human hazard assessment of peroxisome proliferator-induced hepatocarcinogenesis. Human and Experimental Toxicology 13 (Suppl. 2), S1–S118.

Astill, B.D., Deckardt, K., Gembardt, C., Gingell, R., Guest, D., Hodgson, J.R., Mellert, W., Murphy, S.R., Tyler, T.R., 1996. Prechronic toxicity studies on 2-ethylhexanol in F334 rats and B6C3F1 mice. Fundamental and Applied Toxicology 29 (1), 31–39.

Barber, E.D., Astill, B.D., Moran, E.J., Schneider, B.F., Gray, T.J.B., Lake, B.G., Evans, JG., 1987. Peroxisome induction studies on seven phthalate esters. Toxicology and Industrial Health 3, 7–24.

Blanco, M., Urios, A., Martinez, A., 1998. New Escherichia coli WP2 tester strains highly sensitive to reversion by oxidative mutagens. Mutation Research 413, 95–101.

Borchert, P., Wislocki, P.G., Miller, J.A., Miller, E.C., 1973. Metabolism of the naturally occurring hepatocarcinogen safrole to 1-hydroxysafrole and the electrophilic reactivity of 1-acetoxysafrole. Cancer Research 33, 575–589.

Borghoff, S.J., Short, B.G., Swenberg, J.A., 1990. Biochemical mechanisms and pathobiology of nephropathy. Annual Reveiws of Pharmacology and Toxicology 30, 349–367.

Bounds, S.V., Caldwell, J., 1996. Pathways of metabolism of [1′-^{14}C] *trans*-anethole in the rat and mouse. Drug Metabolism and Disposition 24, 717–724.

Burdock, G.A., Wagner, B.M., Smith, R.L., Munro, I.C., Newberne, P.M., 1990. 15 GRAS Substances. Food Technology 44 (2), 78–86.

Caldwell, J., Sutton, J.D., 1988. Influence of dose size on the disposition of *trans*-[methoxy-^{14}C]anethole in human volunteers. Food and Chemical Toxicology 26, 87–91.

Caldwell, J., 1991. *Trans*-anethole-another nongenotoxic rodent carcinogen. Presented at the Toxicology Forum, July 15–19, Aspen, CO, pp. 616–639.

Caldwell, J., Marshall, A.D., Reed, P., Bounds, S.V.J., 1991. Metabolic and mechanistic studies to underpin the safety assessment of *trans*-anethole. Unpublished interim report of the Anethole Task Force of the FEMA.

Caldwell, J., 1992. Problems and opportunities in toxicity testing arising from species differences in xenobiotic metabolism. In: Chambers, P.L., Chambers, C.M., Bolt, H.M., Preziosi, P. (Eds.), Toxicology from Discovery to Experimentation to the Human Perspective. Elsevier, New York, pp. 651–659.

Caldwell, J., Chan, V.S.W., Marshall, A.D., Hasheminejad, G., Bounds, S.V.J., 1992. 1′-Hydroxylation is the only metabolic pathway of simple alkenylbenzenes involved in their genotoxicity. The Toxicologist 12, 56.

Cramer, G.M., Ford, R.A., Hall, R.L., 1978. Estimation of toxic hazard—a decision tree approach. Food and Cosmetic Toxicology 16, 255–276.

D'Agostini, F.D., Balansky, R.M., Camoirano, A., De Flora, S., 2000. Interactions between N-acetylcysteine and ascorbic acid in modulating mutagenesis and carcinogenesis. International Journal of Cancer 88, 702–707.

Doull, J., Cattley, R., Elcombe, C., Lake, B.G., Swenberg, J., Wilkinson, C., Williams, G., van Gemert, M., 1999. A cancer risk assessment of Di(2ethylhexyl)phthalate: Application of the New US EPA Risk Assessment Guidelines. Regulatory Toxicology and Pharmacology 29, 327–357.

EC, 2000. Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council. OJ L 180, 19.7.2000, p. 8.

ECETOC, 1992. Hepatic Peroxisome Proliferation. Monograph 17. European Centre for Ecotoxicity and Toxicology of Chemicals, Brussels. Self-published.

Environmental Protection Agency (EPA), 1991. Risk assessment form. α_2—globulin association with chemically induced renal toxicity and neoplasia in the mail rat. Publication no. EPA/625/3-91010F.

Flavor and Extract Manufacturers Association, 1978. Scientific Literature Reviews on Generally Recognized as Safe (GRAS) food ingredients-Alicyclic compounds of carbon, hydrogen, and oxygen in flavor use, vol. 1–3. Published by the US Food and Drug Administration.

FMI, 1998. The Food Marketing Industry Speaks. Food Marketing Institute, Washington, DC.

Gardner, I., Bergin, P., Stening, P., Kenna, J.G., Caldwell, J., 1995. Protein adducts derived from methyleugenol. International Society for the Study of Xenobiotics International Meeting 8, 208.

Gardner, I., Bergin, P., Stening, P., Kenna, J.G., Caldwell, J., 1996. Immunochemical detection of covalently modified protein adducts in livers of rats treated with methyleugenol. Critical Reviews in Toxicology 9 (4), 713–721.

Gilroy, A.H., Hastwell, R.M., McGregor, D.B., Riach, C.G., 1978. Testing for mutagenic activity of six compounds with *Salmonella typhimurium* and further testing of one of the compounds with *Escherichia coli*. Project No. 410168, Inveresk Research International, Edinburgh, Scotland. Unpublished report.

Hall, R.L., 1976. Estimating the distribution of daily intakes of certain GRAS substances. Comm. On GRAS list survey-Phase III. Nat'l. Acad. Of Sciences/Natl. Res. Council. Washington, DC.

Hall, R.L., Ford, R.A., 1999. Comparison of two methods to assess the intake of flavoring substances. Food Additives Contaminants 16, 481–495.

Hallagan, J.B., Hall, R.L., 1995. FEMA GRAS—A GRAS assessment program for flavor ingredients. Regulatory Toxicilogy and Pharmacology 21, 422–430.

Haseman, J.K., Winbush, J.S., O'Donnell Jr., M.W., 1986. Use of dual control groups to estimate false positive rates in laboratory animal carcinogenicity studies. Fundamental and Applied Toxicology 7, 573–584.

Haseman, J.K., Zeiger, E., Shelby, M.D., Margolin, B.H., Tennant, R.W., 1990. Predicting rodent carcinogenicity from four in vitro assays: An evaluation of 114 chemicals studied by the national Toxicology Program. Journal of the American Statistical Association 85, 964–972.

Haseman, J.K., Bourbina, J., Eustis, S.L., 1994. Effect of individual housing and other experimental design factors on tumor incidence in $B6C3F_1$ mice. Fundamental and Applied Toxicology 23 (1), 44–52.

Haseman, J.K., Hailey, J.R., Morris, R.W., 1998. Spontaneous neoplasm incidences in Fischer 344 rats and $B6C3F_1$ mice in two-year carcinogenicity studies: A National Toxicology Program update. Toxicologic Pathology 26 (3), 428–441.

Heck, J.D., Vollmuth, T.A., Cifone, M.A., Jagannath, D.R., Myhr, B., Curren, R.D., 1989. An evaluation of food flavoring ingredients in a genetic toxicity screening battery. Toxicologist 9 (1), 257.

Hildebrand, H., Hartmann, E., Popp, A., Bomhard, E., 1997. Quantitation of α_2-microglobulin after administration of structurally divergent chemical compounds. Archives of Toxicology 71, 351–359.

Hiramoto, K., Sekiguchi, K., Ayuha, K., Aso-o, R., Moriya, N., Kato, T., Kikugawa, K., 1996a. DNA breaking activity and mutagenicity of soy sauce: characterization of the active components and identification of 4-hydroxy-5-methyl-3(2H)-furanone. Mutation Research 359, 119–132.

Hiramoto, K., Aso-o, R., Ni-iyama, H., Hikage, S., Kata, T., Kikugawa, K., 1996b. DNA strand break by 2,5-dimethyl-4-hydroxy-3(2H)-furanone, a fragrant compound in various foodstuffs. Mutation Research 359, 17–24.

Hirose, M., Shibata, M., Hagiwara, A., Imaida, K., Ito, N., 1981. Chronic toxicity of butylated hydroxytoluene in Wistar rats. Food and Chemical Toxicology 19, 147–151.

Hoechst, A.G., 1991. Unveröffentl. Unters. (Ber.-Nr. 91.0246). Unpublished report.

IARC, 1989. IARC Monographs Evaluating Carcinogenic Risks to Humans, vol. 47. Int'l Agency of Research on Cancer, Lyon, France, pp. 157–169.

IARC, 1996. Hypolipidaemic drugs. In: IRAC Monograph on the Evaluation of Carcinogenic Risk to Humans, vol. 66. Int'l Agency for Research in Cancer, Lyon, France, pp. 390–485.

Ichinotsubo, D., Mower, H., Mandel, M., 1981. Mutagen Testing of a Series of Paired Compounds with the Ames Salmonella Testing System. In Prog. in Muta. Res., vol. 1, Eval. of Short-Term Tests for Carcin., 298–301.

JECFA, 1997. Evaluation of certain food additives and contaminants. Forty-sixth Report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization, WHO Technical Report Series 868.

Kelly, C.M., Bolte, H.F., 2003. St 07 C99: a 24-month dietary carcinogenicity study in rats. Final report. Study No. 99-2644. Huntington Life Sciences, East Millstone NJ. Private communication to the Flavor and Extract Manufacturers Association.

Keyhanfar, F., Caldwell, J., 1996. Factors affecting the metabolism of cinnamyl anthranilate in the rat and mouse. Food and Chemical Toxicology 34, 241–249.

King, T., Faccini, J.M., Jachbaur, J., Perraud, J., Monro, A.M., 1979. 3-Generation and chronic toxicity study in rats. Pfizer Central Research. Unpublished report.

Lambe, J., Cadby, P., Gibney, M., 2002. Comparison of stochastic modelling of the intakes of intentionally added flavouring substances with theoretical added maximum daily intakes (TAMDI) and maximized survey-derived daily intakes (MSDI). Food Additives and Contaminants 19 (1), 2–14.

Lawrence, M., Shu, C.K., 1993. Essential Oils as Components of Mixtures: Their Method of Analysis and Differentiation. In: Ho, C.T., Manley, C.M. (Eds.), Flavor Measurement. Marcel Dekker Inc., New York.

Lehman-McKeeman, L.D., Caudill, D., Takigiku, R., Schneider, R.E., Young, J.A., 1990. Comparative disposition of d-limonene in rats and mice: relevance to male rat specific nephrotoxicity. Toxicology Letters 53, 193–195.

Lhoste, E.F., Roebuck, B.D., Brinck-Johnsen, T., Longnecker, D.S., 1987a. Effect of castration and hormone replacement on azaserine-induced pancreatic carcinogenesis in male and female Fischer rats. Carcinogenesis 8, 699–703.

Lhoste, E.F., Roebuck, B.D., Stern, J.E., Longnecker, D.S., 1987b. Effect of orchiectomy and testosterone on the early stages of azaserine-induced pancreatic carcinogenesis in the rat. Pancreas 2, 38–43.

Li, X., Hiramoto, K., Yoshida, M., Kato, T., Kikugawa, K., 1998. Identification of 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) and 4-hydroxy-2(or5)-ethyl-5(or2)-methyl-3(2H)-furanone (HEMF) with DNA breaking activity in soy sauce. Food and Chemical Toxicology 36, 305–314.

Lijinsky, W., Reuber, M.D., 1987. Chronic carcinogenesis studies of acrolein and related compounds. Toxicology and Industrial Health 3 (3), 337–345.

Longnecke, D.S., 1987. Interface between adaptive and neoplastic growth in the pancreas. Gut 28, 253–258.

Longnecker, D.S., Sumi, C., 1990. Effects of sex steroid hormones on pancreatic cancer in the rat. International Journal of Pancreatology 7, 159–165.

Lu, Y., Onda, M., Uchida, E., Yamamura, S., Yanagi, K., Matsushita, A., Kobayashi, T., Fukuhara, M., Tajiri, T., 2000. The cytotoxic effects of bile acids in crude bile on human pancreatic cancer cell lines. Surgery Today 30, 903–909.

Lucas, C.D., Putnam, J.M., Hallagan, J.B., 1999. Flavor and Extract Manufacturers Association (FEMA) of the United States 1995 poundage and Technical Effects Update Survey. Washington DC.

Madyastha, K.M., Raj, C.P., 1990. Biotransformation of R-(+)-Pulegone and Menthofuran in vitro: Chemical basis for toxicity. Biochemical and Biophysicical Research Communications 173, 1086–1092.

Madyastha, K.M., Raj, C.P., 1991. Evidence for the formation of a known toxin, p-cresol, from menthofuran. Biochemical and Biophysical Research Communications 177, 440–446.

Madyastha, K.M., Raj, C.P., 1992. Metabolic fate of menthofuran in rats: novel oxidative pathways. Drug Metabolism and Disposition 20, 295–301.

Madyastha, K.M., Raj, C.P., 1993. Studies on the Metabolism of a Monoterpene Ketone, R-(+)-Pulegone—A Hepatotoxin in Rat: Isolation and Characterization of New Metabolites. Xenobiotica 23, 509–518.

Malley, L.A., Carakostas, M., Hansen, J.F., Rusch, G.M., Kelly, D.P., Trochimowicz, H.J., 1995. Two-year inhalation toxicity study in rats with hydrochlorofluorocarbon 123. Fundamental and Applied Toxicology 25, 101–114.

Maronpot, R.R., Haseman, J.K., Boorman, G.A., Eutis, S.E., Rao, G.N., Huff, J.E., 1987. Liver lesions in B6C3F1 mice: The National

Toxicology Program, experience and position. Archives of Toxicology (Suppl. 10), 10–26.

Marrapodi, M., Chiang, J., 2000. Peroxisome proliferator-activated receptor α (PPAR.α) and agonist inhibit cholesterol 7α-hydroxylase gene (CYP7A1) transcription. Journal of Lipid Research 41, 514–520.

Marshall, A.D., Caldwell, J., 1992. Influence of modulators of epoxide metabolism on the cytotoxicity of *trans*-anethole in freshly isolated rat hepatocytes. Food and Chemical Toxicology. 30, 467–473.

Marshall, A.D., Caldwell, J., 1996. Lack of influence of modulators of epoxide metabolism on the genotoxicity of *trans*-anethole in freshly isolated rat hepatocytes assessed with the unscheduled DNA synthesis assay. Food and Chemical Toxicology 34, 337–345.

Martinez, A., Urios, A., Blanco, M., 2000. Mutagenicity of 80 chemicals in Escherichia coli tester strains IC203, deficient in OxyR, and its oxy R^+ parent WP2 uvrA/pKM101: detection of 31 oxidative mutagens. Mutation Research 467, 41–53.

McClanahan, R.H., Thomassen, D., Slattery, J.T., Nelson, S.D., 1989. Metabolic activation of (R)-(+)-pulegone to a reactive enonal that covalently binds to mouse liver proteins. Chemical and Research Toxicology 2, 349–355.

McComb, D.J., Kovacs, K., Beri, J., Zak, F., 1984. Pituitary adenomas in old Sprague-dawley rats: a histologic, ultrastructural, and immunocytochemical study. Journal of the National Cancer Institute 73 (5), 1143–1166.

Meijer, J., Lundqvist, G., Williams, G.M., 1987. Comparison of the sex and subcellular distributions, catalytic and immunochemical reactivities of hepatic epoxide hydrolases in seven mammalian species. European Journal of Biochemistry 167, 269–279.

Minnema, D.J., 1997. 90-Day subchronic dietary toxicity study of *trans*-anethole in mice and rats. Unpublished draft final report to FEMA. Convance Laboratories, Vienna, Va.

Miller, E.C., Swanson, A.B., Phillips, D.H., Fletcher, T.L., Liem, A., Miller, J.A., 1983. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenybenzene derivatives related to safrole and estragole. Cancer Research 43, 1124–1134.

Miller, E.C., Miller, J.A., Boberg, E.W., Delclos, K.B., Lai, C.-C., Fennell, T.R., Wiseman, R.W., Liem, A., 1985. Sulfuric acid esters as ultimate electrophilic and carcinogenic metabolites of some alkenylbenzenes and aromatic amines in mouse liver. Carcinogenesis 10, 93–108.

Miller, B.M., Pujadas, E., Gocke, E., 1995. Evaluation of the micronucleus test in vitro using Chinese hamster cells: Results of four chemicals weakly positive in the in vivo micronucleus test. Environmental and Molecular Mutagenesis 26 (3), 240–247.

Miyagawa, M., Takasawa, H., Sugiyama, A., Inoue, Y., Murata, T., Uno, Y., Yoshikawa, K., 1995. The in vivo–in vitro replicative DNA synthesis (RDS) test with hepatocytes prepared from male B6C3F1 mice as an early prediction assay for putative nongenotoxic (Ames-negative) mouse hepatocarcinogens. Mutation Research 343, 157–183.

Moorthy, B., Madyastha, K.M., 1989. Pulegone mediated hepatotoxicity: Evidence for covalent binding of R(+)-[(14)C]pulegone to microsomal proteins in vitro. Chemico-Biological Interactions 72, 325–333.

Moorthy, B., Madyastha, P., Madyastha, K.M., 1989a. Metabolism of a monoterpene ketone, R-(+)-pulegone a hepatotoxin in rat. Xenobiotica 19, 217–224.

Moorthy, B., Madyastha, P., Madyastha, M., 1989b. Hepatotoxicity of pulegone in rats: Its effects on microsomal enzymes. In Vivo Toxicology 55, 327–337.

Munday, R., Kirkby, W.W., 1973. Biological evaluation of flavour cocktail III. 1-Year feeding study in rats. Unpublished report.

Munro, I.C., Ford, R.A., Kennepohl E, Sprenger, J.G., 1996. Thresholds of toxicological concern based on structure-activity relationships. Drug Metabolism Reviews 28 (1/2), 209–217.

National Academy of Sciences (NAS), 1970. Evaluating the safety of food chemicals. Washington, DC.

National Academy of Sciences (NAS), 1975. Evaluating the safety of food chemicals. Washington, DC.

National Academy of Sciences (NAS), 1982. Evaluating the safety of food chemicals. Washington, DC.

National Academy of Sciences (NAS), 1987. Evaluating the safety of food chemicals. Washington, DC.

National Cancer Institute (NCI), 1978. Bioassay of DL-menthol for possible carcinogenicity. NCI-CG-TR-98, PB-288761.

National Cancer Institute (NCI), 1980a. Bioassay of benzoin for possible carcinogenicity. NCI-CG-TR-204; NTP-809; PB-80-217953.

National Cancer Institute (NCI), 1980b. Bioassay of cinnamyl anthranilate for possible carcinogenicity. NCI-CG-TR-196; NTP-80-10.

National Research Council (NRC), 1996. Carcinogens and Anticarcinogens in the Human Diet. A comparison of naturally occurring and synthetic substances. National Academy Press, Washington DC, pp. 91–92.

National Toxicology Program (NTP), 1979. Bioassay of butylated hydroxytoluene (BHT) for possible carcinogenicity (CAS No. 128-37-0). NCI-CG-TR-150. NIH Publication No. 79-1706.

National Toxicology Program (NTP), 1982. Carcinogenesis bioassay of allyl isothiocyanate (CAS No. 57-06-7) in F344/N rats and B6C3F1 mice (gavage studies). NTP-81-36. NIH Publication No. 83-1790. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1983a. Carcinogenesis studies of eugenol. NTP-TR-223; PB-84-1779. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1983b. National Toxicology Program Technical Report on the Carcinogenesis studies of allyl isovalerate in F344/N Rats and B6C3F_1 Mice (Gavage Studies). US Department of Health and Human Services, Public Health Service, National Institutes of Health. National Toxicology Program Technical Report No. 253.

National Toxicology Program (NTP), 1986a. Carcinogenesis studies of ethyl acrylate in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-259; PB-87-2515. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1986b. Toxicology and carcinogenesis studies of benzyl acetate (CAS No. 140-11-4) in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-250. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1986c. Toxicology and carcinogenesis studies of isophorone in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-291; NIH Publication No. 86-2547. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1987. Carcinogenesis studies of food grade geranyl acetate (71% geranyl acetate, 29% citronellyl acetate) (CAS No. 105-87-3) in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-252; PB-88-2508. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1988. Toxicology and carcinogenesis studies of malonaldehyde, sodium salt (3-hydroxy-2-propenal, sodium salt) (CAS No. 24382-04-5) in F344/N rats and B6C3F1 mice (gavage studies). NTP TR-331. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1990a. Toxicology and carcinogenesis studies of d-carvone (CAS No. 2244-16-8) in B6C3F1 mice and toxicologystudies in F344/N rats (gavage studies). NTP-TR-381; PB-90-2836. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1990b. Toxicology and carcinogenesis studies of benzaldehyde in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-378; NIH Publication No. 90-2833. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1990c. Toxicology and carcinogenesis studies of furfural (CAS No.98-01-1) in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-382. Nat'l. Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1990d. Carcinogenicity and toxicology studies of d-limonene in F344/N Rats and B6C3F1 mice. NTP-TR-347. US Dept of Health and Human Services. NIH Publication No. 90-2802 National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1990e. Toxicology and carcinogenesis studies of α-methylbenzyl alcohol (CAS No. 98-85-1) in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-369; NIH Publication No. 89-2824. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1992. Toxicology and carcinogenesis studies of γ-butyrolactone (CAS No. 96-48-0) in F344/N rats and B6C3F1 mice (gavage studies). NTP-TR-406; NIH Publication No. 92-3137. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1993a. Toxicology and carcinogenesis studies of benzyl acetate (CAS No. 140-11-4) in F344/N rats and B6C3F1 mice (feed studies). NTP TR-431. Nat'l. Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1993b. Toxicology and carcinogenesis studies of 3,4-di-hydrocoumarin (CAS No. 119-84-6) in F344/N rats and B6C3F1 mice (gavage studies). NTP TR-423. NIH Publication No. 93-3154. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1993c. Toxicology and carcinogenesis studies of coumarin in F344/N rats and B6C3F mice (gavage studies). NTP-TR-422; NIH Publication No. 93-3153, Research Triangle Park, NC.

National Toxicology Program (NTP), 1997a. Toxicology and carcinogenesis studies of Butyl benzyl phthalate in F344/N rats (feed studies). NTP-TR- 458; NIH Publication No. 97-3374. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1997b. Effect of dietary restriction on toxicology and carcinogenesis studies in F344/N rats and $B6C3F_1$ mice. NTP-TR -460: NIH Publication No. 97-3376. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1997c. Toxicology and carcinogenesis studies of pyridine (CAS No. 110-86-1) in F344/N rats, Wistar rats, and B6C3F1 mice (drinking water studies). NTP-TR-470. NIH Publication No. 98-3960. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 1999. Toxicology and carcinogenesis studies of isobutyraldehyde (CAS No. 78-84-2) in F344/N rats and B6C3F1 mice (inhalation studies). NTP-TR-472; NIH Publication No. 99-3962. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 2002a. Toxicity studies of pulegone in B6C3F1 mice (Gavage studies). Battelle Research Laboratories, Study No. G004164-X. Unpublished report.

National Toxicology Program (NTP), 2002b. Toxicity studies of pulegone in F344/N rats (Gavage studies). Battelle Research Laboratories, Study No. G004164-W. Unpublished report.

National Toxicology Program (NTP), 2003a. Report on the toxicology and carcinogenesis studies of citral (microencapsulated) (CAS No. 5392-40-5) in F344/N rats and $B6C3F_1$ mice (feed studies). Tech. Rept. Series 505. National Toxicology Program, Research Triangle Park, NC.

National Toxicology Program (NTP), 2003b. Toxicology and carcinogenesis studies of 2,4-hexadienal (89% trans,trans isomer, CAS No. 142-83-6; 11% cis,trans isomer) in F344 rats and B6C3F1 mice (gavage studies). NTP-TR-509. NIH Publication No. 01-4443. National Toxicology Program, Research Triangle, NC.

National Toxicology Program (NTP), 2004. Toxicology and carcinogenesis studies of *trans*-cinnamaldehyde (microencapsulated) (CAS No. 14371-10-9) in F344/N rats and B6C3F1 Mice (feed studies). NTP-TR-514; NIH Publication No. 02-4448 National Toxicology Program, Research Triangle, NC.

Newberne, P.M., Carlton, W.W., Brown, W.R., 1989. Histopathological evaluation of proliferative liver lesions in rats fed *trans*-anethole in chronic studies. Food and Chemical Toxicology 27 (1), 21–26.

Newberne, P., Smith, R.L., Doull, J., Goodman, J.I., Munro, I.C., Portoghese, P.S., Wagner, B.M., Weil, C.S., Woods, L.A., Adams, T.B., Hallagan, J.B., Ford, R.A., 1998. GRAS Flavoring Substances 18. Food Technology 52 (9), 66, 68, 70, 72, 74, 76, 79–92.

Newberne, P., Smith, R.L., Doull, J., Goodman, J.I., Munro, I.C., Portoghese, P.S., Wagner, B.M., Weil, C.S., Woods, L.A., Adams, T.B., Lucas, C.D., Ford, R.A., 1999. The FEMA GRAS assessment of *trans*-anethole used as a flavoring substance. Food and Chemical Toxicology 37, 789–811.

Nijssen, B., van Ingen-Visscher, K., Donders, J., 2003. Volatile Compounds in Food 8.1. Centraal Instituut Voor Voedingsonderzioek. TNO. Zeist, The Netherlands. Available from: <http://www.voeding.tno.nl/vcf/VcfNavigate.cfm>.

Oser, B.L., Hall, R., 1977. Criteria employed by the expert panel of FEMA for the GRAS evaluation of flavouring substances. Food Technology 15, 457–466.

Obourn, J.D., Frame, S.R., Chiu, T., Solomon, T.E., Cook, J.C., 1997a. Evidence that A8947 enhances pancreas growth via a trypsin inhibitor mechanism. Toxicology and Applied Pharmacology 146, 116–126.

Obourn, J.D., Frame, S.R., Bell Jr., R.H., Longnecker, D.S., Elliott, G.S., Cook, J.C., 1997b. Mechanisms for the pancreatic oncogenic effects of the peroxisome proliferator Wyeth-14,643. Toxicology and Applied Pharmacology 145, 425–436.

OECD, 1981. Organisation for Economic Co-operation and Development. http://www.oecd.org/.

Palmer, C.A.N., Hsu, M.-H., Muerhoff, A.S., Griffith, K.J., Johnson, E.F., 1994. Interaction of the peroxisome proliferator-activated receptor A with the retinoid X receptor A unmasks a cryptic peroxisome proliferator response element that overlaps an ARP-1-binding site in the CYP4A6 promoter. Journal of Biological Chemistry 269, 18083–18089.

Palmer, C.A.N., Hsu, M.-H., Griffith, K.J., Raucy, J.L., Johnson, E.F., 1998. Peroxisome proliferator activated receptor—a expression in human liver. Molecular Pharmacology 53, 14–22.

Parent, R.A., Caravello, H.E., Long, J.E., 1992. Two-year toxicity and carcinogenicity study of acrolein in rats. Journal of Applied Toxicology 12 (2), 131–139.

Peters, J.M., Cattley, R.C., Gonzalez, F.J., 1997. Role of PPARα in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator WY-14, 643. Carcinogenesis 18, 2029–2033.

Phillips, D.H., Miller, J.A., Miller, E.C., Adams, B., 1981. Structures of the DNA-adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1′-hydroxyestragole. Cancer Research 41, 176–186.

Phillips, D.H., Reddy, M.V., Randerath, K., 1984. 32P-Post-labeling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5, 1623–1628.

Randerath, K., Haglund, R.E., Phillips, D.H., Reddy, M.V., 1984. 32P-Post-labeling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613–1622.

Reddy, J.K., Qureshi, S.A., 1979. Tumorigenicity of the hypolipidaemic peroxisome proliferator ethyl-α-chlorophenoxyisobutyrate (clofibrate) in rats. British Journal of Cancer 40, 476.

Reddy, J.K., Azarnoff, D.L., Hignite, C.E., 1980. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. Nature 283, 397–398.

Reddy, J.K., Lalwai, N.D., 1983. Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. CRC Critical Reviews in Toxicology 12, 1–58.

Reddy, J.K., Reddy, M.K., Usman, M.I., Lalwani, N.D., Rao, M.S., 1986. Comparison of hepatic peroxisome proliferative effect and its implication of hepatocarcinogenicity of phthalate esters, di(2-ethylhexyl) phthalate, and di(2-ethylhexyl) adipate with a hypolipidemic drug. Environmental Health Perspectives 65, 317–327.

Roscher, R., Schwab, W., Schreier, P., 1997. Stability of naturally occurring 2,5-dimethyl-4-hydroxy-3[2H]furanone derivatives. Zeitschrift fur LebensmitteIntersuchung und Forschung 204, 434–441.

Rozman, K.K., Kerecsam, L., Viluksela, M.K., Osterle, D., Demi, E., Viluksela, M., Stahl, B.U., Greim, H., Doull, J., 1996. Toxicologist view of cancer risk assessment. Drug Metabolism and Review 28, 29–52.

Rulis, A.M., Hattan, D.G., Morgenroth, V.H., 1984. FDA's priority-based assessment of food additives. I. Preliminary results. Regulatory, Toxicology and Pharmacology 26, 44–51.

Sangster, S.A., Caldwell, J., Smith, R.L., Farmer, P.B., 1984a. Metabolism of anethole. I. Pathways of metabolism in the rat and mouse. Food and Chemical Toxicology 22, 695–706.

Sangster, S.A., Caldwell, J., Smith, R.L., 1984b. Metabolism of anethole. II. Influence of dose size on the route of metabolism of *trans*-anethole in the rat and mouse. Food and Chemical Toxicology 22, 707–713.

Sangster, S.A., Caldwell, J., Hutt, A.J., Anthony, A., Smith, R.L., 1987. The metabolic disposition of [methoxy-^{14}C]-labeled *trans*-anethole, estragole and p-propylanisole in human volunteers. Xenobiotica 17, 1223–1232.

Shelby, M.D., Erexson, G.L., Hook, G.L., Tice, R.R., 1993. Evaluation of a three-exposure mouse bone marrow micronucleus protocol: Results with 49 chemicals. Environmental and Molecular Mutagenesis 21 (2), 160–179.

Shirai, T., Hagiwara, A., Kurata, Y., Shibata, M., Fukushima, S., Ito, N., 1982. Lack of carcinogenicity of butylated hydroxytoluene on long-term administration to B6C2F1 mice. Food and Chemical Toxicology 20, 861–865.

Smart, R.C., 1994. Usefulness and limitations of short-term toxicity tests. In: Hodgson, E., Levi, P. (Eds.), Biochemical Toxicology, Second ed. Appleton and Lange, Norwalk, CN.

Smith, R.L., Ford, R.A., 1993. Recent progress in consideration of flavoring ingredients under the Food Additives Amendment. 16 GRAS Substances. Food Technology 47, 104–117.

Smith, R.L., Newberne, P., Adams, T.B., Ford, R.A., Hallagan, J.B.FEMA Expert Panel, 1996. GRAS flavoring substances 17. Food Technology 10, 72–81.

Smith, R.L., Doull, J., Feron, V.J., Goodman, J.I., Munro, I.C., Newberne, P.M., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Adams, T.B., McGowen, M.M., 2001. GRAS flavoring Substances—The 20th publication by the Expert Panel of the Flavor and Extract Manufacturers Association on recent progress in the consideration of flavoring ingredients generally recognized as safe under the Food Additives Amendment. Food Technology 55 (12), 34–55.

Smith, R.L., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Munro, I.C., Newberne, P.M., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Adams, T.B., 2002a. The FEMA GRAS assessment of pyrazine derivatives used as flavor ingredients. Food and Chemical Toxicology 40, 429–451.

Smith, R.L., Adams, T.B., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Rogers, A.E., Caldwell, J., Sipes, I.G., 2002b. Safety assessment of allylalkoxybenzene derivatives used as flavoring substances—methyleugenol and estragole. Food and Chemical Toxicology 40, 851–870.

Smith, R.L., Cohen, S., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Adams, T.B., 2004. Safety evaluation of natural flavour complexes. Toxicology Letters 149, 197–207.

Smith, R.L., Cohen, S.M., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Hall, R.L., Higley, N.A., Lucas-Gavin, C., Adams, T.B., 2005. A procedure for the safety evaluation of natural flavor complexes used as ingredients in food: essential oils. Food Chemical Toxicology 43, 345–363.

Spindler, P., Madsen, C., 1992. Subchronic toxicity study of peppermint oil in rats. Toxicology Letters 62, 215–220.

Strasser Jr., J., Charbonneau, M., Borghoff, S.J., Turner, M.J., Swenberg, J.A., 1988. Renal protein droplet formation in male Fischer 344 rats after isophorone (IPH) treatment. Toxicologist 8, 136.

Sumi, C., Longnecker, D.S., Roebuck, B.D., Brinck-Johnsen, T., 1989. Inhibitory effects of estrogen and castration on the early stage of pancreatic carcinogenesis in Fisher rats treated with azaserine. Cancer Research 49, 2332–2336.

Svoboda, D.J., Azarnoff, D.L., 1979. Tumors in male rats fed ethyl chlorophenoxyisobutyrate, a hypolipidemic drug. Cancer Research 39, 3419–3428.

Takayama, S., Kuwabara, N., 1982. Long-term study on the effect of caffeine in Wistar rats. Gann 73, 365–371.

Tennant, R.W., Margolin, B.H., Shelby, M.D., Zeiger, E., Haseman, J.K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S.A., Anderson, B., Minor, R., 1987. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. Science 236, 933–941.

Thomassen, D., Slattery, J.T., Nelson, S.D., 1988. Contribution of menthofuran to the hepatotoxicity of pulegone: assessment based on matched area under the curve and on matched time course. Journal of Pharmacology Experimental Theraputics 224, 825.

Thomassen, D., Slattery, J.T., Nelson, S.D., 1990. Menthofuran-dependent and independent aspects of pulegone hepatotoxicity: Roles of glutathione. Journal of Pharmacology and Experimental Theraputics 253, 567.

Thomassen, D., Pearson, P.G., Slattery, J.T., Nelson, S.D., 1991. Partial characterization of biliary metabolites of pulegone by tandem mass spectrometry. Drug Metabolism and Disposition 19, 997.

Thomassen, D., Knebel, N., Slattery, J.T., McClanahan, R.H., Nelson, S.D., 1992. Reactive intermediates in the oxidation of menthofuran by cytochrome P-450. Chemical Research Toxicology 5, 123–130.

Tian, Q., Li, G., Shen, J., Wang, Y., 1992. Genotoxic study of furaneol on mice germ cells. Weisheng Duilxue Zazhi 6, 26–28.

Truhau, R., Le Bourhis, B., Attia, M., Glomot, R., Newman, J., Caldwell, J., 1989. Chronic toxicity/carcinogenicity study of *trans*-anethole in rats. Food and Chemical Toxicology 27, 11–20.

United States Department of Agriculture, (USDA) 1972. Food and nutrient intake of individuals in the United States. Household food consumption survey 1965–1966. Report No. 11. US Government Printing Office, Washington, DC.

Viswalingam, A., Caldwell, J., Fournel, S., Schladt, L., Oesch, F., 1988. Hepatic effects of cinnamyl anthranilate resemble those of a peroxisome proliferator in mouse, but not in rat. British Journal of Pharmacology 93, 32.

Waddell, W.J., 2002. Forum Thresholds of carcinogenicity of flavors. Toxicological Sciences 68, 275–279.

Waddell, W.J., 2003a. Threshold for carcinogenicity of N-nitrosodiethylamine for esophageal tumors in rats. Food and Chemical Toxicology 41, 739–741.

Waddell, W.J., 2003b. Threshold of carcinogenicity in the ED01 study. Toxicological Sciences 72, 158–163.

Waddell, W.J., 2003c. Threshold in chemical carcinogenesis: What are animal experiments telling us? Toxicologic Pathology 31 (3), 260–262.

Waddell, W.J., 2003d. Comparison of human exposures to selected chemicals with thresholds for NTP carcinogenicity studies in rodents. Human and Experimental Toxicology 22, 501–506.

Webb, D.R., Ridder, M., Alden, C.L., 1989. Acute and subchronic nephrotoxicity of d-limonene in Fischer 344 rats. Food and Chemical Toxicology 27 (10), 639–649.

Wiseman, R.W., Miller, E.C., Miller, J.A., Liem, A., 1987. Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J × C3H/HeJF1 mice. Cancer Research 47, 2275–2283.

Wolf, M.A., Rowe, V.K., McCollister, D.D., Hollingsworth, R.L., 1956. Toxicological studies of certain alkylated benzenes and benzene. Archives of Industrial Health 14, 387–398.

Woods, L.A., Doull, J., 1991. GRAS evaluation of flavoring substances by the Expert Panel of FEMA Regulatory. Toxicology and Pharmacology 14, 48–58.

Xing, B., Liu, K., Li, Y., Yao, A., Li, Y., Zhi, X., Zhang, X., Zheng, A., 1988. Mutagenic studies on HDMF. Zhonghua Yufangyixue Zazhi 22, 85–87.

Zeiger, E., Haseman, J.K., Shelby, M.D., Margolin, B.H., Tennant, R.W., 1990. Evaluation of four in vitro genetic toxicity tests for predicting rodent carcinogenicity: Confirmation of earlier results with 41 additional chemicals. Environmental and Molecular Mutagenesis 16 (Supplement 18), 1–14.