GRAS Flavoring Substances 17

The 17th publication by the Flavor and Extract Manufacturers' Association's Expert Panel on recent progress in the consideration of flavoring ingredients generally recognized as safe under the Food Additives Amendment

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The Flavor and Extract Manufacturers' Association of the United States (FEMA) in 1960 established an independent Expert Panel of toxicologists, biochemists, and other scientists to review the safety of flavor ingredients under the authority of Section 201(s) of the Federal Food, Drug, and Cosmetic Act (Oser and Hall, 1977; Oser and Ford, 1991). This provision, commonly known as the generally recognized as safe (GRAS) provision (Hallagan and Hall, 1995a), exempts from the food additive provisions of the Act substances that can be "generally recognized, by experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures . . . to be safe under the conditions of intended use."

This is the 17th publication by the FEMA Expert Panel. It includes the results of the Panel's review of 19 new flavor ingredients (Table 1 on p. 73, Table 2 on p. 78). It also includes the Panel's conclusion that additional average usual and maximum use levels in imitation dairy products of 1.0 and 5.0 ppm, respectively, for sodium (±)2-(4-methoxyphenoxy)propanoate (FEMA No. 3773) and 0.0001 and 0.01 ppm, respectively, for 4-methoxy-2-methyl-2-butanethiol (FEMA No. 3785) would not alter the GRAS status of these materials. As with previous reports, this publication also describes the results of other recent Expert Panel activities.

Recent Developments in the Flavor Industry

The flavor industry is dynamic; changes in technology and use patterns are common. The Expert Panel routinely reviews recent developments to determine the possible impact on the FEMA GRAS program. For example, a critical part of the GRAS provision is that safety is determined "under the conditions of intended use." Thus, when new uses of a FEMA GRAS flavor ingredient are developed, the substance must be reevaluated in light of those new uses.

A significant recent development in the flavor industry is the production of flavor ingredients using biotechnology. An approach for assessing the safety of flavor ingredients produced using genetically modified organisms and a mechanism for integrating this approach into the FEMA GRAS program was described by Hallagan and Hall (1995b), who relied heavily on the approach developed by the International Food Biotechnology Council (IFBC, 1990).

The FEMA Expert Panel uses a decision-tree approach to assess the safety of flavor ingredients produced using genetically modified organisms (GMOs). This approach consists of several levels (Hallagan and Hall, 1995b):

The first level consists of an evaluation of the identity of the substance, including an examination of its regulatory status; i.e., is it currently permitted for use in food? If so, what are the specifications for the substance, and are they met by the material produced through the use of a GMO? If the substance produced through a GMO is the same as the conventionally produced substance, the assessment is greatly simplified. In fact, if the substance is currently permitted for use in food by virtue of the existence of an appropriate prior safety assessment, the evaluation would essentially be complete. In addition to determining the identity of the substance, the first level consists of evaluating the method of production employing a GMO, specifications for the substance produced using a GMO, and the safety of constituents produced using a GMO compared to conventional methods of production.

In the second level, the Panel evaluates specific issues associated with genetic modification, including whether the microbe ends up in the flavor ingredient, whether there are antibiotic resistance concerns, and whether there are safety concerns associated with the vector and DNA insert code. The Panel's evaluation is performed within the context of its usual inquiry into the method of production of a conventionally produced substance, to ensure that no deleterious contaminants are present in the final product. In general, the issues evaluated by the Panel are consistent with the issues identified by the Food and Drug Administration in its approval of recombinant chymosin (21 CFR 184.1685; Flamm, 1991).

Once the Panel has resolved issues associated with the GMO used in the production of the flavor ingredient, it applies the criteria employed in a GRAS assessment (Woods and Doull, 1991; Hallagan and Hall, 1995a) if it is necessary to do so, such as when the flavor ingredient is not currently permitted for use in food. If the Panel determines that the flavor ingredient is GRAS, then the result of its evaluation is published, as in this article.

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In the Panel's view, the GRAS assessment of a flavor ingredient produced by modern biotechnology should be no different from the safety assessment of a conventionally produced flavor ingredient once issues associated with the GMO are resolved. The Panel's approach to flavor ingredients produced using GMOs is largely consistent with the "vertical" approach advocated by Miller and Gunary (1993).

The Panel performed a GRAS assessment of Thaumatin B-Recombinant (rThaumatin) produced through the use of a GMO and concluded that the substance is GRAS (Table 1). Plant-derived thaumatin was previously determined to be GRAS (Oser et. al., 1984), and rThaumatin was determined to be a highly purified form of plant-derived thaumatin. The Panel fully evaluated the production process for rThaumatin, including the development of the GMO. The applicant also provided toxicology data on rThaumatin which were consistent with those obtained previously for plant-derived thaumatin and did not lead to the identification of any safety concerns.

Review of New Toxicological Information

From its inception, the Panel has maintained a constant surveillance of scientific data on flavor ingredients previously judged to be GRAS to assure that they continue to meet the criteria for GRAS status (Hall and Oser, 1970). To conduct comprehensive and systematic reviews of FEMA GRAS flavor ingredients, the Panel routinely consults new information on mechanisms of toxicity, pharmacokinetics and metabolism, exposure, new methods of toxicology testing, and relevance of animal testing to humans.

As a part of the first such review, initiated in 1975 and referred to as GRAS affirmation (GRASa), FEMA, in cooperation with the National Academy of Sci-

Table 1—Primary Names and Synonyms. *Primary names, in capital letters, and synonyms, in lower case, are listed alphabetically*

FEMA No.	Flavor Ingredient
3797	4-ACETOXY-2,5-DIMETHYL-3(2H)-FURANONE 3(2H)-Furanone, 4-(acetyloxy)-2,5-dimethyl-
3798	2,4-DIHYDROXYBENZOIC ACID Benzoic acid, 2,4-dihydroxy- 4-Carboxyresorcinol 2,4-DHBA β-Hydroxysalicylic acid β-Resorcinolic acid
3799	1,2-DIMETHOXYBENZENE Benzene, 1,2-dimethoxy- Catechol dimethyl ether Veratrol-E
3800	4-ETHYLOCTANOIC ACID Octanoic acid, 4-ethyl-
3801	ETHYL VANILLIN β-D-GLUCOPYRANOSIDE Benzaldehyde, 3-ethoxy-4-(β-D-glucopyranosyloxy)- 3-Ethoxy-4-(β-glucopyranosyloxy)benzaldehyde Glucoethylvanillin
3802	5-HYDROXY-2-DODECENOIC ACID LACTONE δ-2-Dodecenolactone 6-Heptyl-5,6-dihydro-2-pyrone 5-Heptyl-2-pentene-5-olide 2H-Pyran-2-one, 6-heptyl-5,6-dihydro-
3803	4-HYDROXY-3-METHYLOCTANOIC ACID LACTONE 5-Butyldihydro-4-methylfuran-2(3H)-one 2(3H)-Furanone, 5-butyldihydro-4-methyl- β-Methyl-γ-octalactone Octanoic acid, 4-hydroxy-3-methyl-, lactone Whiskey lactone
3804	2-ISOPROPYL-N,2,3-TRIMETHYLBUTYRAMIDE Butanamide, N,2,3-trimethyl-2-(1-methylethyl)- N,2,3-Trimethyl-2-isopropylbutanamide
3805	*l*-MENTHOL ETHYLENE GLYCOL CARBONATE Carbonic acid, 2-hydroxyethyl 5-methyl-2-(1-methylethyl) cyclohexyl ester, [1R-(1α,2β,5α)] Frescolat, type MGC Menthol glycol carbonate
3806	*l*-MENTHOL 1- and 2-PROPYLENE GLYCOL CARBONATE Carbonic acid, menthyl ester, monoester with 1,2-propanediol Carbonic acid, 2-hydroxypropyl *l*-menthyl ester Frescolate, type MPC
3807	*l*-MENTHONE 1,2-GLYCEROL KETAL 1,4-Dioxaspiro[4,5]decane-2-methanol, 9-methyl-6-(1-methylethyl)-, *l*-isomer Frescolat, racemic Frescolat, type MGA 9-Methyl-6-(1-methylethyl)-1,4dioxaspiro[4,5]decan-2-methanol, *l*-isomer
3808	*d,l*-MENTHONE 1,2-GLYCEROL KETAL 1,4-D oxaspiro[4,5]decane-2-methanol, 9-methyl-6-(1-methylethyl)-, *dl*-isomer 9-Methyl-6(1-methylethyl)-1,4-dioxaspiro-[4,5]decane-2-methanol, *dl* isomer
3809	*cis*- and *trans*-MENTHONE-8-THIOACETATE (1R-*cis* and *trans*)-2-(1-Acetylthio-1-methylethyl)-5-methylcyclohexanone Ethanoic acid, S-[1-methyl-1-(4-methyl-2-oxocyclohexyl)-ethyl] ester, *cis* and *trans* isomers
3810	MONO-MENTHYL SUCCINATE Butanedioic acid, mono[5-methyl-2-(1-methylethyl)cyclohexyl] ester, [1R-(1α,2β,5α)]-
3811	NEOHESPERIDIN DIHYDROCHALCONE 3,5-Dihydroxy-4-(3-hydroxy-4-methoxy-hydrocinnamoyl)phenyl-2-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside Neohesperidin DHC NHDC 1-Propanone,1-[4-[[2-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)
3812	SODIUM 3-METHOXY-4-HYDROXYCINNAMATE Ferulic acid, sodium salt 3-(4-Hydroxy-3-methoxyphenyl)propenoic acid, sodium salt Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, monosodium salt Sodium ferulate
813	TAURINE 2-Aminoethanesulfonic acid Ethanesulfonic acid, 2-amino-
3814	THAUMATIN B-RECOMBINANT Contour rThaumatin
3815	VANILLYL ETHYL ETHER 4-(Ethoxymethyl)-2-methoxyphenol Ethyl 4-hydroxy-3-methoxybenzyl ether Phenol, (4-ethoxymethyl)-2-methoxy- VEE

ences, undertook a comprehensive survey of the usage of the existing FEMA GRAS substances. Concurrently, FEMA was contracted by FDA to prepare Scientific Literature Reviews (SLRs) on groups of structurally related substances containing data relevant to the safety evaluation of flavor ingredients.

As the result of GRASa, approximately 1,270 substances were affirmed as GRAS, and approximately 30 additional substances required more data to support the GRAS decision. FEMA sponsored studies to obtain the necessary data on all but three of these 30 substances. There was a lack of interest in any future use of these three materials, and therefore the requested data were not acquired to complete their safety evaluation. These three substances—2-methyl-5-vinylpyrazine (FEMA No. 3211), o-vinylanisole (FEMA No. 3248), and musk ambrette (FEMA No. 2758)—were removed from the GRAS list (Oser et al., 1984); the other 27 were affirmed as GRAS after the required studies had been completed.

In 1993, the Panel again undertook a comprehensive review of all of the flavor ingredients previously determined to be GRAS. As part of the GRAS reaffirmation (GRASr) process, the Panel initiated a program to revise the FEMA SLRs to incorporate recent advances in science and technology. As part of the GRASr process, an Interpretive Summary is prepared for each individual flavor ingredient. This is an integration of relevant scientific information on metabolism, pharmacology, toxicology, and exposure that forms the basis for the Panel's conclusions of GRAS for a flavor ingredient. A comprehensive summary is also produced for each group of structurally related flavor ingredients.

By 1994, the Panel completed the GRASr process for approximately 400 aliphatic acyclic substances used as flavor ingredients. During 1995, an additional 124 substances (119 aliphatic alicyclic substances and 5 fused-ring lactone derivatives) were reaffirmed as GRAS. The Panel plans to publish the results of the GRASr program in a series of publications in the peer-reviewed literature (e.g., Adams et al., 1996).

The following substances have had new toxicological findings published since their initial GRAS review.

Menthone (FEMA No. 2667)

Menthone is an alicyclic monoterpenoid ketone. Two common stereoisomers which occur in nature are *l*-menthone and *d*-isomenthone. The *l*-isomer and the racemic mixture of menthone are used as a flavor ingredient in foods up to an average maximum level of 70 ppm. Based on a reported annual volume of 13,300 kg (NAS, 1987), the estimated daily per capita intake ("eaters only") of menthone from use as a flavor ingredient is 42 μg/kg. (The intake calculation used for menthone is as follows: Intake (μg/kg) = [(annual volume, kg) × (1 × 10^9 (μg/kg)]/ [(population × 0.6 × 365 days)/60 kg], where the population (10%, "eaters only") = 24 × 10^6; 0.6 represents the assumption that only 60% of the flavor volume was reported in the survey (NAS, 1987); the average adult body weight in the U.S. is 60 kg.)

Menthone is a constituent of peppermint oil (15–30%) and spearmint oil and occurs naturally in other foods such as rum, nutmeg, and cocoa (CIVO-TNO, 1994). Its intake from consumption of peppermint oil is approximately eight times its intake from use as a flavor ingredient (Stofberg and Kirschman, 1985).

Three short-term studies, originating from the same laboratory, on peppermint oil or peppermint oil components (e.g., menthone, pulegone, and menthol) have reported "cyst-like spaces" in the white matter of the cerebellum. Groups of male and female rats were administered 0, 200, 400, or 800 mg/kg/day menthone (Madsen et al., 1986); 0, 10, 40, or 100 mg/kg/day peppermint oil (Thorup et al., 1983a); 0, 20, 80, or 160 mg/kg/day pulegone (FEMA No. 2963; Thorup et al., 1983b); or 0, 200, 400, and 800 mg/kg/day menthol (FEMA No. 2665; Thorup et al., 1983b) by gavage daily for 28 days. At the two highest dose levels in the peppermint oil (40 and 100 mg/kg/day) and pulegone (80 and 160 mg/kg/day) studies and at all dose levels in the menthone study, the authors reported "cyst-like spaces" in the white cerebellar matter. This effect was not observed in the menthol study, even though menthol is metabolized to menthone.

The two pathologists on the FEMA Expert Panel reviewed the original rat brain histology slides from the three studies and additional data and made the following observations:

1. The three reports of "cyst-like spaces" in the white matter of the cerebellum were not accompanied by any evidence of cellular reaction in tissue adjacent to the spaces. A subsequent 90-day study with dose levels of 0, 10, 40, or 100 mg/kg/day peppermint oil in rats also showed no evidence of cellular reaction in adjacent tissue. Despite the extended dosing period, there was no significant difference in the appearance and the extent of "cyst-like spaces" between the 90-day and 28-day studies (Spindler and Madsen, 1992).

2. Attempts to reproduce the "cyst-like spaces" in rat white cerebellar tissue, even when higher dose levels (150 and 500 mg/kg/day) of peppermint oil were administered daily for 5 weeks to the same strain of rat (Mengs and Stotzem, 1989), have failed to confirm results of the three original 28-day studies and the followup 90-day study (Spindler and Madsen, 1992). In addition, no evidence of "cyst-like spaces" was observed in the white matter of the cerebellum of dogs administered peppermint oil in gelatin capsules daily at dose levels of 25 or 125 mg/kg for 5 weeks (Mengs and Stotzem, 1989).

3. A study with peppermint oil completed in 1992 using a brain perfusion method did not reveal any evidence of "cyst-like spaces" in the cerebellum of any of the rats (Olsen, 1994). Following reexamination of the slides and consideration of all available data, the Expert Panel concluded that the weight of evidence strongly suggests that the rat cerebellar "cyst-like spaces" originally reported with peppermint oil, pulegone, and menthone were artifacts arising from inadequate preparation and fixation of the cerebellar tissue.

Menthone was reaffirmed as GRAS in 1995 based on recognized pathways of its metabolic detoxication and those of menthol (Williams 1940; Neubeuer, 1901; Hamalainen, 1912; Yamaguchi et al., 1994); its very low level of flavor use; evaluation of the reports of neurotoxicity, including the artifactual nature of results reported in short-term studies of peppermint oil and its constituents, including menthone; the safety factor calculated from dose levels that produced no effects in a 5-week or a 90-day study for peppermint oil which contains menthone or in a chronic dietary study for the structurally related alcohol *d,l*-menthol, which metabolizes to menthone (NCI, 1978); and its very low acute oral toxicity (Levenstein, 1973; Igimi and Ide, 1974). This evidence of safety is supported by the occurrence of menthone as a natural component of traditional foods.

Allyl Isovalerate (FEMA No. 2045)

Allyl isovalerate is an aliphatic acyclic ester formed from allyl alcohol and isovaleric acid (FEMA No. 3102). Allyl isovalerate is used as a flavor ingredient in foods up to an average maximum level of 40 ppm. Based on a reported annual volume of 0.9 kg (NAS, 1987), the estimated daily per capita intake ("eaters only") is 3 ng/kg (see section on menthone for calculation of intake).

A bioassay on allyl isovalerate was conducted by the Southern Research Institute, Birmingham, Ala., under contract to the National Toxicology Program (NTP) during January 1979 to January 1981 (NTP, 1983). In the standard NTP protocol, groups of 50 F344 rats and B6C3F1 mice of both sexes were administered

allyl isovalerate via corn oil gavage at doses of 0, 31, or 62 mg/kg/day for 5 days per week for 103 weeks. The NTP report concluded: "Under the conditions of these 2-year studies, allyl isovalerate was carcinogenic for F344/N rats and B6C3F1 mice, causing increased incidences of hematopoietic system neoplasms (mononuclear-cell leukemia in male rats and lymphoma in female mice)."

There was a statistically significant ($p<0.05$) increase in the incidence of hematopoietic neoplasms in rats and mice. However, hematopoietic tumors occur at a high and variable rate in both sexes of F344 rats and B6C3F1 mice. Statistical analysis should apply a significance level of 1% ($p<0.01$) to account for the high background incidence of these common tumors in both mice and rats. This is consistent with the proposed guidelines (Haseman et al., 1986), which consider a compound to exhibit carcinogenic potential if the highest dose is associated with an increased incidence of a common tumor that is significant at $p<0.01$, or an increased incidence in a rare tumor at $p<0.05$.

Based on pair-wise comparisons of the incidence of hematopoietic tumors in the NTP study by a Fisher exact test ($p<0.05$), significance levels were >1% ($p>0.01$; i.e., 0.030 for high-dose male F344 rats and 0.093 for high-dose female B6C3F1 mice). Therefore, the incidence of commonly observed hematopoietic tumors is *not* statistically significant for either high-dose rats or mice at the 1% level. Statistical analysis of data for the incidence of hematopoietic tumors in independent control groups of rats and mice ($p=0.0255$ for male F344 rats and 0.0315 for female B6C3F1 mice) confirms the need for the 1% significance level (Haseman et al., 1986).

From a comparison with historical data, the slight but statistically significant (at the 5% level) increase in the incidence of specific types of hematopoietic tumors at the highest dose level in the allyl isovalerate-treated rats (males: control, 2%; 62 mg/kg, 14%; females: controls, 8%; 62 mg/kg, 18%) and mice (males: control, 8%; 62 mg/kg, 16%; females: controls, 22%; 62 mg/kg, 36%) falls within the historical range of control groups administered corn oil via gavage in other NTP studies of these two strains of rodents.

For example, the incidence of mononuclear cell leukemia in historical control male rats treated only with corn oil via gavage ranges from a low of 4% to a high of 24%, and leukemia incidence in female rats ranges from 4 to 28% (NTP, 1983). In more recent studies (Furosemide), NTP has reported leukemia incidences of 46% and 16% in control male and female rats, respectively (NTP, 1989). The historical control incidence of lymphoma ranges from a low of 8.3% to a high of 22% in male B6C3F1 mice, and 14% to 31% in female mice (NTP, 1983). Again, except for the high-dose female mice, the historical control incidence of lymphoma in B6C3F1 mice includes the range of incidences reported in the control and treated groups in the NTP study on allyl isovalerate. Because the incidence of hematopoietic neoplasms in the F344 rat and the B6C3F1 mouse is a common phenomenon, study results which show statistical significance (only at $p<0.05$) for these types of neoplasms must be cautiously interpreted (Haseman et al., 1986).

To further explore the increased incidence of mononuclear cell leukemia in rats and malignant lymphoma in mice reported in the NTP study, and the pancytopenia described in isovaleric acidemia (Cohn et al., 1978), NTP conducted a 14-day gavage study using allyl isovalerate to determine if there would be toxic effects to the bone marrow in B6C3F1 mice and F344 rats (Hong et al., 1988). B6C3F1 mice were provided 0, 31, 62, or 125 mg/kg/day, 5 days/wk for two weeks; F344 rats were dosed similarly providing 0, 31, 62, 125, or 250 mg/kg/day,5 days/wk for two weeks.

Allyl isovalerate had no effect on either total bone marrow cellularity or hematology in mice or rats. Significant decreases in mice of pluripotent hematopoietic stem cells (CFU-S) and granulocyte-macrophage progenitors (CFU-GM) in the bone marrow were reported. Host resistance following challenge with either *Listeria monocytogenes* or malaria (*Plasmodium yoelli*) did not demonstrate significant differences between treated and control mice, nor were there other effects on the immune system. The immunologic studies described by Hong et al. (1988) have not been validated and have little value other than for experimental interest. The infectious disease challenges demonstrate a lack of effect by allyl isovalerate on host resistance in B6C3F1 mice or F344 rats.

Allyl isovalerate was not mutagenic in the NTP studies for *Salmonella* tester strains (TA98, TA100, TA1535, TA1537) with or without S-9 activation. The lack of genotoxic activity of allyl isovalerate and the lack of any carcinogenic response in sensitive target organs such as the liver, even in the highly sensitive B6C3F1 mouse, further suggest that the finding of increased hematopoietic system neoplasms in the NTP study was a chance occurrence resulting from the high, but variable, spontaneous incidence of these types of tumors. The lack of hematopoietic neoplasms in animals maintained on drinking water containing allyl alcohol (Lijinsky and Reuber, 1987) or acrolein (Lijinsky and Reuber, 1987; Parent et al., 1992) for two years also supports the conclusion that hemopoietic neoplasms occur spontaneously in F344 rats and B6C3F1 mice.

In summary, the NTP bioassay of allyl isovalerate reported an increased incidence of mononuclear cell leukemia in rats and lymphomas in mice. However, based on (1) the available toxicology data on allyl isovalerate, (2) the observation that the incidences of hematopoietic neoplasms reported in the NTP study were not statistically significant at the 1% level ($p<0.01$; Haseman et al., 1986) and were within the range of incidence reported in historical controls, (3) the lack of genotoxic activity of allyl isovalerate, and (4) the lack of carcinogenic activity of allyl isovalerate and other allyl esters used as flavor ingredients, it is concluded that the results of the NTP study do not provide evidence of a potential carcinogenic effect in humans. It is notable that administration of allyl isovalerate in the NTP study was associated with a statistically significant decrease in the incidence of tumors in liver, lung and thyroid in male mice and the pituitary in female mice. The nominal no observed adverse effect levels (NOAELs) of the toxicity studies are variable, but this may reflect an adaptive action by the animal.

The relevance of the findings of the NTP study to the safety assessment of the use of allyl isovalerate as a flavor ingredient is particularly doubtful given that the dose levels of 31 mg/kg/day and 62 mg/kg/day of allyl isovalerate used in the rat and mouse 2-year bioassay are >10,000,000 times the daily per capita intake ("eaters only") of 3 ng/kg from use of allyl isovalerate as a flavor ingredient. This large margin of safety combined with the available toxicology data, including the results of the chronic NTP study, do not justify a modification of the current GRAS status of allyl isovalerate under conditions of intended use.

Allyl isovalerate was reaffirmed as GRAS in 1995 based on its mode of hydrolysis (Anders, 1989; Heymann, 1980; Butterworth et al., 1975), the metabolism of allyl alcohol and isovaleric acid (Racker, 1955; Serafini-Cessi, 1972; Patel et al. 1980; Voet and Voet, 1990), its very low level of flavor use, the safety factor calculated from results of subchronic and chronic studies (NTP, 1983), and its low acute oral toxicity (Moreno, 1977; NTP, 1983).

Dihydrocoumarin (FEMA No. 2381)

Dihydrocoumarin (i.e., 3,4-dihydrocoumarin) is used as a flavor ingredient in foods up to an average maximum level of 164 ppm. Based on a reported annual

volume of 3,750 kg (NAS, 1987), the estimated daily per capita intake ("eaters only") of dihydrocoumarin is 12 μg/kg (see menthone section for calculation of intake). Dihydrocoumarin has not been reported to occur naturally in food (CIVO-TNO, 1994).

NTP (1993) conducted a bioassay of dihydrocoumarin using the standard NTP protocol with F344 rats and B6C3F1 mice of both sexes. Rats and mice received dihydrocoumarin in corn oil by gavage five days per week for 103 weeks, providing daily dose levels of 0, 150, 300, or 600 mg/kg to rats and 0, 200, 400, or 800 mg/kg to mice. The NTP report concluded:

"Under the conditions of these 2-year gavage studies, there was some evidence of carcinogenicity of 3,4-dihydrocoumarin in male F344 rats based on the increased incidence of renal tubule adenomas and focal hyperplasia. The transitional cell carcinomas in two 600 mg/kg males may also have been chemical related. There was no evidence of carcinogenic activity of 3,4-dihydrocoumarin in female F344 rats receiving 150, 300, or 600 mg/kg. There was no evidence of carcinogenic activity of 3,4-dihydrocoumarin in male B6C3F1 mice receiving 200, 400, or 800 mg/kg. There was some evidence of carcinogenic activity in female B6C3F1 mice based on increased incidences of hepatocellular adenoma, and hepatocellular adenoma and carcinoma (combined)."

In a dose-related manner, decreased survival rates were reported only in male rats at all dose levels, with the majority of deaths occurring after week 92. Only 2 of 60 males rats survived to study termination. The decreased survival was attributed to a progressive degenerative nephropathy leading to renal failure. In a related stop-exposure, evaluation groups of male rats received 600 mg/kg dihydrocoumarin by gavage for 9 or 15 months (NTP, 1993), at which time half the animals were necropsied while the remainder (i.e., the stop-exposure group) received corn oil until death or the end of the study. Nephropathy was significantly greater for the stop-exposure group than for males sacrificed at 9 or 15 months. (Stop-exposure evaluations are intended to determine the progression or regression of chemical-related lesions during the recovery period.) These findings indicate that this form of nephropathy is a progressive degenerative disease associated with the aging process. Any chemically related nephrotoxicity observed is probably irreversible, especially in the male sex of F344 rats. Female rats and mice of both sexes exhibited no significant change in survival rates, mean body weight changes, and no clinical findings related to the administration of dihydrocoumarin.

Microscopic examination of combined single and step sections of the kidneys of male rats revealed a statistically significant ($p<0.05$) dose-related increase in renal tubule hyperplasia (control, 1/50; 150 mg/kg, 5/48; 300 mg/kg, 6/47; 600 mg/kg, 8/50). The incidence of renal tubule adenomas in the treated groups of male rats was not significantly different ($p<0.05$) than in the control group (control,1/50; 150 mg/kg, 1/48; 300 mg/kg, 3/47; 600 mg/kg, 6/50), but the total incidence of renal tubule adenomas in all groups of treated male rats (10/145) and in the high-dose group (6/50) exceeds the range in NTP historical controls (8/1,019). Renal transitional cell carcinomas were reported in two male rats that died early in the study (at days 444 and 528) at the 600 mg/kg dose level, but there was no evidence of transitional cell carcinomas in the majority (86%) of males surviving longer than 528 days. Step-sections showed no additional evidence of treatment-related response of the renal transitional cells.

Although treated female rats also exhibited increased nephropathy at the two highest dose levels, they did not experience a significant increase in either renal tubule hyperplasia or renal tubule neoplasms. The incidence of renal tubule hyperplasia and neoplasms in male mice was not statistically significant and not dose-related (controls 0/50; 200 mg/kg, 1/51; 400 mg/kg, 2/51; 800 mg/kg, 1/49). There was no incidence of renal tubule hyperplasia or renal tubule neoplasms in female mice. Additionally, no renal histopathology was observed in dietary studies providing similar dose levels of dihydrocoumarin to rats or dogs for periods up to 2 years (Hagan et al., 1967; Trubeck Labs, 1957). Therefore, the renal hyperplastic and neoplastic effects are sex and species specific.

Chronic renal nephropathy and an increased incidence of focal hyperplasia and renal tubule neoplasms are well-recognized age-related phenomena in the male F344 rat. The male rat kidney has been shown to be a unique target organ for the carcinogenic effects of a variety of chemical substances (EPA, 1991; Burdock et al., 1990). The Expert Panel concludes that the renal effects of dihydrocoumarin in the male rat are a species- and sex-specific phenomenon and reflect the sensitivity of the male rat kidney to chronic progressive nephropathy, focal hyperplasia, and specific tumorigenic responses. The occurrence of transitional cell carcinomas in two male rats cannot be assumed to be related to the daily administration of dihydrocoumarin, since there was no evidence of renal transitional cell changes in longer-lived animals at any dose level

The high background incidence of hepatocellular adenomas and carcinomas in both control and treated groups of male and female mice demonstrate the sensitivity of the B6C3F1 mouse liver to neoplastic findings which is more pronounced for male B6C3F1 mice (Maronpot et al., 1986; Haseman et al., 1986). In the NTP study, the incidence of adenomas was greater in control or treated males (control 29/50; 200 mg/kg, 23/51; 400 mg/kg, 36/51; 800 mg/kg, 31/50) than in any group of control or treated females (control 10/51; 200 mg/kg, 20/50; 400 mg/kg, 22/50; 800 mg/kg, 20/52); this demonstrates the increased sensitivity of the male mouse liver. The incidence of hepatocellular adenomas in all groups of treated female mice was significantly greater than in the control group, but the incidence was not dose-related.

The incidence of hepatocellular carcinomas was not significantly different between treated and control groups of male mice (control 11/50; 200 mg/kg, 11/51; 400 mg/kg, 22/50; 800 mg/kg, 20/52), but, again, the incidence of carcinomas in control males was greater than in any group of treated females (control 3/51; 200 mg/kg, 2/50; 400 mg/kg, 4/50; 800 mg/kg, 6/52). The incidence of hepatocellular carcinomas in groups of treated female mice was not significantly different from that in the control group (control 3/51; 200 mg/kg, 2/50; 400 mg/kg, 4/50; 800 mg/kg, 6/52). These neoplastic findings are consistent with the historically high levels of background hepatocellular neoplasms in B6C3F1 mice (Maronpot and Boorman, 1982; Maronpot et al., 1986; Haseman et al., 1986).

The Expert Panel considers that the observations of hepatic neoplasms in the NTP mouse bioassay are not relevant to the safety of dihydrocoumarin when it is used as a flavor ingredient. This conclusion is consistent with that reached by others (Nutrition Foundation, 1983; FDA, 1994) and is based on the high incidence of spontaneous hepatocellular neoplasms (adenomas and carcinomas) in the strain of mice studied, the absence of consistent dose-response data, the lack of hepatocellular neoplastic effects in rats, and the relatively high dose levels (800 mg/kg/day) administered, compared to intake levels (0.012 mg/kg/day) from use as a flavor ingredient.

The Expert Panel also reviewed genotoxicity data and concluded, on the basis of the weight of evidence, that dihydrocoumarin was not mutagenic (Prival et al., 1982; Haworth et al., 1983; Heck et al., 1989; NTP, 1993) and that the isolated positive results from an SCE-CHO assay (NTP, 1993) performed at high

solution concentrations of dihydrocoumarin were not evidence of a genotoxic potential. The negative response in the *Salmonella* mutagenicity assay (Ashby and Tenant, 1991) supports the Panel's conclusion that dihydrocoumarin exhibits little potential for interaction with DNA.

Dihydrocoumarin was reaffirmed as GRAS in 1995 based on its presumed mode of lactone ring hydrolysis and subsequent oxidative metabolism to carboxylic acids; its low level of flavor use; the safety factor calculated from results of subchronic (Lake et al., 1994) and chronic studies (Hagan et al., 1967); its lack of tumorigenic, genotoxic, and mutagenic potential (Prival et al., 1982; Haworth et al., 1983; Heck et al., 1989; NTP, 1993) and its very low acute oral toxicity (Moreno, 1972; Jenner et al., 1964; Levenstein, 1953).

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—References continued on page 81, Table 2 on next page

Table 2—**GRAS Flavoring Ingredients and Usage Levels (usual ppm/average maximum ppm).** *FEMA average maximum levels on which the Expert Panel based its judgments that the substances are generally recognized as safe for their intended uses*

FEMA No.	Substance	Baked goods	Frozen dairy	Meat products	Soft candy	Gelatin & puddings	Soups	Snack foods	Nonalcoholic beverages	Alcoholic beverages	Other uses
3797	4-Acetoxy-2,5-dimethyl-3 (2H)furanone	5/10	—	2/5	2/5	2/5	1/2	2/5	—	—	hard candy 2/5 confection frosting 2/5 imitation dairy 2/5
3798	2,4-Dihydroxybenzoic acid	—	450/450	100/100	500/500	500/500	250/250	500/500	500/500	500/500	breakfast cereals 500/500 fats oils 500/500 milk products 100/100 cheese 500/500 fruit ices 500/500 poultry 500/500 egg products 500/500 fish products 500/500 processed vegetables 100/100 condiment relish 500/500 jam jelly 500/500 sweet sauce 500/500 nut products 500/500 reconstituted vegetable 500/500 gravies 500/500 imitation dairy 500/500 chewing gum 3333/3333 sugar substitutes 12500/125,000 instant coffee tea 125/125 seasonings flavors 25000/25000
3799	1,2-Dimethoxybenzene	0.3/3.0	—	—	0.5/5.0	0.3/3.0	—	—	0.1/1.0	0.2/2.0	milk products 0.1/1.0 hard candy 0.5/5.0 chewing gum 15/30
3800	4-Ethyloctanoic acid	—	—	1.0/10	—	—	0.1/1.0	0.1/1.0	—	—	milk products 0.1/1.0 cheese 1.0/10 gravies 0.2/2.0
3801	Ethyl vanillin β-D-glucopyranoside	15/150	—	—	—	—	—	3.0/20	—	—	hard candy 5.0/25 instant coffee tea 5.0/50
3802	5-Hydroxy-2-dodecenoic acid lactone	0.5/5.0	0.1/5.0	0.1/5.0	3.0/10	0.1/10	0.1/5.0	1.0/15	1.0/5.0	2.0/5.0	breakfast cereals 1.0/5.0 other grains 1.0/5.0 fat oils 5.0/15 milk products 0.5/5.0 cheese 0.5/5.0 fruit juice 0.1/5.0 fruit ices 0.1/5.0 poultry 0.1/5.0 egg products 0.1/5.0 fish products 0.1/5.0 processed vegetables 0.1/5.0 condiment relish 0.1/5.0 confection frosting 3.0/25 jam jelly 0.1/5.0 sweet sauce 0.1/5.0 nut products 0.1/5.0 reconstituted vegetable 0.1/5.0 gravies 2.0/25 imitation dairy 2.0/25 hard candy 1.0/10 chewing gum 2.0/15 granulated sugar 0.1/5.0 sugar substitutes 0.1/5.0 instant coffee tea 2.0/15 seasonings flavors 0.1/5.0

—Table 2 continued on p. 80

Table 2—**GRAS Flavoring Ingredients and Usage Levels (usual ppm/average maximum ppm)**, *continued*

FEMA No.	Substance	Baked goods	Frozen dairy	Meat products	Soft candy	Gelatin & puddings	Soups	Snack foods	Nonalcoholic beverages	Alcoholic beverages	Other uses
3803	4-Hydroxy-3-methyl-octanoic acid lactone	—	—	—	—	—	—	—	0.5/3.0	1.0/5.0	hard candy 1.0/5.0 chewing gum 2.0/10
3804	2-Isopropyi-N,2,3-trimethyl-butyramide	—	—	—	—	—	—	—	—	3.0/8.0	confection frosting 50/150 hard candy 50/50 chewing gum 750/3000
3805	*l*-Menthol ethylene glycol carbonate	—	—	—	—	500/2000	—	—	—	—	confection frosting 500/2000 hard candy 500/2000 chewing gum 5000/20,000
3806	*l*-Menthol 1-and 2-propylene glycol carbonate	—	—	—	—	—	—	—	—	—	hard candy 1000/3000 chewing gum 5000/10,000
3807	*l*-Menthone 1,2-glycerol ketal	50/80	—	—	50/80	—	—	—	10/15	—	chewing gum 500/800
3808	*d,l*-Menthone 1,2-glycerol ketal	50/80	—	—	50/80	—	—	—	10/15	—	soft candy 50/80 chewing gum 500/800
3809	*cis*- and *trans*-Menthone-8-thioacetate	1.0/10	0.2/10	—	1.0/10	0.5/5.0	—	—	0.1/0.3	0.2/5.0	breakfast cereals 0.5/5.0 fats oils 3.0/40 milk products 0.2/10 cheese 0.5/5.0 fruit juice 0.2/10 fruit ices 0.2/10 processed vegetables 0.1/2.0 confection frosting 1.0/10 jam jelly 0.5/10 sweet sauce 0.2/5.0 reconstituted vegetable 0.1/2.0 imitation dairy 0.2/10 hard candy 1.0/10 chewing gum 5.0/20
3810	Mono-menthyl succinate	300/900	70/210	—	200/600	150/450	—	—	40/120	40/120	milk products 60/180 fruit ices 70/210 confection frosting 200/600 jam jelly 150/450 hard candy 200/600 chewing gum 1250/3750
3811	Neohesperidin dihydrochalcone	—	2.0/3.0	—	2.0/3.0	2.0/3.0	1.0/2.0	—	2.0/3.0	—	fats oils 4.0/4.0 milk products 2.0/3.0 fruit juice 2.0/3.0 fruit ices 1.0/2.0 processed vegetables 2.0/3.0 condiment relish 2.0/3.0 jam jelly 2.0/3.0 sweet sauce 2.0/3.0 imitation dairy 3.0/4.0 hard candy 2.0/4.0 chewing gum 4.0/5.0
3812	Sodium 3-methoxy-4-hydroxycinnamate	100/100	—	—	—	100/100	—	—	400/400	—	sweet sauce 300/300 sugar substitutes 2000/2000
3813	Taurine	250/250	—	585/585	—	—	500/500	2100/2100	30/30	—	breakfast cereals 1000/1000 fats oils 565/565 cheese 630/630 poultry 550/550 egg products 190/190 fish products 190/190

									processed vegetables 200/200
									condiment relish 1125/1125
									sweet sauce 375/375
									nut products 640/640
									reconstituted vegetable 375/375
									gravies 375/375
									imitation dairy 190/190
									sugar substitutes 3750/3750
									seasonings flavors 70000/70000
3814	Thaumatin B-recombinant	1.0/1.0	1.0/2.0	2.0/2.0	2.0/5.0	1.0/2.0	2.0/5.0	1.0/2.0	5.0/10	5.0/10	breakfast cereals 1.0/2.0
											milk products 3.0/6.0
											cheese 10/20
											fruit juice 2.0/5.0
											fruit ices 2.0/5.0
											poultry 2.0/5.0
											egg products 2.0/5.0
											fish products 5.0/10
											processed vegetables 2.0/5.0
											condiment relish 1.0/2.0
											confection frosting 2.0/5.0
											jam jelly 2.0/5.0
											sweet sauce 2.0/5.0
											nut products 5.0/10
											reconstituted vegetable 2.0/5.0
											gravies 2.0/5.0
											imitation dairy 10/15
											hard candy 2.0/5.0
											chewing gum 150/200
											sugar substitutes 2.0/5.0
											instant coffee tea 2.0/5.0
											seasonings flavors 0.5/1.0
3815	Vanillyl ethyl ether	5.0/15	3.0/5.0	—	5.0/10	—	—	5.0/15	3.0/5.0	3.0/5.0	breakfast cereals 5.0/10
											milk products 2.0/5.0
											confection frosting 5.0/20
											alcoholic beverage 3.0/5.0
											chewing gum 10/20
											instant coffee tea 3.0/10

(Continued from page 77)

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