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Review

The FEMA GRAS assessment of α,β-unsaturated aldehydes and related substances used as flavor ingredients

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ABSTRACT

This publication is the 12th in a series of safety evaluations performed by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA). In 1993, the Panel initiated a comprehensive program to re-evaluate the safety of more than 1700 GRAS flavoring substances under conditions of intended use. Since then, the number of flavoring substances has grown to more than 2200 chemically-defined substances. Elements that are fundamental to the safety evaluation of flavor ingredients include exposure, structural analogy, metabolism, toxicodynamics and toxicology. Scientific data relevant to the safety evaluation for the use of aliphatic, linear α,β-unsaturated aldehydes and structurally related substances as flavoring ingredients are evaluated. The group of substances was reaffirmed as GRAS (GRASr) based, in part, on their self-limiting properties as flavoring substances in food; their low level of flavor use; the rapid absorption and metabolism of low in vivo concentrations by well-recognized biochemical pathways; adequate metabolic detoxication at much higher levels of exposure in humans and animals; the wide margins of safety between the conservative estimates of intake and the no-observed-adverse effect levels determined from subchronic and chronic studies. While some of the compounds described here have exhibited positive *in vitro* genotoxicity results, evidence of in vivo genotoxicity and carcinogenicity occurs only under conditions in which animals are repeatedly and directly exposed to high irritating concentrations of the aldehyde. These conditions are not relevant to humans who consume α,β-unsaturated aldehydes as flavor ingredients at low concentrations distributed in a food or beverage matrix.

Contents

Abbreviations: ABS, chromosomal aberrations; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; BSA, bovine serum albumin; dG, deoxyguanosine; FEMA, Flavor and Extract Manufacturers Association; FPG, formamidopyrimidine DNA glycosylase; GRAS, generally recognized as safe; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; K_M, Michaelis constant; LDL, low density lipoprotein; LPO, lipid peroxidation; MN, micronuclei; NOEL, no-observed-effect level; PCE, polychromatic erythrocyte; PCNA, proliferating cell nuclear antigen; PUFA, polyunsaturated fatty acids; SCE, sister chromatid exchange; UDS, unscheduled DNA synthesis; V_{max}, maximum reaction rate.

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1. Introduction

This review presents the key scientific data relevant to the safety evaluation for the flavor use of 91 aliphatic linear α,β-unsaturated aldehydes, and related alcohols, acetals, carboxylic acids, and esters. All aldehydes in this group contain α,β-unsaturation or extended conjugation (2,4-dienals and trienals). This group contains alcohols and esters that hydrolyze to alcohols which are then readily and sequentially oxidized to yield the corresponding α,β-unsaturated aldehydes and carboxylic acids. This group also contains acetals which readily hydrolyze into α,β-unsaturated aldehydes and alcohols that are converted to α,β-unsaturated aldehydes and esters that further hydrolyze to linear α,β-unsaturated carboxylic acids. Summarily, the group contains flavoring substances that may hydrolyze and/or oxidize to yield linear α,β-unsaturated aldehydes, α,β-unsaturated aldehydes with extended conjugation, and corresponding α,β-unsaturated carboxylic acids (see Table 1).

2. Flavor use, natural occurrence in food, and endogenous formation

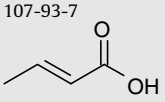
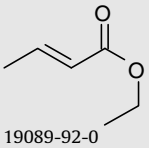
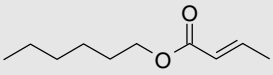
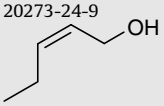
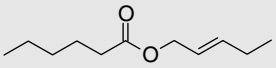
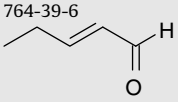
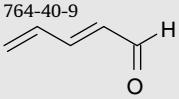
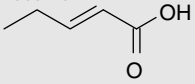
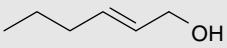
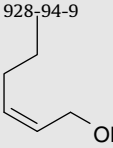
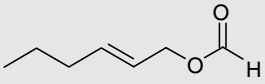
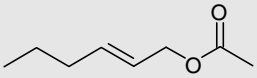
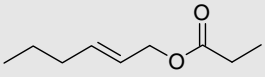
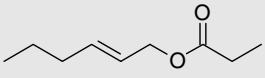
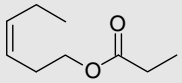
Approximately 66% of the reported total annual volume (14,278 kg) reported in the most recent survey (Gavin et al., 2008) of 91 flavor ingredients in this group, is accounted for by *trans*-2-hexenal, its corresponding alcohol, acetate ester, and ethyl acetal. *trans*-2-Hexenal (No. 21) has a reported annual volume of use as a flavor ingredient in the USA of 6183 kg. It also is ubiquitous in the food supply as a naturally occurring substance (Nijssen et al., 2006). Formed mainly by the action of lipoxygenases on unsaturated fatty acids in plants (Almosnino and Belin, 1991; Andrianarison et al., 1991), it is a constituent of most fruits and vegetables, occurring at concentrations in the range from 0.01 to 20 ppm (Nijssen et al., 2006). These levels approximate mean added usual use levels as a flavor ingredient. An estimated daily intake from consumption of fruits and vegetables has been reported to be between 31 and 165 μg/kg bw (Schuler and Eder, 1999). It also occurs widely in non-alcoholic (e.g., tea) and alcoholic beverages (e.g., wine) and in cooked meats as a result of lipid thermal breakdown.

The combined daily *per capita* intake of *trans*-2-hexenal from foods that contain *trans*-2-hexenal is calculated to be approximately 2390 μg/person per day (see Table 2). The highest intake of *trans*-2-hexenal occurs from consumption of bananas. The estimated daily *per capita* intake of *trans*-2-hexenal from intentional addition as a flavor ingredient is 57 μg/person per day. Intake of *trans*-2-hexenal from consumption of traditional foods exceeds intake as an added flavoring substance by a factor ≈40 (Stofberg and Kirschman, 1985; Stofberg and Grundschober, 1987; Gavin et al., 2008; Nijssen et al., 2006; United States Department of Agriculture Economic Research Service) (see Tables 1 and 2).

Other members in this group of α,β-unsaturated aldehydes and alcohols that show significant reported annual volumes as flavor ingredients include ethyl *trans*-2-*cis*-4-decadienoate (No. 79,746 kg/yr), a key component of pear aroma, and *trans*-2-*cis*-6-nonadienal (No. 62,971 kg/yr), a substance commonly recognized as violet leaf aldehyde that is characteristic of cucumber. Substances in this group that have intakes from traditional food that far exceed that from flavor use (i.e., consumption ratio >1) include 2-decenal (No. 70), 2-dodecenal (No. 86), 2-hexen-1-ol (No. 9), 2-hexenal (No. 21), 2-hexen-1-yl acetate (No. 12), *trans*-2-heptenal (No. 40), 2-nonenal (No. 57), 2-octenal (No. 47), 2-pentenal (No. 6), *trans*-2-nonen-1-ol (No. 55), 2-undecenal (No. 83), 2,4-pentadienal (No. 7), *trans*,*trans*-2,4-hexadienal (No. 30), 2,4-heptadienal (No. 42), *trans*,*trans*-2,4-octadienal (No. 49), 2,6-nonadien-1-ol (No. 60), 2,4-nonadienal (No. 59), nona-2-*trans*-6-*cis*-dienal (No. 62); 2-*trans*,4-*trans*-decadienal (No. 73), ethyl *trans*-2-*cis*-4-decadienoate (No. 79), and 2,4-undecadienal (No. 84).

Dienals have been detected in many of the same foods as their α,β-unsaturated aldehyde homologues (apples, grapes, broccoli, chicken, tea and beer) (Nijssen et al., 2006). Compared to *trans*-2-hexenal, *trans*,*trans*-2,4-hexadienal (No. 30) has been detected at lower levels in a more limited number of foods. Higher homologous dienals and trienals (>C_9) have been detected in heated

Table 1
Identity and exposure data for α,β-unsaturated aldehydes and related substances used as flavor ingredients

Flavoring ingredient	FEMA number	CAS number and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eater only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				μg/d	μg/kg bw/day		
1. (*E*)-2-Butenoic acid	3908	107-93-7 	40[d]	7	0.1	+	NA
2. Ethyl *trans*-2-butenoate	3486	10544-63-5 	43	5	0.09	+	NA
3. Hexyl 2-butenoate	3354	19089-92-0 	0.4[e]	0.05	0.0009	+	NA
4. (*Z*)-2-Pentenol	4305	20273-24-9 	5	1	0.01	+	NA
5. Pent-2-enyl hexanoate	4191	74298-89-8 	3[d]	0.5	0.01	+	NA
6. 2-Pentenal	3218	764-39-6 	11	1	0.02	60	5
7. 2,4-Pentadienal	3217	764-40-9 	1[f]	0.2	0.003	4	4
8. 2-Pentenoic acid	4193	13991-37-2 	0.01[d]	0.02	0.0003	+	NA
9. 2-Hexen-1-ol	2562	2305-21-7 	2634	322	5	7989	3
10. (*Z*)-2-Hexen-1-ol	3924	928-94-9 	17	2	0.03	+	NA
11. (*E*)-2-Hexenyl formate	3927	53398-78-0 	0.3	0.04	0.0006	+	NA
12. 2-Hexen-1-yl acetate	2564	10094-40-3 	518	63	1	424	0.8
13. *trans*-2-Hexenyl propionate	3932	53398-80-4 	151	18	0.3	+	NA
14. (*Z*)-3 & (*E*)-2-Hexenyl propionate	3778	33467-74-2 53398-80-4  	30	4	0.06	+	NA

(*continued on next page*)

Table 1 (*continued*)

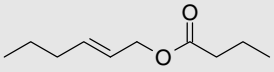
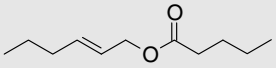
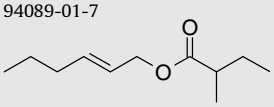
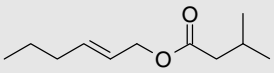
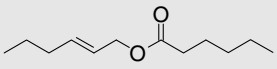
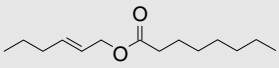
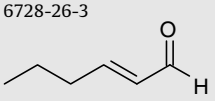
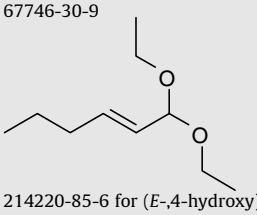
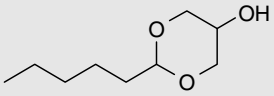
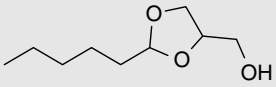
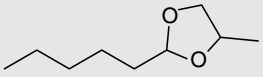
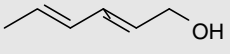
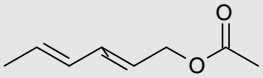
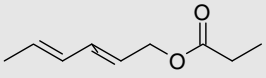
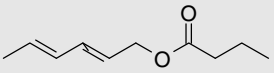
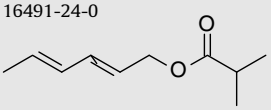
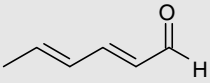
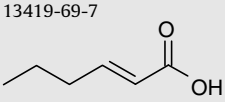
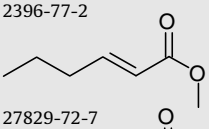
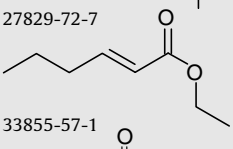
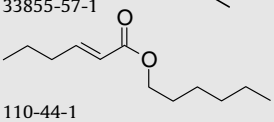
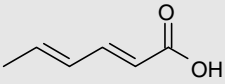
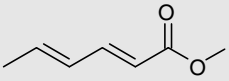
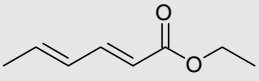
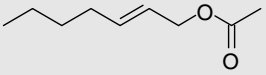
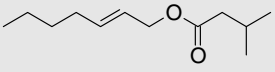
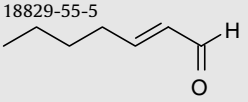
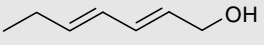
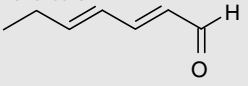
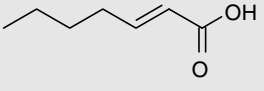
Flavoring ingredient	FEMA number	CAS number and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eater only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/day		
15. *trans*-2-Hexenyl butyrate	3926	53398-83-7 	0.1	0.02	0.0003	+	NA
16. *trans*-2-Hexenyl pentanoate	3935	56922-74-8 	1	0.1	0.002	–	NA
17. *trans*-2-Hexenyl 2-methylbutyrate	4274	94089-01-7 	0.2	0.04	0.0006	–	NA
18. *trans*-2-Hexenyl isovalerate	3930	68698-59-9 	0.03	0.004	6.E-05	+	NA
19. (*E*)-2-Hexenyl hexanoate	3983	53398-86-0 	1	0.1	0.002	+	NA
20. 2-Hexenyl octanoate	4135	85554-72-9 	0.1	0.02	0.0003	–	NA
21. 2-Hexenal	2560	6728-26-3 	6183	756	13	246,550[g]	40
22. (*E*)-2-Hexenal diethyl acetal	4047	67746-30-9 	129	16	0.3	–	NA
23. *trans*-2-Hexenal glyceryl acetal	4273	214220-85-6 for (*E*-,4-hydroxy)  897672-50-3 (*E*,5-hydroxy) 	50[d]	9	0.1	–	NA
24. *trans*-2-Hexenal propylene glycol acetal	4272	94089-21-1 	50[d]	9	0.1	–	NA
25. (*E*,*E*)-2,4-Hexadien-1-ol	3922	111-28-4 	10	1	0.02	+	NA
26. 2,4-Hexadienyl acetate	4132	1516-17-2 	0.08	0.01	0.0002	–	NA
27. 2,4-Hexadienyl propionate	4131	16491-25-1 	0.3	0.03	0.0005	–	NA

Table 1 (*continued*)

Flavoring ingredient	FEMA number	CAS number and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eater only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/day		
28. 2,4-Hexadienyl butyrate	4133	16930-93-1 	1	0.1	0.002	–	NA
29. 2,4-Hexadienyl isobutyrate	4134	16491-24-0 	57	7	0.1	–	NA
30. *trans*,*trans*-2,4-Hexadienal	3429	142-83-6 	6	0.8	0.01	1	0.2
31. *trans*-2-Hexenoic acid	3169	13419-69-7 	379	46	0.8	+	NA
32. Methyl 2-hexenoate	2709	2396-77-2 	88	11	0.2	+	NA
33. Ethyl *trans*-2-hexenoate	3675	27829-72-7 	0.1	0.02	0.0003	+	NA
34. Hexyl *trans*-2-hexenoate	3692	33855-57-1 	2	0.2	0.004	+	NA
35. (*E*,*E*)-2,4-Hexadienoic acid	3921	110-44-1 	229	28	0.5	+	NA
36. Methyl sorbate	3714	689-89-4 	0	0	0	+	NA
37. Ethyl sorbate	2459	2396-84-1 	0.9	0.1	0.002	+	NA
38. Hept-*trans*-2-en-1-yl acetate	4125	16939-73-4 	0.08	0.01	0.0002	–	NA
39. Hept-2-en-1-yl isovalerate	4126	253596-70-2 	44	5	0.09	–	NA
40. *trans*-2-Heptenal	3165	18829-55-5 	6	0.8	0.01	7614	1269
41. 2,4-Heptadien-1-ol	4127	33467-79-7 	0.07	0.01	0.0001	+	NA
42. 2,4-Heptadienal	3164	4313-03-5 	19	2	0.039	872	46
43. (*E*)-2-Heptenoic acid	3920	10352-88-2 	18	2	0.04	+	NA

(*continued on next page*)

Table 1 (*continued*)

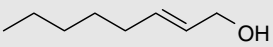
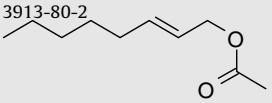
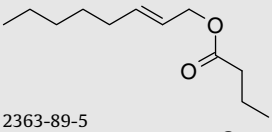
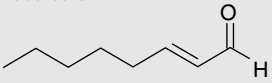
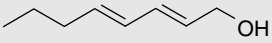
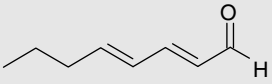
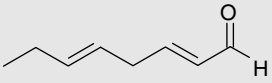
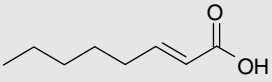
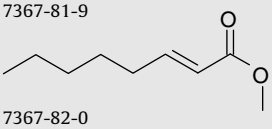
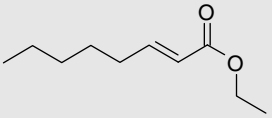
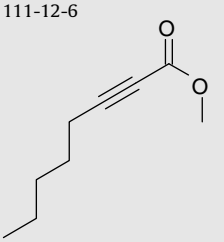
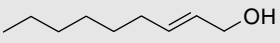
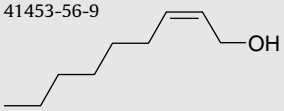
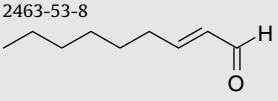
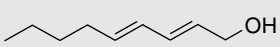
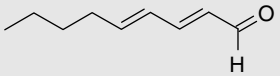
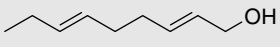
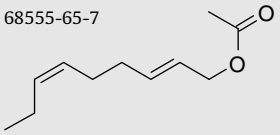
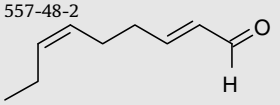
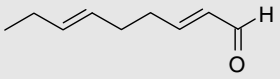
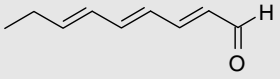
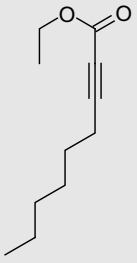
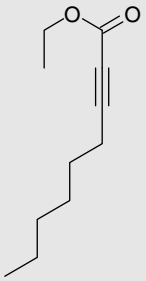
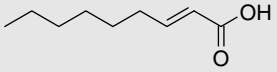
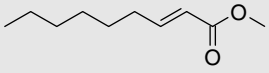
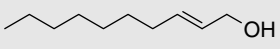
Flavoring ingredient	FEMA number	CAS number and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eater only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/day		
44. (*E*)-2-Octen-1-ol	3887	18409-17-1 	0.4	0.05	0.0008	+	NA
45. *trans*-2-Octen-1-yl acetate	3516	3913-80-2 	13	2	0.03	–	NA
46. *trans*-2-Octen-1-yl butanoate	3517	84642-60-4 	2	0.3	0.004	–	NA
47. 2-Octenal	3215	2363-89-5 	3	0.4	0.007	2046	682
48. (*E*,*E*)-2,4-Octadien-1-ol	3956	18409-20-6 	0.05	0.01	0.0001	–	NA
49. *trans*,*trans*-2,4-Octadienal	3721	30361-28-5 	0.6	0.07	0.001	0.7	1
50. 2-*trans*,6-*trans*-Octadienal	3466	56767-18-1 	0.05[e]	0.007	0.0001	–	NA
51. (*E*)-2-Octenoic acid	3957	1871-67-6 	10	2	0.03	+	NA
52. Methyl *trans*-2-octenoate	3712	7367-81-9 	2	0.3	0.005	+	NA
53. Ethyl *trans*-2-octenoate	3643	7367-82-0 	0.7	0.08	0.001	+	NA
54. Methyl 2-octynoate	2729	111-12-6 	225	28	0.5	–	NA
55. *trans*-2-Nonen-1-ol	3379	31502-14-4 	0.2[e]	0.03	0.0004	1.4	7
56. *cis*-2-Nonen-1-ol	3720	41453-56-9 	3	0.3	0.005	+	NA
57. 2-Nonenal	3213	2463-53-8 	4	0.5	0.008	5413	1353

Table 1 (*continued*)

Flavoring ingredient	FEMA number	CAS number and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eater only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/day		
58. 2,4-Nonadien-1-ol	3951	62488-56-6 	3	0.4	0.006	+	NA
59. 2,4-Nonadienal	3212	6750-03-4 	10	1	0.02	189	19
60. 2,6-Nonadien-1-ol	2780	7786-44-9 	42	5	0.09	50	1
61. (*E,Z*)-2,6-Nonadien-1-ol acetate	3952	68555-65-7 	3	0.4	0.007	–	NA
62. Nona-2-*trans*-6-*cis*-dienal	3377	557-48-2 	971	119	2	3265	27
63. 2-*trans*-6-*trans*-Nonadienal	3766	17587-33-6 	0.01	0.002	3.E-05	+	NA
64. Nona-2,4,6-trienal	4187	57018-53-8 	0.02[d]	0.004	0.00006	+	NA
65. Methyl 2-nonynoate	2726	111-80-8 	295	36	0.6	–	NA
66. Ethyl 2-nonynoate	2448	10031-92-2 	4	0.5	0.009	–	NA
67. (*E*)-2-Nonenoic acid	3954	14812-03-4 	0.1	0.01	0.0002	+	NA
68. Methyl 2-nonenoate	2725	111-79-5 	1159	142	2	–	NA
69. (*E*)-2-Decenol	4304	22104-80-9 	1[d]	0.2	0.0003	+	NA

(*continued on next page*)

Table 1 (*continued*)

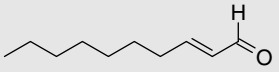
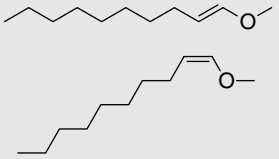
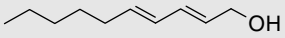
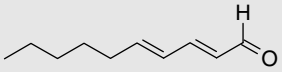
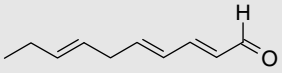
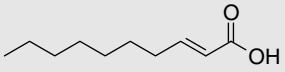
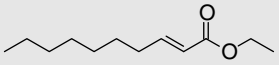
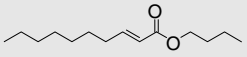
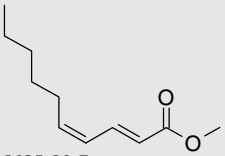
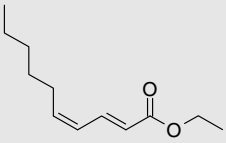
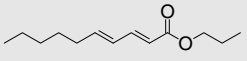
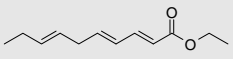
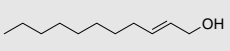
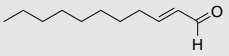
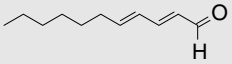
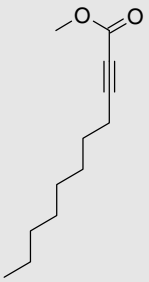
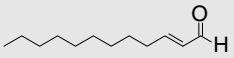
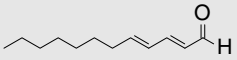
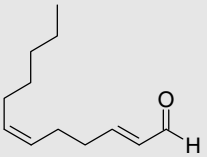
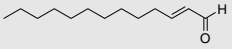
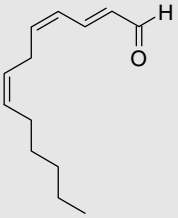
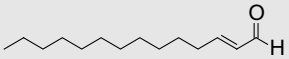
Flavoring ingredient	FEMA number	CAS number and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eater only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/day		
70. 2-Decenal	2366	3913-71-1 	68	8	0.1	34,111	502
71. *trans*- and *cis*-1-Methoxy-1-decene	4161	79930-37-3 	1	0.1	0.002	–	NA
72. (*E*,*E*)-2,4-Decadien-1-ol	3911	18409-21-7 	21	3	0.04	+	NA
73. 2-*trans*,4-*trans*-Decadienal	3135	25152-84-5 	121	15	0.2	33,414	276
74. 2,4,7-Decatrienal	4089	51325-37-2 	0.08	0.01	0.0002	+	NA
75. (*E*)-2-Decenoic acid	3913	334-49-6 	0.06	0.01	0.0001	+	NA
76. Ethyl *trans*-2-decenoate	3641	7367-88-6 	0.1	0.02	0.0003	+	NA
77. Butyl 2-decenoate	2194	7492-45-7 	2[e]	0.3	0.004	–	NA
78. Methyl (*E*)-2-(*Z*)-4-decadienoate	3859	4493-42-9 	0.4	0.05	0.0008	+	NA
79. Ethyl *trans*-2-*cis*-4-decadienoate	3148	3025-30-7 	746	91	2	455	0.6
80. Propyl 2,4-decadienoate	3648	84788-08-9 	1[d]	0.2	0.003	–	NA
81. Ethyl 2,4,7-decatrienoate	3832	78417-28-4 	0.003	0.0004	6.E-06	+	NA
82. 2-Undecen-1-ol	4068	37617-03-1 	0.3	0.03	0.0006	+	NA

Table 1 (*continued*)

Flavoring ingredient	FEMA number	CAS number and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eater only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/day		
83. 2-Undecenal	3423	2463-77-6 	4	0.5	0.008	14,168	3542
84. 2,4-Undecadienal	3422	13162-46-4 	5	0.6	0.01	4	0.8
85. Methyl 2-undecynoate	2751	10522-18-6 	0.2[f]	0.04	0.0006	–	NA
86. 2-Dodecenal	2402	4826-62-4 	18	2	0.04	64	3
87. *trans,trans*-2,4-Dodecadienal	3670	21662-16-8 	0.002	0.0002	3.E-06	–	NA
88. 2-*trans*-6-*cis*-Dodecadienal	3637	21662-13-5 	0.1[f]	0.02	0.0003	+	NA
89. 2-Tridecenal	3082	7774-82-5 	3	0.4	0.006	+	NA
90. 2-*trans*-4-*cis*-7-*cis*-Tridecatrienal	3638	13552-96-0 	0.05[f]	0.009	0.0001	+	NA
91. Tetradec-2-enal	4209	51534-36-2 	0.6	0.07	0.0012	+	NA

[a] Intake (µg/person/day) calculated as follows: [(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days)], where population (10%, "eaters only") = 28×10^6 for the USA; where correction factor × 0.6 for NAS surveys and 0.8 for the Lucas et al. (1999) and Gavin et al. (2008) USA surveys representing the assumption that only 60% and 80% of the annual flavor volume, respectively was reported in the poundage surveys Gavin et al. (2008), Lucas et al. (1999), and NAS (1970, 1982, 1987). Intake (µg/kg bw/day) calculated as follows: [(µg/person/day)/body weight], where body weight × 60 kg. Slight variations may occur from rounding.

[b] Quantitative data for the United States reported by Stofberg and Grundschober (1987).

[c] The consumption ratio is calculated as follows: (annual consumption via food, kg)/(most recent reported volume as a flavoring substance, kg); NA = data not available.

[d] The volume cited is the anticipated volume, which was the maximum amount of flavor estimated to be used annually by the manufacturer at the time the material was proposed for use.

[e] Lucas et al. (1999).

[f] NAS (1970, 1982).

[g] Consumption ratio for 2-hexenal calculated according to Table 2.

Table 2
Consumption of *trans*-2-hexenal from foods

Food	Annual consumption of this food in the USA (kg/yr)	Concentration of *trans*-2-hexenal in food (mg/kg food)[c]	Annual consumption of *trans*-2-hexenal via this food in the USA (kg)[d]
Apple	1,863,000,000[a]	0.585	1090
Apple juice	736,000,000[a]	0.6	442
Apricot	253,000,000[b]	35.75	9045
Banana	2,346,000,000[a]	76	178296
Cherry	161,000,000[a]	0.06525	11
Grapefruit juice	276,000,000[a]	0.07	19
Grape	920,000,000[a]	0.5	460
Melon	2,185,000,000[a]	0.17	371
Peach	805,000,000[a]	0.102	82
Pear	276,000,000[a]	0.027	7
Raspberry	50,600,000[a]	0.0275	1
Strawberry fruit	368,000,000[a]	6.6	2429
Cabbage (cooked)	1,081,000,000[a]	0.07	76
Cucumber	989,000,000[a]	1.8	1780
Potato	13,110,000,000[a]	0.015	197
Bell pepper	368,000,000[a]	0.006	2
Tomato	7,084,000,000[a]	6.5	46046
Butter (heated)	460,000,000[a]	8.4	3864
Fish	1,357,000,000[a]	0.008	11
Chicken	5,796,000,000[a]	0.13	753
Beer	21,620,000,000[a]	0.002	43
Black tea	75,900,000[a]	16.5	1252
Tea	195,500,000[a]	6.46	1263
Potato chips (American)	437,000,000[a]	0.04	17
Mango	36,800,000[a]	0.8	29
Fig	13,800,000[a]	0.005	0.07
Artichoke	46,000,000[a]	0.00027	0.01
Radish	115,000,000[b]	65	7475
Kiwifruit	18,400,000[a]	13.9	256
Turnip	92,000,000[b]	3	276
		Total	246,550

[a] From Stofberg and Grundschober (1987).
[b] United States Department of Agriculture Economic Research Service, http://www.ers.usda.gov/United States Department of Agriculture Economic Research Service, http://www.ers.usda.gov/.
[c] From Maarse et al. (1999).
[d] Annual consumption of food in the USA (kg/yr) × Concentration of 2-hexenal in food (mg/kg) × (1×10^{-6} kg/mg) = Annual consumption of 2-hexenal *via* this food in the USA (kg).

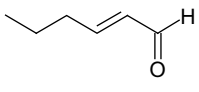
products such as peanuts and chicken (Maarse et al., 1999) and in plants as breakdown products from thermal degradation of polyunsaturated fatty acids (Almosnino and Belin, 1991; Andrianarison et al., 1991).

The dienal with the highest annual volume of use, *trans*-2-*cis*-6-nonadienal (No. 62), has been detected in at least 25 foods, primarily vegetables and cooked meats (chicken), at natural concentrations in the range from <1 ppb to 6 ppm (Nijssen et al., 2006). Based only on its natural presence in cucumber at 6 ppm, the estimated average daily intake of *trans*-2-*cis*-6-nonadienal (≈1 mg/person per day) from consumption of cucumber (Stofberg and Grundschober, 1987) is at least 10 times the daily *per capita* intake ("eaters only") from use as an added flavor ingredient (0.119 mg/person per day).

In addition to food and flavor sources, aliphatic linear 2-alkenals such as 2-butenal, 2-hexenal[1], 2-nonenal and 2,4-alkadienals such as 2,4-decadienal are produced endogenously in animals as products of lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFA) (Frankel et al., 1987). As products of LPO decomposition, endogenous alkenals such as butenal or acrolein form exocyclic propane/etheno-DNA adducts (Bartsch, 1999) and protein adducts (Chen et al., 2000). Alkenal-DNA adducts are increased in humans consuming high levels of polyunsaturated fatty acids (ω-6) (Fang et al., 1996), in patients with metal (iron or copper) storage diseases associated with liver cancer (Nair et al., 1999), and in breast tissue of breast cancer patients (Wang et al., 1996). One of the most abundant LPO aldehyde products is malondialdehyde which forms deoxyguanosine adducts (1–120/10^8 nucleotides) in the liver, colon, breast, pancreas and white blood cells of healthy humans (Marnett, 2002). The adduct is mutagenic in bacteria and has been shown to be repaired by nucleotide excision repair pathways (Marnett, 1999).

Endogenous aldehydes also form carbonylated protein adducts. Protein adducts increase during aging and marked increases have been reported in numerous pathological conditions (e.g., atherosclerosis, rheumatoid arthritis, and metal storage diseases) (Shingarpure et al., 2001). Often drugs or other agents that induce LPO form reactive aldehydes. Rats given doses of 10 mg/kg of doxorubicin showed increased heart and plasma levels of aliphatic alkenals prior to cardiotoxicity and plasma aldehyde (*trans*-2-heptenal, *trans*-2-nonenal, and 4-hydroxy-2-nonenal) concentrations increased to 3.5 times control levels (Luo et al., 1999).

Based on their mode of absorption, distribution, and metabolism, the potential for dietary aldehydes to form protein and DNA adducts is not as significant as that of aldehydes formed endogenously in a target organ. The high oxidation potentials of linear aliphatic alcohols and aldehydes at low concentrations (see below) in body fluids following absorption suggest that plasma levels of dietary aldehydes are significantly decreased prior to and during first-pass hepatic metabolism.

[1]


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3. Hydrolysis, absorption, distribution, excretion, and metabolism

3.1. Hydrolysis

3.1.1. Acetals

The three acetals (Nos. 22–24) in this group all hydrolyze to a common aldehyde, *trans*-2-hexenal, and to ethanol, glycerol, or propylene glycol (Knoefel, 1934; Morgareidge, 1962). *In vitro* studies in simulated gastric fluid show that 1,1-dimethoxyethane[2], acetal[3], and related acetals are hydrolyzed within 1–5 h, and to a lesser degree in simulated intestinal fluid (Morgareidge, 1962). In a study in which rabbits were administered 1,1-dimethoxyethane, acetal, and other aliphatic acetals in aqueous suspension by stomach tube indicated that the acetals undergo rapid hydrolysis in the stomach (see Fig. 1) (Knoefel, 1934). It was also reported that those acetals that are resistant to acid hydrolysis also show lower levels of narcotic effects, which are observed at high dose levels (Knoefel, 1934). Aliphatic acetals would be expected to undergo similar hydrolysis in humans.

A study investigated the feasibility of using acetals as pro-drugs. 2-Propylpentanal acetals were synthesized and their metabolic conversion to valproic acid (2-propylpentanoic acid), which acts as an anticonvulsant, was determined. Both 2-propylpentanoic acid and 2-propylpentanol were identified in the supernatant and microsomal fractions of rat liver that had been incubated with the dimethyl, diethyl, and di-isopropyl acetals of 2-propylpentanal. The appearance of valproic acid occurs at a rate of 470 nmol/g liver/15 min and the rate of 2-propylpentanol formation is 410 nmol/g liver/15 min. These studies provide evidence that the *in vivo* hydrolysis of dimethoxy-, diethoxy-, and diisopropyl-2-propylpentanal acetals yields the corresponding alcohols and parent aldehyde, 2-propylpentanal (Vicchio and Callery, 1989).

Results from experimental studies support the conclusion that acetals are readily hydrolyzed in the digestive system—the acidic environment of the stomach, intestinal fluid, and the liver—to yield alcohol and aldehyde products. The three acetals of *trans*-2-hexenal (No. 21) are expected to hydrolyze to 2-hexenal and ethanol, glycerol, or propylene glycol. The absorption, distribution, and excretion of these acetal metabolites have been studied from a number of perspectives in rodents and humans (Hald and Jacobsen, 1948; Wallgren and Barry, 1970; Halsted et al., 1973; Lame and Segall, 1986; Mitchell and Petersen, 1987).

3.1.2. Esters

Similar to the acetals discussed above, α,β-unsaturated esters (Nos. 2, 3, 5, 11–20, 26–29, 32–34, 36–40, 45, 46, 52–54, 61, 65, 66, 68, 76–81, 85) undergo rapid hydrolysis to their component alcohols and carboxylic acids in reactions that are principally catalyzed by carboxylesterases (see Fig. 2) (Heymann, 1980; Anders, 1989; Graffner-Nordberg et al., 1998). A number of β-carboxylesterase isoenzymes have been identified and their substrate specificities have been correlated with the structures of the alcohol and carboxylic acid components (e.g., R and R', see Fig. 2). In general, α,β-unsaturated esters are more rapidly hydrolyzed than their saturated analogues (Heymann, 1980).

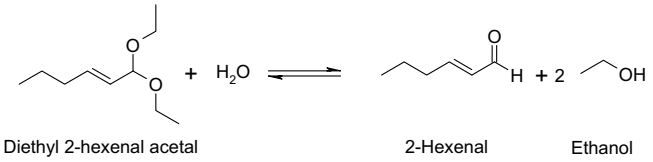


Fig. 1. Acetal hydrolysis in rabbits (Knoefel, 1934).

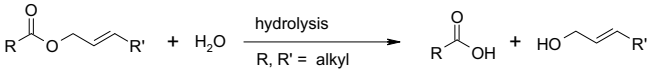
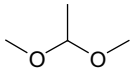
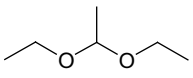


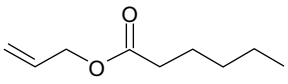
Fig. 2. Ester hydrolysis.

Allyl (i.e., 2-propenyl) esters hydrolyze readily under both *in vivo* and *in vitro* conditions to produce allyl alcohol and their component carboxylic acids (Silver and Murphy, 1978; Butterworth et al., 1975; Grundschober, 1977; Longland et al., 1977). 2-Propenyl hexanoate[4] was rapidly hydrolyzed in artificial pancreatic juice ($t_{1/2}$ = 1.98 min), rat liver ($t_{1/2}$ = 3.96 s), and rat small intestinal mucosa ($t_{1/2}$ = 0.096 s), but it was hydrolyzed more slowly in artificial gastric juice ($t_{1/2}$ = 1,120 min) (Longland et al., 1977). Allyl tiglate (i.e., 2-propenyl (*E*)-2-methyl-2-butenoate), at a concentration of 400 μl/l, was completely hydrolyzed in pig jejunum in less than 2 h at pH 7.5 (Leegwater and van Straten, 1974; Grundschober, 1977). Results from studies with the allyl esters of acetic, propionic, hexanoic, isobutyric, isovaleric, and 2-ethyl hexanoic acids indicate that the *in vitro* hydrolysis of straight-chain allyl esters is approximately 100 times faster than that of their branched-chain counterparts (Butterworth et al., 1975). The inhibition by known carboxylesterase inhibitors (i.e., triorthotolyl phosphate and *S*,*S*,*S*-tributylphosphotrithioate) of the hydrolysis of allyl esters supports the conclusion that hepatic carboxylesterases catalyze these reactions (Silver and Murphy, 1978).

Studies have provided evidence that the esters formed from aliphatic alcohols and α,β-unsaturated carboxylic acids are hydrolyzed similarly to esters formed from α,β-unsaturated alcohols and saturated carboxylic acids. Acrylate (i.e., 2-propenoate) esters (1 μM/ml) were incubated with rat liver, kidney and lung homogenates (see Table 3) (Miller et al., 1981). While the relative rates of hydrolysis for methyl acrylate[5], ethyl acrylate[6], and butyl acrylate[7] in tissue homogenates were similar, the hydrolysis rate in the liver homogenate was approximately 20 times faster than in the kidney and lung homogenates (Miller et al., 1981). In a related *in vitro*

[2]


[3]


[4]


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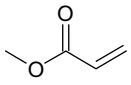

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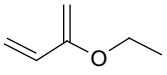

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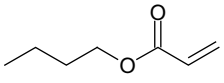


Table 3
Hydrolysis of acrylate esters (nmol/min) (Miller et al., 1981)

	Methyl acrylate[5]	Ethyl acrylate	Butyl acrylate[7]
Liver homogenate	17.2	26.8	23.6
Kidney homogenate	0.6	0.9	–
Lung homogenate	1.2	1.3	–
Whole blood	11.0	12.0	9.4

experiment with rat blood, the three esters rapidly disappeared, and the half-life for ethyl acrylate was estimated to be 15 min (Miller et al., 1981). When incubated *in vitro* in the presence of enzyme-saturating levels of rat nasal mucus carboxylesterase, short-chain acrylate esters (i.e., methyl acrylate V_{max} 0.241 mM/min; ethyl acrylate V_{max} 0.568 mM/min) hydrolyze more rapidly than butyl acrylate (V_{max} 0.141 mM/min) (Stott and McKenna, 1985). Rat nasal mucus carboxylesterase is reported to have a specific activity that is equivalent to that of rat liver carboxylesterase, and greater than that of the carboxylesterases found in rat kidney, lung or blood (Stott and McKenna, 1985).

Similar to their short chain analogues, in simulated digestive environments, longer chain unsaturated esters also have been reported to readily hydrolyze. In simulated pancreatic fluid at 37 °C, 100% hydrolysis of *trans*-2-hexenyl propionate (No. 13) to the corresponding alcohol, 2-hexen-1-ol (No. 9), and propionic acid occurred within 2 h of incubation (Bennett, 1998). Similarly, varying concentrations of a related unsaturated ester, *cis*-3-hexenyl propionate,[8] were shown to be hydrolyzed completely to *cis*-3-hexenol within 2 h under the same experimental conditions (Bennett, 1998). In a similar experiment, a series of aliphatic flavoring esters were incubated in artificial pancreatic fluid (Buck and Renwick, 2000). In the presence of pancreatin, the half lives (based upon the loss of parent ester) for geranyl formate, geranyl acetate, geranyl butyrate and neryl acetate were approximately 0.1, 0.2, 0.03, and 0.2 min, respectively (Buck and Renwick, 2000). Collectively, these results confirm that aliphatic unsaturated esters undergo rapid hydrolysis to their corresponding alcohols and acids.

3.2. Absorption, distribution, metabolism and excretion

3.2.1. Oxidation of alcohols to aldehydes

In vitro experiments with human liver microsomes have confirmed that the oxidation of aliphatic unsaturated alcohols is catalyzed by a number of isoforms of NAD^+/NADH-dependent alcohol dehydrogenase (ADH) (Pietruszko et al., 1973). In studies using human and horse ADH, a correlation between the alcohol substrate chain length (C_1–C_6) and enzyme binding affinity was observed, but the determined V_{max} values (maximum reaction rate) for all substances tested were essentially the same, regardless of the chain length or exact structure. This indicates that the rate-limiting step in the catalysis is not alcohol substrate binding or release. In the same study, comparison of K_M values for a number of saturated and unsaturated alcohols indicated that the 2-alkenols exhibited increased enzyme binding affinity compared to their corresponding saturated analogues (Klesov et al., 1977). In studies investigating the catalysis of alcohol oxidation by NAD^+/NADH-dependent ADH in human liver microsomes, 2-hexen-1-ol (No. 9) exhibited the lowest K_M and highest V_{max} when compared to six homologous saturated linear aliphatic alcohols (Pietruszko et al., 1973).

3.2.2. Oxidation of aldehydes to carboxylic acids

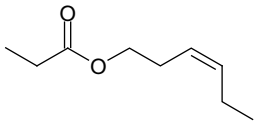
A superfamily of 19 aldehyde dehydrogenases (ALDH) that catalyzes the oxidation of a wide variety of linear aliphatic aldehydes, including α,β-aliphatic unsaturated aldehydes, to carboxylic acids has been identified, and their chromosome locations have been mapped (Lame and Segall, 1986; Feldman and Weiner, 1972). ALDH1A and ALDH2 are isoforms that are constitutively expressed in the cytosol and mitochondria, respectively, with the highest levels found in liver cells. ALDH1A is an effective catalyst in the oxidation of longer chain aldehydes (Vidal et al., 1998) while ALDH2 acts as a primary enzyme in the detoxication of reactive substrates. For instance, ALDH2 has been shown to rapidly oxidize 4-hydroxy-2-nonenal in hepatocytes (Siems et al., 1997). Another ALDH variant, the mitochondrial NAD^+-dependent ALDH1B1, is found throughout human tissue and has been shown to effectively oxidize short chain aldehydes (Stewart et al., 1995). Similarly, microsomal NAD^+-dependent ALDH3A2 performs a housekeeping function in all human tissues and catalyzes the rapid oxidation of medium and long-chain aliphatic unsaturated aldehydes (Kelson et al., 1997). In addition to the ALDH family, both cytochrome P450 and aldehyde oxidase have been reported to oxidize aliphatic α,β-unsaturated aldheydes (Raner et al., 1997; Wolpert et al., 1973; Beedham, 1988).

Analogous to other α,β-unsaturated aldehydes, the highest volume flavoring agent in this group, *trans*-2-hexenal (No. 21), is readily oxidized *in vitro* to *trans*-2-hexenoic acid (No. 31) in the cytosoiic fraction of mouse liver cells (Lame and Segall, 1986) and by isoenzymes of rat ALDH present in mitochondrial, cytosolic, and microsomal fractions (Mitchell and Petersen, 1987). In general, the members of the ALDH superfamily demonstrate higher catalytic activity *in vitro* for higher molecular weight and more lipophilic aldehydes (Nakayasu et al., 1978). Prior to absorption, 15% of a 100 mg/kg bw dose of *trans*-2-nonenal (No. 57) given to rats was oxidized to *trans*-2-nonenoic acid (No. 67) (Grootveld et al., 1998). A broad assortment of oxidative enzymes found in many locations throughout the body, including a large number of ALDH isoenzymes, cytochrome P450, and aldehyde oxidase, rapidly convert α,β-unsaturated aldehydes to their corresponding α,β-unsaturated carboxylic acids.

Linear α,β-unsaturated aldehydes, carboxylic acids and their corresponding alcohols and esters are rapidly absorbed, distributed, metabolized and excreted in the urine and, to a lesser extent, in the feces. In *in vivo* experiments with *trans*-2-nonenal (No. 57) and *trans*-2-pentenal (No. 6), male Wistar albino rats were administered a bolus dose of 100 mg/kg bw of one of the aldehydes by gavage in unheated olive oil. A control group of rats received only the unheated olive oil. Urine samples were collected prior to and after administration. ^{1}H NMR analysis indicated that both *trans*-2-nonenal and *trans*-2-pentenal entered systemic circulation from the gastrointestinal tract, and were metabolized in the fatty acid pathway or were conjugated with glutathione to yield the C-3 mercapturate conjugate that is excreted mainly in the urine within 24 hours. Trace amounts of *trans*-2-nonenal and *trans*-2-pentenal were detected in the feces (Grootveld et al., 1998).

The most extensively studied α,β-unsaturated ester with regards to absorption, distribution, and excretion is ethyl acrylate (ethyl 2-propenoate), which is readily absorbed, hydrolyzed, further metabolized and excreted. *In vivo* experiments in rats indicate rapid absorption and distribution in all major tissues (Ghanayem et al., 1987). Gavage administration of 100 to 400 mg (2,3-^{14}C)-ethyl acrylate/kg bw to rats resulted in >90% absorption within four hours. Tissue distribution analysis showed that the radioactivity

[8]



levels were highest in the forestomach, glandular stomach, intestine, liver and kidney. A dose-dependent concentration gradient was observed in all tissues, although in the forestomach, lower concentrations of radioactivity were observed in rats administered 400 mg (2,3-^{14}C)-ethyl acrylate/kg bw compared to rats administered 200 mg/kg bw at the same time interval (Ghanayem et al., 1987). In all cases, the majority of administered radiolabeled ethyl acrylate was excreted as CO_2.

Similar results were observed in a metabolic study where male Sprague–Dawley rats (3 rats/dose) were given a single oral dose of 2, 20, or 200 mg (2,3-^{14}C)-ethyl acrylate/kg bw (DeBethizy et al., 1987). After 3 days, distribution of the radioactive label and total radioactivity was determined. The total radioactivity recovered was inversely proportional to dose and ranged from 73% to 108% (see Table 4). The major mode of elimination of the radiolabel was as $^{14}CO_2$, which accounted for roughly 52–61% of the total recovered radioactivity. No dose-dependence was observed on the rate of expiration of radioactively labeled CO_2; within 10 h after dosing 45–60% of the total $^{14}CO_2$ recovered had been collected. Approximately 10–15% of the distributed radioactivity was found in the major tissues (i.e., liver, stomach, gastrocnemius muscle, and epididymal fat) after 72 h. Levels of radioactive metabolites recovered in excretia and tissues were inversely proportional to dose (DeBethizy et al., 1987).

Efficient absorption and rapid metabolic clearance were observed following oral administration to male and female F344 rats of a bolus dose of ethyl acrylate. Sixty minutes after a 200 mg/kg dose was given by gavage, no ethyl acrylate was detected in peripheral blood (limit of detection was 1 μg/ml) (Frederick et al., 1992; NTP, 1986a). Half-lives of ethyl acrylate in male and female F344 rats were reported to be longest in forestomach tissue (74 and 94 min, respectively), 64 and 62 min in glandular stomach tissue, 49 and 68 min in stomach contents, and 14 and 11 min in blood (NTP, 1986a).

These experiments provide support for the conclusion that 2-alkenals, 2-alkenoic acids and related 2-alken-1-ols and esters are rapidly absorbed and metabolized.

3.2.3. Metabolism of aliphatic linear α,β-unsaturated carboxylic acids

3.2.3.1. Oxidation in the fatty acid pathway and tricarboxylic acid cycle. Linear α,β-unsaturated acids, such as *trans*-2-hexenoic acid, participate directly in fatty acid metabolism. In the fatty acid pathway, the α,β-unsaturated carboxylic acid is condensed with coenzyme A (CoA), and the resulting *trans*-Δ^2-enoyl CoA ester product is converted to the 3-ketothioester and undergoes subsequent β-cleavage to produce acetyl-CoA (Nelson and Cox, 2000). The other product of this cascade is a new thioester reduced by 2 carbons relative to the α,β-unsaturated acid starting material. The β-cleavage process continues in this manner until the unsaturated position is reached. For α,β-unsaturated acids, where the site of unsaturation begins at an even-numbered carbon (using IUPAC numbering), fragmentation to acetyl-CoA eventually results in a Δ^2-enoyl CoA that is itself a substrate for further fatty acid oxidation. In cases where the carbon–carbon double bond is *cis*, it is first isomerized to its *trans* analogue *via* the 3-hydroxyacyl-CoA epimerase. The *trans* form then enters the fatty acid oxidation pathway. Eventually, even-numbered carbon acids are completely converted to acetyl-CoA units. Odd-numbered carbon acids are similarly cleaved, but yield acetyl-CoA units and a single propionyl-CoA unit. Both acetyl-CoA and propionyl-CoA can be completely metabolized in the citric acid cycle to yield carbon dioxide and water, or can act as building blocks in anabolic processes (Nelson and Cox, 2000).

The results from experiments using radiolabeled α,β-unsaturated acids indicate that α,β-unsaturated acids metabolize primarily to carbon dioxide and water in the fatty acid pathway and tricarboxylic acid cycle. After single oral doses of (1-^{14}C) (*E*,*E*)-2,4-hexadienoic acid (either 40 or 3000 mg/kg bw) were given to female mice, 77–85% was eliminated as expired (^{14}C) carbon dioxide within four days, and the majority (88%) of the $^{14}CO_2$ was expired within the first 24 h. Of the original dose, only 4% and 5% was excreted in the urine as (*E*,*E*)-muconic acid[9] (i.e., (*E*,*E*)-2,4-hexadienedioic acid) and unchanged (*E*,*E*)-2,4-hexadienoic acid, respectively, accounting for 0.4% and 0.7% of the total radioactivity administered present in the urine collected over the first 24 h. Of the 40 mg/kg bw dose, only 1% of the administered radioactivity was recovered in the feces (Westoo, 1964). In related experiments, rats administered between 61 and 1213 mg of (1-^{14}C) (*E*,*E*)-2,4-hexadienoic acid/kg bw eliminated >85% as exhaled $^{14}CO_2$ within 10 h, independent of dose, and only approximately 2% of the radioactivity was detected in the urine. No (*E*,*E*)-muconic acid and unchanged (*E*,*E*)-2,4-hexadienoic acid were detected (Fingerhut et al., 1962).

Esters that are formed from α,β-unsaturated carboxylic acids have been demonstrated to undergo similar metabolism. A single oral dose of 200 mg (2,3-^{14}C)-ethyl acrylate (ethyl 2-propenoate)/kg bw was administered to male F344 rats by gavage. After 4 and 24 h, approximately 27% and 70% was expired as $^{14}CO_2$, respectively. After 24 h, a small amount of unchanged ethyl acrylate (1%) was also observed in the expired air. Approximately 10% and 4% of the dose was excreted in the urine and feces within 24 h, respectively, and roughly 4% of the ^{14}C-label derived from ethyl acrylate was excreted in the bile within 6 h after dosing (Ghanayem et al., 1987). These experimental results are consistent with the study discussed above, where the majority of a single oral dose of 2, 20, or 200 mg (2,3-^{14}C)-ethyl acrylate/kg bw given to rats was exhaled as $^{14}CO_2$, with 45–60% of the total $^{14}CO_2$ being recovered within the first 10 h (DeBethizy et al., 1987). Based on these data, it can be concluded that 2,3-alkenols and α,β-unsaturated aldehydes predominantly metabolize via oxidation to yield the corresponding α,β-unsaturated carboxylic acid, and this is then followed by complete metabolism in the fatty acid pathway and the tricarboxylic acid cycle.

3.2.4. Glutathione conjugation of α,β-unsaturated aldehydes

Reaction with glutathione (GSH) is a common mode of detoxication for many metabolites. Two major routes have been identified. Highly reactive metabolites spontaneously react to form glutathione conjugates. Alternatively, the formation of glutathione conjugates can be catalyzed by the glutathione *S*-transferases (GSTs) (Boyland and Chasseaud, 1967; Coles and Ketterer, 1990; Hinchman and Ballatori, 1994; Jakoby, 1978; Mannervik, 1985). The members of the GST superfamily have broad and overlapping substrate specificities, which allows for the detoxication of a wide diversity of chemical substances. From a mechanistic perspective,

Table 4
Distribution and elimination of radioactivity in male rats 72 h following a single oral dose of (2,3-^{14}C)-ethyl acrylate[a] (DeBethizy et al., 1987)

Dose (mg/kg)	CO_2	Urine	Major tissues	Feces	Total recovered
2	61.1	28.4	13.0	5.9	108.4
20	56.8	13.5	14.9	3.7	88.8
200	52.3	8.4	10.4	1.8	72.8

[a] All values reported as percentage of the amount of radiolabel administered.

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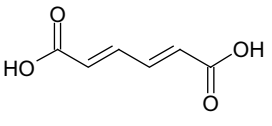

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the formation of most GSH conjugates proceeds *via* nucleophilic attack by GSH on an electrophilic carbon position within a substrate (e.g., at the 3-position on α,β-unsaturated aldehydes). In addition to possessing electrophilic centers, practically all of the substrates that are utilized by GSTs have a common degree of hydrophobicity. The important function of maintaining of adequate levels of GSH in animal cells is carried out by the GSH redox cycle, and adequate supplies of GSH are critical in the detoxication of high *in vivo* concentrations of α,β-unsaturated aldehydes (Nelson and Cox, 2000; Schulz et al., 2000; Reed et al., 1986). The majority of GSH conjugates with α,β-unsaturated aldehydes are formed by the GST-catalyzed addition of GSH across the electrophilic carbon–carbon double bond, but they can also be formed at a lower rate in a non-enzymatic reaction (Eisenbrand et al., 1995; Grootveld et al., 1998).

The formation of GSH conjugates of 2-alkenals is greater in cultured rat hepatocytes that are rich in GSH and GST than human lymphoblastoid cells (Namalva cells) (Eisenbrand et al., 1995). Cells such as human lymphoblastoids with low levels of GSH, GST, and other deactivating enzymes are more susceptible to the cytotoxic effects of 2-alkenals like *trans*-2-hexenal. In both the cultured rat hepatocytes and human lymphoblastoids, the depletion of intracellular GSH was highly correlative with the 2-alkenal consumption (Eisenbrand et al., 1995). Similarly, the level of liver GSH decreased by 75% when 36 μmol/kg bw of the *trans*,*trans*-muconaldehyde[10] was administered by intraperitoneal injection to male rats (Witz, 1989). The conclusions that GSH conjugation plays an important role in detoxication of α,β-unsaturated aldehydes is also consistent with findings in Ames-type genotoxicity experiments, where the presence of GSH reduces the *in vitro* cytotoxicity of α,β-unsaturated aldehydes in *Salmonella typhimurium* TA104 (Marnett et al., 1985).

The smallest α,β-unsaturated aldehyde acrolein (2-propenal) has been demonstrated to metabolize through formation of the GSH conjugate (Penttila et al., 1987; Carmella et al., 2007) or through reaction with other free thiols found in the cell (Ohno et al., 1985). Formation of the acrolein-GSH conjugate occurs both spontaneously and in a GST-catalyzed reaction. The resulting species is then reduced to the 3-hydroxypropyl GSH conjugate that can be excreted as the mercapturic acid or cysteinyl derivatives. Metabolic precursors of acrolein produce the same urinary metabolites. For example, 3-hydroxypropylmercapturic acid (6–11%) was found in the urine of male albino CFE strain of rats given subcutaneous injections of acrolein (606 mg) or metabolic relatives allyl alcohol (613 mg) allyl formate (758 mg), allyl propionate (1500 mg), or allyl benzoate (dose not given) (Kaye, 1973). Similarly, when allyl propionate was given to rats by intraperitoneal injection or gavage, 3-hydroxymercapturic acid was the primary urinary metabolite. Analogous mercapturic acid conjugates have been observed in studies with 2-buten-1-ol, 2-butenal (Gray and Barnsley, 1971), and with the higher homologues discussed below.

When male Wistar albino rats were gavaged with a single 100 mg/kg bw doses of *trans*-2-pentenal or *trans*-2-nonenal, the major metabolites identified in the urine were 3-S-(*N*-acetylcysteinyl)pentan-1-ol and 3-S-(*N*-acetylcysteinyl)nonan-1-ol, respectively, which are the mercapturic acid conjugates of the corresponding alcohols (Fig. 3). Feces of the animals that were

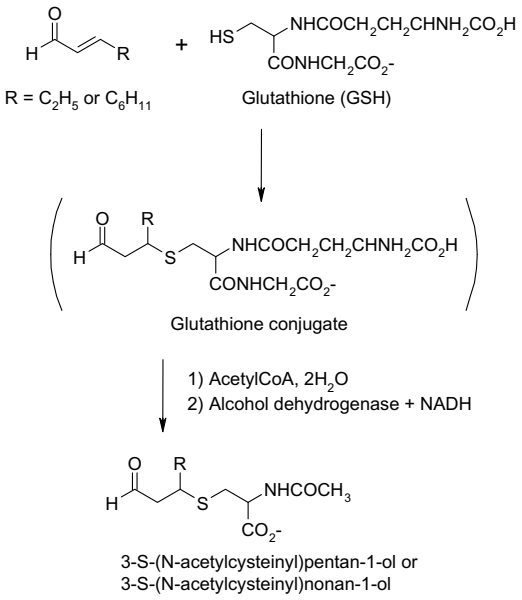

Fig. 3. Glutathione conjugation of α,β-unsaturated aldehydes.

administered 2-nonenal contained trace amounts of the unreacted aldehyde, while the stomach contents that were analyzed 16 h post-dosing contained approximately 15% of the administered dose as *trans*-2-nonenoic acid. In the urine, low levels of glucuronic acid conjugates were detected, which were hypothesized to be produced from a multi-step pathway that includes thiol conjugation followed by oxidation or reduction of the aldehyde functional group and subsequent glucuronic acid conjugation of the resulting carboxylic acid or alcohol, respectively (Grootveld et al., 1998).

Under conditions in which β-oxidation of α,β-unsaturated aldehydes is inhibited from occurring, levels of GSH conjugation are increased. When rats were given a single 100 mg/kg bw intraperitoneal dose of (*E*)-2-propyl-2,4-pentadienoic acid,[11] a sterically hindered α,β-unsaturated acid that is a poor substrate for β-oxidation, the primary excretion product isolated from the urine was the mercapturic acid conjugate (Kassahun et al., 1991). Alternatively, under conditions of oxidative stress (see section below), α,β-unsaturated aldehydes undergo lipid peroxidation prior to reaction with GSH. Within 24 h of receiving a 15 mg/kg bw dose of 5-(H^3)-4-hydroxy-2-hexenal by injection into the hepatic vein, Sprague–Dawley rats eliminated most (>79%) of the radioactivity in the urine as a mercapturic acid metabolite (see Fig. 4). The major excretion product resulted from Michael addition of GSH to the β-position of 4-hydroxy-2-hexenal followed by hemiacetal formation (Winter et al., 1987).

3.2.5. Endogenous formation of α,β-unsaturated aldehydes

3.2.5.1. Glutathione conjugation, oxidative stress, lipid peroxidation, and apoptosis. Oxidative stress occurs when cellular components (i.e., proteins, polypeptides, RNA and DNA bases, and particularly polyunsaturated fatty acid (PUFA) chains of phospholipids in cell membranes) react with free radicals (superoxide, $O_2^{\cdot-}$ and hydro-

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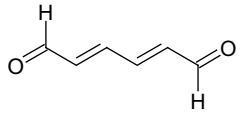

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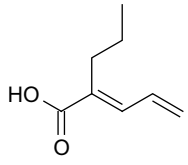

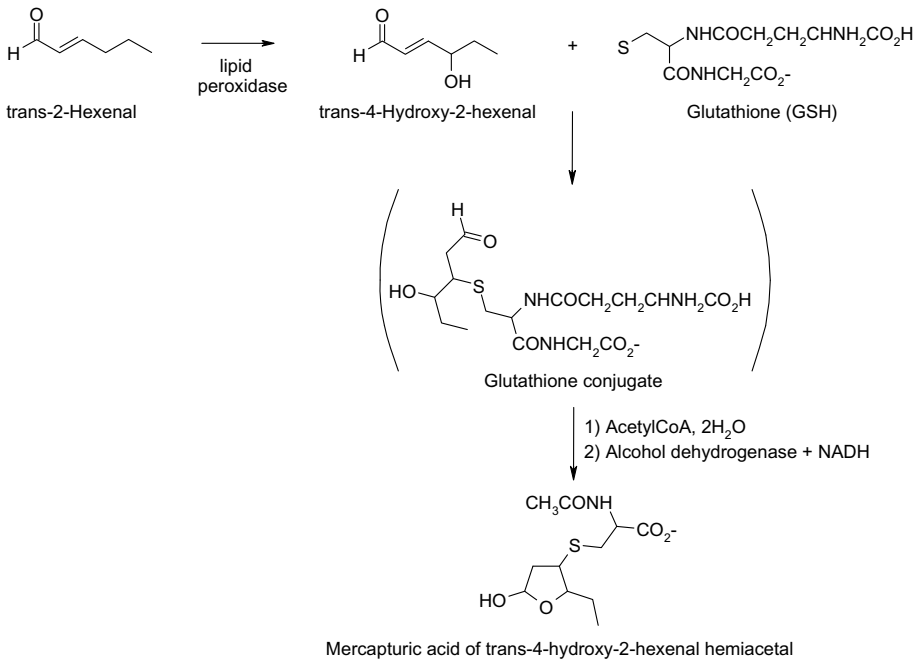

Fig. 4. Glutathione conjugation of α,β-unsaturated aldehydes under conditions of oxidative stress.

xyl, OH·). Two contributing factors leading to oxidative stress are the depletion of cellular GSH and increased lipid peroxidation, which can be directly linked to the formation and dynamic concentration of α,β-unsaturated aldehydes within the cell. Free glutathione at homeostasis concentration, performs a critical antioxidant role by rapidly reacting with unstable free radicals at its free thiol site, in mammalian cells are normally high (1–10 mM) (Armstrong, 1987, 1991). When GSH depletion occurs, oxidative stress results and the unscavenged free radicals react with PUFAs and a variety of aldehyde fragmentation products are produced.

The mechanism of PUFA fragmentation occurs via abstraction of the diallylic hydrogen atom (e.g., the C_{11} hydrogen of 9,12-octadecadienoic acid, linoleic acid). The resulting enolic radical species further reacts to produce an unstable hydroperoxide intermediate that rapidly dissociates to yield an alkoxy radical that then undergoes either β-scission or hydrogen abstraction. β-Scission produces a variety of shortened, conjugated, α,β-unsaturated aldehydes (e.g., 2-butenal, *trans*-2-hexenal, 4-hydroxy-2-nonenal, and 2,4-decadienal). The α,β-unsaturated aldehydes that are formed under oxidative stress conditions are inducers of programmed cell death (Esterbauer et al., 1991; Eckl et al., 1993; Dianzani, 1998). Available data suggests that the formation of α,β-unsaturated aldehydes during lipid peroxidation may be also involved in the pathophysiological effects associated with oxidative stress (Ichihashi et al., 2001). The conversion of PUFAs to shorter chain, reactive unsaturated aldehyde products compromises the structural integrity of the lipid bilayer, leading to membrane leakage, Na^+ influx, K^+ efflux, and cell swelling due to the influx of water.

The dynamic equilibrium between cellular pro-oxidant and antioxidant systems ensures a sufficient supply of GSH for detoxication of α,β-unsaturated aldehydes (Nelson and Cox, 2000). If increases in the concentrations of reactive oxygen species (e.g., superoxide anion $O_2^{\cdot -}$; hydrogen peroxide, H_2O_2; hydroxyl radical, OH·) are not counteracted by concomitant increases in the levels of antioxidant species, oxidative stress can occur, and the intracellular levels of reactive oxygen species rises above a toxic threshold (Schulz et al., 2000). Under conditions of oxidative stress, the ratio of GSH to glutathione disulfide (GSSG) is abnormally altered as GSH is consumed and GSSG is accumulated. Low concentrations of cellular GSH reduce the efficiency of GSH-dependent detoxication pathways and increase the possibility that α,β-unsaturated aldehydes will react with cellular components (i.e., proteins and DNA), ultimately resulting in toxicity and apoptosis (Eder et al., 1993; Ichihashi et al., 2001).

3.2.6. Protein and DNA adduct formation

3.2.6.1. Protein adducts. The ability of endogenous and exogenous sources of α,β-unsaturated aldehydes to form adducts with protein has been studied (Ichihashi et al., 2001). After 24 h incubation with bovine serum albumin (BSA) (1 mg/ml), low concentrations of 2-butenal (crotonaldehyde) (1 or 2.5 mM) produced protein adducts with carbonyl moieties in amounts commensurate with the sum of the histidine and lysine residues on BSA that were identified as being lost by amino acid analysis. At higher concentrations (5, 10, or 20 mM), non-carbonyl and carbonyl adducts were detected. In adducts in which free carbonyls were present, the imidazole nitrogen on histidine and free amine nitrogen on lysine residues present in BSA formed a covalent bond in a Michael-type addition to the *beta* position of 2-butenal.

In the same experiments, a monoclonal antibody with affinity for crotonaldehyde-derived protein adducts was developed to study the endogenous formation of α,β-unsaturated aldehydes and their subsequent reaction with protein (Ichihashi et al., 2001). The elicited antibody was immunoreactive for lysine adducts of 2-butenal, 2-pentenal, and 2-hexenal. In carcinogenicity model studies focusing on the rat kidney, rats were administered Fe^{3+}-nitrilotriacetate by intraperitoneal injection. This material induces acute oxidative damage to renal proximal tubule tissues. The production of immunoreactive material was determined by sacrificing animals at 0, 4, 8, 24, 48, and 72 h, and preparing the excised kidneys for immunohistochemical study. Low concentrations of the aldehyde adduct antibody were detected at times up to and including 24 h. At 48 h, intense immunoreactivities were observed in both the cytoplasm and nuclei. The pattern of distribution and the delay in antibody detection were noted by the authors as being consistent with cytosolic protein adduct formation in the rat

kidney as a result of the formation of membrane lipid peroxidation products (aldehydes) (Ichihashi et al., 2001).

The formation of α,β-unsaturated aldehyde-protein adducts of low density lipoprotein (LDL) resulting from lipid peroxidation has been studied in two experiments. Lipid peroxidation was induced by incubation of PUFAs with an iron/ascorbate free-radical generating system, which were subsequently incubated with BSA. Alternatively, LDL was incubated with 5 μM Cu^{2+}. Incubation of the antibody specific for the crotonaldehyde-lysine protein adduct with either the Cu^{2+}-oxidized LDL product or with the mixture of iron/ascorbate, linolenic acid, BSA indicated that 2-alkenal-lysine modified protein adducts had been formed (Ichihashi et al., 2001). In conclusion, these data provide strong evidence that 2-alkenals that are produced endogenously as a result of lipid peroxidation form adducts with proteins in either Schiff base or Michael addition-type reactions.

3.2.6.2. DNA adducts. Results from*in vitro* studies have indicated that α,β-unsaturated aldehyde-DNA adducts form under conditions of oxidative stress. When a number of α,β-unsaturated aldehydes were incubated with cultured rat hepatocytes and human lymphoblastoid cells, α,β-unsaturated aldehyde-DNA adducts and single strand breaks were reported only after GSH levels had been reduced to roughly 20% of pre-treatment levels. In this experiment, GSH levels in Namalva cells and rat hepatocytes were determined prior to incubation with *trans*-2-hexenal as being approximately 1.6 and 80 nmol/2 × 10^6 cells, respectively. After a one-hour incubation with the aldehyde, the intracellular GSH had reacted with the 2-alkenal to the extent that GSH levels were reduced to approximately 20% of the control values. At this point, DNA single strand breakage and 2-alkenal-DNA adducts were reported The authors concluded that metabolically proficient cells that contain high levels of GSH and GST, such as hepatocyte cells, efficiently protect against the effects of elevated levels of *trans*-2-hexenal and other α,β-unsaturated aldehydes. However, the authors also pointed out that sufficient concentrations of 2-alkenals may exert adverse effects on tissues with which they are in direct contact (e.g., buccal mucosa) (Eisenbrand et al., 1995).

Fluorescence spectroscopy has revealed that 2-alkenals can form DNA adducts both *in vitro* and *in vivo* (Frankel et al., 1987; Eder et al., 1993; Cadet et al., 1999; NTP, 2003). Studies have shown that *trans*-2-hexenal, a common α,β-unsaturated aldehyde product of lipid peroxidation, can react with calf thymus DNA, Namalva cells, and rat colon mucosal cells, to produce low levels of exocyclic 1,N^2-propanodeoxyguanosine at concentrations of 0.2, 0.2, and 0.4 mM, respectively (Golzer et al., 1996). In calf-thymus DNA, crotonaldehyde (0.2 mM) formed adducts at a roughly 10-fold higher rate.

The role of GSH depletion in oxidative DNA breakage has been investigated in V79 cells treated with *trans*-2-hexenal (100 μM), cinnamaldehyde (300 μM) 2,4-hexadienal (300 μM), and 2-cyclohexenone (300 μM). After 1 h incubation, reaction with the four α,β-unsaturated aldehydes resulted in GSH concentrations that were less than 20% of controls, and in all cases DNA damage was reported. At 3 h after initial treatment, however, the levels of DNA damage decreased with a corresponding increase in GST concentrations. Formamidopyrimidine DNA glycosylase (FPG) sensitive sites were detected with *trans*-2-hexenal and cinnamaldehyde, but not with 2,4-hexadienal or 2-cyclohexenone. The authors concluded that cytotoxic and genotoxic cell damage caused by oxidative stress occurs in a dose-dependent manner due to 2-alkenal-related GSH depletion (Janzowski et al., 2003).

The ability of 2-hexenal to form *cis*- and *trans*-isomers of 1,N^2-propanodeoxyguanosine adducts *in vivo* was evaluated in various organs of male Fischer 344 rats (4/group) sacrificed at different intervals (8, 24, 48, and 96 h). A single dose of 500, 200, or 50 mg 2-hexenal/kg bw was given *via* gavage (Schuler and Eder, 1999). Using ^{32}P-post-labeling (detection limit of 0.03 adducts/10^6 nucleotides), no DNA adducts were detected in the organs of untreated or treated rats that were sacrificed 8 h after treatment. The highest levels of DNA adducts were detected 48 h after treatment, and occurred in the forestomach, liver, and esophagus. Levels of adducts 48 h after treatment with 500 mg/kg bw (3.1 adducts/10^6 in the forestomach, 1.7 adducts/10^6 in the liver, and 1.1 adducts/10^6 in the esophagus) were disproportionately greater than those at 200 mg/kg (forestomach, 0.42 adducts/10^6; liver, 0.15 adducts/10^6; esophagus, 0.1 adducts/10^6). The authors concluded that at higher dose levels, 2-hexenal depletes intracellular GSH, and DNA binding occurs with the fraction of the aldehyde that has not been consumed. Adduct levels in animals sacrificed at 4 days were significantly less than those sacrificed at 2 days. Very low levels of DNA adducts were identified in all organs of rats administered 50 mg/kg bw 2-hexenal, and were quantifiable only in the esophagus (0.08 adducts/10^6). The endogenous formation and subsequent reaction of 2-hexenal under conditions of GSH depletion and oxidative stress were not determined or corrected for in this experiment.

In an experiment with 2-butenal that used the same ^{32}P-post-labeling technique and also analyzed for the formation of 2-alkenal DNA adducts in the liver, single oral doses of 0, 200, or 300 mg/kg bw were given by gavage to male Fischer rats (Schuler et al., 1999; Schuler and Eder, 1999). The limit of detection was given as 3 adducts/10^8 nucleotides with a standard deviation of 40%, but the authors reported DNA adducts at two time points for the 200 mg/kg bw dose (1.7 adducts/10^8 nucleotides after 12 h, 2.9 adducts/10^8 after 20 h) and at one time point for the 300 mg/kg bw dose (3.4 adducts/10^8 nucleotides). Repeat dose experiments with rats administered by gavage 1 or 10 mg/kg bw of 2-butenal five times weekly for 6 weeks resulted in detection of DNA adducts (2.0 adducts/10^8 or 6.2 adducts/10^8, respectively). Male rats administered 2-butenal five times weekly for 4 weeks were reported to have DNA adduct levels that were 69% of the peak level measured 24 h after the last dose, and after 2 weeks adduct levels were reduced to 18% of the peak adduct level.

More recently, a sensitive and specific LC/MS/MS method to assay for the *in vivo* formation of DNA adducts of *trans*-2-hexenal in rats has been developed that utilizes isotopically-labeled *trans*-2-hexenal-deoxyguanosine ([$^{13}C_4$,$^{15}N_2$]hex-PdG) as an internal standard (Stout et al., 2006, 2008). In these experiments, groups of male and female F344N rats (5/group) were administered single doses of 0, 200, or 500 mg/kg bw of *trans*-2-hexenal by gavage using the administration regimen in the previously published protocol (Schuler and Eder, 1999), or repeat doses of 0, 10, 30, or 100 mg/kg bw once daily for 5 days or 5 days per week for 4 weeks. The analytical methods used in these experiments allowed for more sensitive quantitation of DNA adducts (~1 adduct/10^8 nucleotides).

In addition to the standard battery of evaluations (i.e., body weight, hematology, clinical chemistry, necropsy and histopathological evaluations), forestomach, glandular stomach, and liver tissues samples were subjected to cell proliferation (PCNA) and DNA adduct analysis. Ulcerative lesions and inflammation of the forestomach were apparent in animals given single doses of 200 or 500 mg/kg bw, and these were accompanied by proliferative hyperplasia in the forestomachs of rats given repeat doses of 100 mg/kg bw. In these animals, clinical chemistry observations supported histopathologic findings. The incidences of epithelial hyperplasia, degeneration and active inflammation of the forestomach in rats given 100 mg *trans*-2-hexenal/kg bw/day for 4 weeks in this study were consistent with those for rats administered 120 mg/kg bw/day of 2,4-hexadienal for 90 days (NTP, 2003).

The histopathologic findings of forestomach damage and hyperplasia correlated with DNA adducts detected using LC/MS/MS. One day after a single dose of 200 mg/kg bw, or one day after repeat dose exposure of 100 mg/kg bw for 1 or 4 weeks, a diasteromeric mixture of *trans*-2-hexenal-deoxyguanosine (dG) adducts was quantifiable only in the forestomach. In all cases, the level of adducts were at or near the limits of quantitation ($1/10^8$), and were not detected in all animals at these dose levels. DNA adducts detected in the forestomach in these experiments were 1–2 orders of magnitude lower than those previously reported (Schuler and Eder, 1999). Adducts were not measured in animals administered 500 mg/kg bw because inflammation resulting in complete or near-complete loss of the forestomach mucosa had occurred. Consistently, DNA adducts were detected only for doses that also resulted in significant damage to the forestomach mucosa or that induced epithelial hyperplasia. No 2-alkenal-DNA adducts were detected in the liver or glandular stomach in any treated rats. Additionally, no DNA adducts were found in the forestomach of rats that received repeated daily doses less than 100 mg/kg bw/day for 28 days. This contrasts with earlier findings, in which liver DNA adducts had been reported in the earlier study (Schuler and Eder, 1999). The authors of the more recent study noted that they used *trans*-2-hexenal of 98% purity while Schuler and Eder (1999) used material of >99% purity, and that it was unclear whether fasted rats were used in the previously published experiments.

The formation of DNA adducts from lipid peroxidation products resulting from exposure to 2,4-hexadienal has also been investigated (Chung et al., 1999). 2,4-Hexadienal was administered by gavage to rats and mice daily 5 days per week for 14 weeks (NTP, 2003). ^{32}P-DNA post-labeling, followed by high performance liquid chromatography, was used to determined the levels of propenal or crotonaldehyde cyclic dG adducts in the liver and forestomach tissue of male rats administered 0 or 90 mg/kg bw of 2,4-hexadienal or mice (forestomach only) administered 0 or 120 mg/kg bw/day. In rats, significant increases in dG adducts (2-butenal) were found in forestomach tissue but no increases in dG adducts were detected in liver tissue. In mice, no significant increase in dG adducts were reported; rather, dG adducts were greater for control mice compared to treated mice. The authors concluded that treatment with 2,4-hexadienal may increase cyclic adduct formation in the rat forestomach DNA.

The relationship between induction of GSH depletion by α,β-unsaturated aldehydes, cytotoxicity, and DNA damage has been investigated. A homologous series (2-hexenal to 2-nonenal) of 2-alkenals. were incubated with V79 Chinese hamster fibroblast cells or Caco-2 human colon adenocarcinoma cells for 1 h. Levels of cytotoxicity were reported as being similar in both cell lines. The longest chain aldehyde, 2-nonenal, was reported as the most cytotoxic. The strong overlap between cytotoxic concentrations and levels leading to DNA damage suggested that cytotoxicity is directly correlated with DNA damage. As with previous *in vitro* assays, after 1 h GSH levels were approximately 20% of controls. The concentrations of α,β-unsaturated aldehydes required for DNA damage were 10-fold higher than those required for GSH depletion, implying that initial cellular GSH depletion is a prerequisite for DNA damage (Glaab et al., 2001).

^{32}P-Post-labeling assays have shown that *trans*-2-hexenal forms cyclic 1,N^2-propanodeoxyguanosine adducts in primary colon mucosa cells from rats and humans at *in vitro* concentrations as low as 0.4 mM. This concentration is higher than the highest identified concentration of *trans*-2-hexenal in flavored foods (14 ppm, 0.14 mM), and it is naturally found in some fruits and vegetables at levels up to 30 ppm (0.3 mM) (Golzer et al., 1996).

In addition to direct reaction with DNA, apoptosis can be induced by α,β-unsaturated aldehydes, leading to DNA fragmentation. In a study with 4-hydroxy-2-nonenal, GSH depletion was dose-and time-dependent and resulted in induction of poly-ADP-ribose polymerase (PARP) cleavage and DNA fragmentation (Ji et al., 2001). Reduced levels of intracellular GSH induces the release of mitochondrial cytochrome c to the cytosol, resulting in activation of a cascade of cytosolic cysteine proteases (i.e., caspases). Caspase-3 activation protein cleavage and PARP, which leads to DNA fragmentation and subsequent cell death (Liu et al., 1996; Li et al., 1997; Zou et al., 1997; Green and Reed, 1998; Cain et al., 1999).

In summary, the available data indicate that high concentrations of α,β-unsaturated aldehydes may deplete intracellular GSH, resulting in oxidative stress and the resultant formation of protein and DNA adducts. Under oxidative stress conditions, 2-alkenals may also be endogenously formed from the lipid peroxidation of membrane PUFAs. However, given the extremely low dietary exposure of humans to α,β-unsaturated aldehydes and the ready metabolism of α,β-unsaturated aldehydes via aldehyde oxidation and glutathione conjugation, there is no significant potential for oxidative stress or DNA adduct formation under conditions of use as flavoring substances.

4. Toxicological studies

Typically, the toxicological studies are organized according to duration (i.e., short-term, long-term, and carcinogenicity), flavor ingredient and then species. However, in the interest of preserving the integrity of the studies performed by the National Toxicology Program (NTP), short-term toxicity and carcinogenicity studies will be discussed in the long-term study section (see Section 4.3) in the sequence in which they were conducted.

4.1. Acute toxicity

Experiments for 18 of the 37 2-alkenal and structurally related alcohols, acids, and esters in this group have been conducted to establish oral LD_{50} values. Rat oral LD_{50} values that range from 767 to >8000 mg/kg bw have been reported for 16 of these substances (Nos. 1, 13, 15, 18, 19, 21, 22, 40, 54, 56, 57, 65, 66, 70, 86, and 89) (Bar and Griepentrog, 1967; Smyth et al., 1970; Gaunt et al., 1971; Moreno, 1972, 1973a,b,c, 1976a, 1977a,b,c, 1978a,b,c, 1979, 1980a,d, 1982). For five of the aliphatic, linear, α,β-unsaturated, di- and trienals and related alcohols, acids, and esters used as flavoring agents (Nos. 30, 35, 42, 62, and 81), oral LD_{50} values in rats are in the range from 300 to 12,500 mg/kg bw (Deuel et al., 1954; Smyth et al., 1954; de Groot et al., 1974; Moreno, 1976b, 1980b,c; Uchida et al., 1985; Driscoll, 1996). Three separate oral LD_{50} values have been reported for *trans,trans*-2,4-hexadienal (No. 30) (300, 730, and <5000 mg/kg bw) (Smyth et al., 1954; de Groot et al., 1974; Moreno, 1980b).

Oral LD_{50} values in mice for four substances (Nos. 21, 45, 46, and 89) range from 1550 to greater than 8000 mg/kg bw (Gaunt et al., 1971; Pellmont, 1974a,b; Moreno, 1980e). Collectively, these data demonstrate that the oral acute toxicity of α,β-unsaturated aldehydes and related alcohols, acids and esters is low. Similarly, mouse oral LD_{50} values for six aliphatic, linear, α,β-unsaturated, di- and trienals and related alcohols, acids and esters (Nos. 36, 37, 60, 80, 88, and 90) range from 1000 to greater than 8,000 mg/kg bw (Sparfel et al., 1968; Edwards, 1973; Pellmont, 1971, 1977; Moreno, 1978d), also indicating that these substances have low acute oral toxicity (see Table 5).

4.2. Short-term studies (see Table 5)

4.2.1. 2-Hexenal (No. 21)

4.2.1.1. Rats. CFW rats (15/sex/group) were given a diet containing 2-hexenal (0, 260, 640, 1600, or 4000 ppm, providing an average

Table 5
Acute and short-term toxicity studies of α,β-unsaturated aldehydes and related substances used as flavor ingredients

Flavoring ingredient		Oral acute studies		Short-term studies			
		Oral LD_{50}mg/kg bw (species)	Reference	Species; sex[a]	Time (days)/ route	NOAEL (mg/kg bw)	Reference
1	(*E*)-2-Butenoic acid	1000 (Rat)	Bar and Griepentrog (1967)				
13	*trans*-2-Hexenyl propionate	>5000[b] (Rat)	Moreno (1976a)				
15	*trans*-2-Hexenyl butyrate	>5000 (Rat)	Moreno (1978a)				
18	*trans*-2-Hexenyl isovalerate	>5000 (Rat)	Moreno (1978b)				
19	(*E*)-2-Hexenyl hexanoate	>5000 (Rat)	Moreno (1978c)				
21	2-Hexenal	850 (Rat)	Moreno (1973a)	Rat; M, F	91 days/Diet	80	Gaunt et al. (1971)
21	2-Hexenal	780 (M) 1130 (F) (Rat)	Gaunt et al. (1971)	Rabbit; F	91 days/Gavage	<200	Gaunt et al. (1971)
21	2-Hexenal	1750 (M) 1550 (F) (Mice)	Gaunt et al. (1971)				
22	(*E*)-2-Hexenal diethyl acetal	860 (Rat)	Moreno (1977a)				
30	*trans*,*trans*-2,4-Hexadienal	300 (Rat)	Moreno (1980b)	Rat; M, F	14/Gavage	7.5[c]	de Groot et al. (1974)
30	*trans*,*trans*-2,4-Hexadienal	730 (Rat)	Smyth et al. (1954)	Rat; M, F	90/Diet	2.23	Mecler and Craig (1980)
30	*trans*,*trans*-2,4-Hexadienal	<5000 (Rat)	de Groot et al. (1974)	Mouse; M,F	16/Gavage	27	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal			Mouse; M, F	98/Gavage	30 (M) NE (F)	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal			Mouse; M, F	728/Gavage	60 (M) 30 (F)	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal			Rat; M, F	16/Gavage	27	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal			Rat; M, F	98/Gavage	15 (M) 60 (F)	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal			Rat; M, F	728/Gavage	NE	NTP (2001)
35	(*E*,*E*)-2,4-Hexadienoic acid	9600 (Rat)	Uchida et al. (1985)	Rat; M, F	90/Diet	2480	Deuel et al. (1954)
35	(*E*,*E*)-2,4-Hexadienoic acid	10,500 (Rat)	Deuel et al. (1954)	Dog; M, F	90/Diet	1333[c]	Deuel et al. (1954)
35	(*E*,*E*)-2,4-Hexadienoic acid	12,500 (Rat)	Uchida et al. (1985)	Mouse; M, F	560/Diet	1400	Hendy et al. (1976)
35	(*E*,*E*)-2,4-Hexadienoic acid			Rat; M, F	730/Diet	750	Gaunt et al. (1975)
35	(*E*,*E*)-2,4-Hexadienoic acid potassium salt			Rat; M	420/Drinking water	300[c]	Dickens et al. (1968)
35	(*E*,*E*)-2,4-Hexadienoic acid potassium salt			Rat; M	420/Diet	50[c]	Dickens et al. (1968)
36	Methyl sorbate	5600 (Mice)	Pellmont (1977)				
37	Ethyl sorbate	>8000 (Mice)	Sparfel et al. (1968)				
40	*trans*-2-Heptenal	1300 (Rat)	Moreno (1980d)				
40	*trans*-2-Heptenal	1300 (Rat)	Moreno (1982)				
42	2,4-Heptadienal	1150 (Rat)	Moreno (1980c)				
45	*trans*-2-Octen-1-yl acetate	>8000 (Mice)	Pellmont (1974a)				
46	*trans*-2-Octen-1-yl butanoate	>8000 (Mice)	Pellmont (1974b)				
54	Methyl 2-octynoate	2500 (Rat)	Moreno (1972)				
54	Methyl 2-octynoate	1530 (Rat)	Bar and Griepentrog (1967)				
56	*cis*-2-Nonen-1-ol	>5000 (Rat)	Mondino (1981)				
57	2-Nonenal	5000 (Rat)	Moreno (1977b)				
60	2,6-Nonadien-1-ol	>5000 (Mice)	Moreno (1978d)				
62	Nona-2-*trans*-6-*cis*-dienal	>5000 (Rat)	Moreno (1976b)				
65	Methyl 2-nonynoate	1180 (M) 870 (F) (Rat)	Freeman (1980)				
65	Methyl 2-nonynoate	2220 (Rat)	Moreno (1973b)				
66	Ethyl 2-nonynoate	2850 (Rat)	Moreno (1973c)				
70	2-Decenal	5000 (Rat)	Moreno (1977c)				
73	2-*trans*,4-*trans*-Decadienal			Mouse; M, F	17/Gavage	400	NTP (1997)
73	2-*trans*,4-*trans*-Decadienal			Mouse; M, F	90/Gavage	100 (M) 200 (F)	NTP (1997)
73	2-*trans*,4-*trans*-Decadienal			Rat; M, F	17/Gavage	400	NTP (1997)
73	2-*trans*,4-*trans*-Decadienal			Rat; M, F	90/Gavage	100	NTP (1997)
73	2-*trans*,4-*trans*-Decadienal			Rat; M, F	90/Diet	33.9	Damske et al. (1980)
80	Propyl 2,4-decadienoate	1000 (Mice)	Pellmont (1971)				
81	Ethyl 2,4,7-decatrienoate	>2000 (Rat)	Driscoll (1996)				
86	2-Dodecenal	>5000 (Rat)	Moreno (1980a)				
88	2-*trans*-6-*cis*-Dodecadienal	5000 (Mice)	Edwards (1973)	Rat; M, F	28/Diet	2.06[d]	Edwards (1973)
89	2-Tridecenal	>5000 (Mice)	Moreno (1980e)				
89	2-Tridecenal	>5000 (Rat)	Moreno (1979)				
90	2-*trans*-4-*cis*-7-*cis*-Tridecatrienal	5000 (Mice)	Edwards (1973)	Rat; M, F	28/Diet	33[d]	Edwards (1973)

[a] M = Male; F = Female. If not listed, sex was not specified in the report.
[b] This does not represent a true LD_{50} as this is the only dose evaluated at which a 100% mortality was reported.
[c] Study performed with either a single dose or multiple doses that produced no adverse effect. The value is therefore not a true NOEL, but is the highest dose level tested that produced no adverse effects. The actual NOEL may be higher.
[d] The substance was administered as a component of a mixture.

daily intake of 0. 13, 32, 80, and 200 mg/kg bw, respectively) for 13 weeks (Gaunt et al., 1971; FDA, 1993). For each animal body weights and food intake were measured weekly. Blood samples were collected at week 6 and at the termination of the study. Similarly, urine samples were collected at week 7 and again at the end of the study period. At termination, rats were euthanized and given

gross examinations. Several organs were weighed (brain, pituitary, thyroid, heart, liver, spleen, adrenal glands, kidneys, and gonads) and tissue samples taken from these organs and the lymph nodes, thymus, urinary bladder, stomach, duodenum, ileum, colon, caecum, rectum, pancreas, uterus, and skeletal muscle of control and high-dose rats were stained for microscopic examination.

No differences between control and treated rats were observed in general health and behavior. A small, statistically insignificant decrease in growth rate of the 200 mg/kg bw group animals was reported, but this was due to a 10% reduction in food intake probably due to decreased palatability of the diet. High-dose (200 mg/kg bw) males had significantly reduced urine specific gravity, which was the only effect reported in urinalysis. Statistically significant, but not dose-dependent decreases were reported in male rats in levels of hemoglobin at 80 mg/kg bw and in the levels of erythrocytes at 32 and 80 mg/kg bw. However, no corresponding increase in kidney weights, or changes in histology indicative of damage were reported. Treated female rats had no reported changes in hematology. In treated females at every dose level, a 20–30% increase in relative and absolute ovary weights that was not dose-dependent was reported relative to control animals, but no accompanying ovarian histologic changes were reported. To confirm these results, additional groups of female rats (10/group) were fed 2-hexenal (0 or 4000 ppm, corresponding to approximately 0 or 200 mg/kg bw) for 13 weeks, using either the 2-hexenal sample previously used or a differently sourced sample. In this follow-up study, no effects that were reported in the first study were observed using either sample of 2-hexenal, and no adverse effects were observed during histopathological examination of the ovaries, uterus, pituitary, and adrenal glands. The increased ovary weights that were reported in the first study were thus considered not to be treatment related. The no-observed-effect level (NOEL) for 2-hexenal in rats based on these findings is approximately 80 mg/kg bw (Gaunt et al., 1971).

More recently, a 28-day gavage study that was designed to evaluate toxicity, DNA binding, and cell proliferation following exposure to *trans*-2-hexenal was conducted in rats (Stout et al., 2008). Rats were administered single doses of *trans*-2-hexenal (0, 200, or 500 mg/kg bw), were given repeat doses of 0, 10, 30, or 100 mg/kg bw for 5 days, or were given the same repeat doses 5 days a week for 4 weeks. In the 100 mg/kg bw repeat dose group, a significant decrease in body weights was observed at the end of the 4th week of exposure. After either 1 or 4 weeks of exposure, no hematological or biochemical changes were reported at any of the repeat dose levels. Two animals in the 4 weeks, 30 mg/kg bw dose group exhibited stomach edema, which was also seen in the majority of rats given 100 mg/kg *trans*-2-hexenal for either 1 week or 4 weeks. Animals given 100 mg/kg bw repeat doses were reported to have gross lesions that were less severe than those that resulted from a single dose of 200 mg/kg bw. These lesions were mostly edematous in nature, although a few cases of ulceration were also reported. Histopathological examination of the liver, forestomach, and glandular stomach of rats showed lesions of the stomach in both the 1 and 4 week repeat-dose groups. No significant histopathological changes to animals dosed with 10 mg/kg were found after 1 week. Minimal, focal or multifocal hyperplasia was reported in three of the five animals exposed to 30 mg/kg bw for 1 week. In animals given the 100 mg/kg bw dose for 1 week, moderate to severe diffuse mucosal hyperplasia (epithelialization) was observed, with dysplasia in all rats and hyperkeratosis in three rats. Additionally, chronic-active ulcers with submucosal edema, fibroblast proliferation and infiltrates of neutrophils, macrophages and lymphocytes were reported in two rats from this group. Exposure to *trans*-2-hexenal at 10 mg/kg bw for 4 weeks resulted in minimal, multifocal mucosal hyperplasia. Administration of 30 mg/kg of *trans*-2-hexenal for 4 weeks resulted in mild-to-moderate, multifocal-to-diffuse mucosal hyperplasia. Exposure to oral doses of 100 mg/kg for 4 weeks produced either moderate-to-severe, diffuse mucosal hyperplasia in 4 rats, and in 1 rat, severe diffuse hyperplasia (epithelialization) with dysplasia was observed. In these animals, damage to the gastric ridge in exposed rats was not distinct from damage to the forestomach.

4.2.1.2. Rabbits. A group of 10 female white New Zealand rabbits was administered by gavage 200 mg/kg bw of *trans*-2-hexenal daily for 13 weeks (Gaunt et al., 1971). A control group was administered only the corn oil vehicle daily by gavage for 13 weeks. One animal died early in the study, but this was attributed to gavage error. Body weights were measured weekly throughout the study. At the end of the study, animals were euthanized, gross examinations performed, and blood samples for hematological evaluation were obtained. The brain, pituitary gland, thyroid, heart, liver, spleen, adrenal glands, kidneys, and gonads were weighed. From these organs, as well as the lymph nodes, thymus, urinary bladder, stomach, duodenum, ileum, colon, cecum, rectum, pancreas, uterus, and skeletal muscle, tissue sections were collected and stained.

Compared to controls, treated rabbits gained less weight during the early portion of the study (through week 4), but this was not a statistically significant effect. The stomachs of treated rabbits were significant ($p < 0.001$) increased in weight relative to control animals, but no other differences in organ weights, including the ovaries, were reported. In three of the rabbits administered *trans*-2-hexenal, hemorrhage and small acute stomach ulcers were found upon microscopic examination. The authors concluded that these effects were due to gavage administration that produced a high concentration (600 mg/100 ml) of an irritating aldehyde into the stomach, which resulted in conditions amenable for gastric mucosa ulceration. This conclusion was supported by the lack of ulcerative effects observed in the stomachs of rats given *trans*-2-hexenal in the diet. No other histopathological changes were noted. Hemoglobin concentrations were decreased in treated rats, resulting in mild anemia, but the authors associated these effects with the presence of stomach ulcers.

4.2.2. trans,trans-2,4-Hexadienal (No. 30)

4.2.2.1. Rats. Rats (5/sex/dose group) were administered 0, 0.75, or 7.5 mg *trans*,*trans*-2,4-hexadienal/kg bw/day in corn oil *via* gavage 6 days a week for 14 days. Animal body weights and food and water intake that were recorded after the first and second weeks showed no differences between treated and control animals. Hemoglobin levels determined at day 14, were not significantly different in treated rats relative to the control group. At the conclusion of the study, animals were necropsied, and no treatment-related abnormalities were observed by macroscopic examination. Liver weights of male rats given 0.75 mg/kg bw/day were significantly increased relative to control animals, but this was not found for the 7.5 mg/kg bw/day group. The increased liver weight in the lower dose group was not accompanied by any histopathological findings in the liver or kidneys. Therefore, under the conditions of this study 7.5 mg *trans*,*trans*-2,4-hexadienal/kg bw/day was established by the authors as the NOEL, which was the highest dose tested (de Groot et al., 1974).

Charles River rats (24/sex/dose group) were fed 0 (basal diet control) or 2.23 mg *trans*,*trans*-2,4-hexadienal/kg bw/day, 7 days a week, for 13 weeks. For both control and test group, animals were observed daily for mortality and signs of toxicity. At the 6th and 12th weeks of the study, hematological and blood chemistry evaluations and urinalyses were conducted. At week 6, males and females demonstrated transient decreases ($p < 0.05$) in blood glucose levels and increases ($p < 0.05$) in blood urea nitrogen levels, respectively, compared to the control group, but these effects were not observed at week 12. No significant differences in body

weight or food and water consumption were found between treated and control animals. At the conclusion of the exposure period rats were necropsied. Gross and histopathological examinations revealed no treatment-related lesions, and no differences between treated and control animal organ weights were found. The authors reported a NOEL of 2.23 mg *trans*,*trans*-2,4-hexadienal/kg bw/day (Mecler and Craig, 1980).

4.2.3. (E,E)-2,4-Hexadienoic acid (No. 35)

4.2.3.1. Rats. (*E*,*E*)-2,4-Hexadienoic acid (sorbic acid) was given in the diet at 0%, 0.5%, 1%, 2%, 4%, or 8% to groups of Sherman rats (5/sex/concentration level) for a period of 13 weeks. These dietary concentrations correspond to daily intakes of 0, 320, 630, 1260, 2480, or 5060 mg/kg bw, respectively (Deuel et al., 1954). No animals died during the course of the study, and no differences in weight gain and food consumption were reported in any of the treated animals relative to the controls. Histopathological examination of necropsied animals did not reveal any abnormalities in treated rats. In the rats given 5060 mg/kg bw, a slight increase in relative liver weight was reported compared to the controls, but this was not accompanied by any histopathological liver changes. Animals administered 4% (*E*,*E*)-2,4-hexadienoic acid (2480 mg/kg bw/day) showed no treatment-related effects (Deuel et al., 1954).

4.2.3.2. Dogs. Two male and one female puppy were fed a diet containing 4% (*E*,*E*)-2,4-hexadienoic acid for 13 weeks. No significant differences between treated and control animals in either food consumption or body weight gain were reported. Histopathological examination at the termination of the exposure period did not reveal any treatment-related lesions. The authors concluded that a diet containing 4% (*E*,*E*)-2,4-hexadienoic acid, which corresponds to a daily intake of 1,333 mg/kg bw/day (FDA, 1993), does not produce adverse effects in dogs (Deuel et al., 1954).

4.2.4. trans-2,trans-4-Decadienal (No. 73)

4.2.4.1. Mice. 2,4-Decadienal (0, 45, 133, 400, 1200, or 3600 mg/kg bw) in corn oil was administered daily by gavage to groups of $B6C3F_1$ mice (5/sex/dose), 5 days per week, for a period of 17 days. All animals in the highest dose group, and 1 male and 1 female given 1200 mg/kg bw died prior to termination of the study. Both males and females given 1200 mg/kg bw had significant decreases in body weight gain relative to the control group. All treated animals exhibited clinical effects of diarrhea and lethargy. In the two highest dose groups, males and females had ruffled fur and a thin appearance, and in the highest dose group ataxia and abnormal breathing were reported. At doses of 400 mg/kg bw/day or less, no treatment-related clinical signs of toxicity or effects on organ weights were reported. Gross pathological and microscopic evaluation revealed ulceration in the forestomachs of males and females in exposed to 1200 mg/kg bw. As no treatment-related effects were observed in the 400 mg/kg bw/day groups, it was established as the NOEL in male and female mice (NTP, 1997).

2,4-Decadienal (0, 50, 100, 200, 400, or 800 mg/kg bw/day) in corn oil was administered to groups of $B6C3F_1$ mice (10/sex/dose) by gavage 5 days per week for 13 weeks. The survival of treated animals at any dose was not different than controls. In males in the 800 mg/kg bw/day dose group, a lower rate of body weight gain was reported in comparison to the controls. In animals of both sexes at the 400 and 800 mg/kg bw/day dose levels, increased salivation following dose administration was first observed during week 7 of the study and continued intermittently through week 10 of the study, but not thereafter. Females in the 200, 400, and 800 mg/kg bw/day groups were reported as lethargic at week 12. Hematology and organ weights showed no treatment-related effects at any dose level. Histological evaluation revealed minimal-to-mild epithelial hyperplasia, inflammation, and edema in the forestomach of treated animals in the 200 (males only), 400, and 800 mg/kg bw/day groups. NOEL values of 100 and 200 mg/kg bw/day were established for male and female $B6C3F_1$ mice, respectively (NTP, 1997).

4.2.4.2. Rats. 2,4-Decadienal (0, 45, 133, 400, 1,200, or 3,600 mg/kg bw/day) in corn oil was administered by gavage to groups of F344/N rats (5/sex/dose) 5 days per week for 17 days. All the animals in the highest dose group died before the study was completed, but no early deaths were reported at any other dose level. In comparison to the controls, males and females given 1200 mg/kg bw had a statistically significant decrease in body weight gain. At this same dose level, diarrhea was reported in both sexes. Upon necropsy gross pathological and microscopic evaluation of the forestomach of rats of both sexes given 1200 mg/kg bw/day revealed ulceration. No treatment-related effects were observed in the 400 mg/kg bw/day dose groups and therefore, it was established as the NOEL in F344/N rats (NTP, 1997).

In a 13-week study, groups of F344/N rats (10/sex/dose) were administered 2,4-decadienal (0, 50, 100, 200, 400, or 800 mg/kg bw/day) in corn oil by gavage 5 days per week. There were no treatment-related effects on the survival of the rats at any dose level. In comparison to the controls, rats of both sexes administered 200, 400, and 800 mg/kg bw/day doses showed a lower rate of body weight gain throughout the study. In both sexes in the 200, 400, and 800 mg/kg bw/day dose groups, salivation was reported prior to and following administration of 2,4-decadienal, but in the 50 and 100 mg/kg bw/day groups, this effect was sporadic and only occurred immediately after administration. The severity of the salivation was dose-dependent, and became less prevalent in the lower dose groups in the last weeks of the study. Beginning at week 7 and until the termination of the study, animals in the 200, 400, and 800 mg/kg bw/day dose groups displayed post-administration lethargy. This effect was sporadic in the lower dose groups, and in all animals the severity was reported to be dose-dependent and acute (i.e., recovery within minutes). At necropsy, histological evaluation of male and female rats in the 400 and 800 mg/kg bw/day dose groups revealed minimal or mild forestomach epithelial hyperplasia (NTP, 1997). Due to the sporadic and acute nature of the salivation and lethargy effects in the lower dose groups (i.e., 50 and 100 mg/kg bw/day) and their speedy recovery, the NOEL for the 13-week study in rats was 100 mg 2,4-decadienal/kg bw/day.

2,4-Decadienal was fed as part of the diet to groups of Charles River rats (6/sex/group) for 13 weeks, providing daily exposures of 0, 3.39, 10.70, or 33.90 mg/kg bw. Daily observations were made for mortality and general signs of toxicity. No significant differences between test and control animals were reported after weekly measurements of body weights and food consumption. At week 6, females in the 10.70 and 33.90 mg/kg bw dose groups were reported to have a decrease in hemoglobin and hematocrit values, but at week 12 these were comparable to the controls. Histopathological evaluations of major tissues from all high-dose animals and from half of the controls, and of livers and kidneys of all study animals, showed no treatment-related effects. At the conclusion of the study body and organ weights of animals at any dose level were not significantly different from the controls (Damske et al., 1980).

4.2.5. 2-trans-6-cis-Dodecadienal (No. 88) and 2-trans-4-cis-7-cis-tridecatrienal (No. 90)

4.2.5.1. Rats. A maltodextrin-microencapsulated mixture of 2-*trans*-6-*cis*-dodecadienal and 2-*trans*-4-*cis*-7-*cis*-tridecatrienal (0.2, 0.4, 1.0, 2.0, 4.0, 10.0, or 20.0 ppm and 3.2, 6.4, 16, 32, 64, 160, or 320 ppm, respectively) was added to the diet of rats (6/sex/dose) for 4 weeks. Food consumption measurements

indicated that the highest dose levels provided intakes of 1.93 and 2.06 mg 2-*trans*-6-*cis*-dodecadienal/kg bw/day for males and females, respectively, and 30.9 and 33 mg 2-*trans*-4-*cis*-7-*cis*-tridecatrienal/kg bw/day for males and females, respectively. A control group of 12 rats was fed a diet with maltodextrin only. No significant effects on body weight gain, food consumption and utilization, organ weights, clinical chemistry or macroscopic pathology were reported in either sex at any dose level. The NOEL was reported to be the highest dose tested (1.93 and 2.06 mg 2-*trans*-6-*cis*-dodecadienal/kg bw/day for males and females, respectively, and 30.9 and 33 mg 2-*trans*-4-*cis*-7-*cis*-tridecatrienal/kg bw/day for males and females, respectively) (Edwards, 1973).

4.3. Long-term studies of toxicity and carcinogenicity (see Table 5)

4.3.1. trans,trans-2,4-Hexadienal (No. 30)

4.3.1.1. Mice. In a preliminary dose range-finding study, groups of B6C3F$_1$ mice (5/sex/group) were administered 2,4-hexadienal (0, 3, 9, 27, 80, or 240 mg/kg bw/day) in corn oil by gavage 5 days per week over 16 days for a total of 12 doses (NTP, 2003). In addition to twice daily observations, body weights and clinical findings were recorded at the onset of the study, on day 8, and at the end of the study. In females given 240 mg/kg bw, clinical signs of toxicity including lethargy, ruffled fur and convulsions were accompanied by a 20% mortality rate and significant weight loss. No deaths or clinical signs of toxicity occurred in animals at any other dose level, and no treatment-related differences in organ weights were reported at any dose. Gross pathological evaluation upon necropsy revealed ulceration and/or necrosis of the forestomach in mice of both sexes treated with 240 mg 2,4-hexadienal/kg bw/day. In both sexes at the 80 mg/kg bw/day dose level, mild epithelial hyperplasia and hyperkeratosis were reported. Based on these results, a NOEL of 27 mg 2,4-hexadienal/kg bw/day was established in male and female mice (NTP, 2003).

In a follow-up dose range-finding study, 2,4-hexadienal (0, 7.5, 15, 30, 60, or 120 mg/kg bw/day) in corn oil was administered by gavage, 5 days per week, for 14 weeks, to groups of B6C3F$_1$ mice (10/sex/group) (NTP, 2003). General health observations were made twice daily, and clinical findings were recorded weekly. At the end of the exposure period, all treated animals were necropsied, and complete histopathology was performed on animals from the high-dose and control groups. Three male mice died during the study, but these deaths were attributed to dosing errors, and no test substance-related effects on survival were reported. Body weight gains in both sexes at all doses were comparable to controls. At week 7 of the study, males in the 60 and 120 mg/kg bw dose group males and females in the 120 mg/kg bw dose group had increased salivation, and in weeks 9 and 10 these same groups exhibited anal wetness. Hematology results for all dosed and control groups showed no treatment-related or biologically significant differences. In males given 60 mg/kg bw/day, absolute and relative liver (liver wt./body wt.) weights ($p \leqslant 0.05$) were greater than those of control animals, In females given 60 mg/kg bw absolute liver weights ($p \leqslant 0.01$) were greater than those of control animals, and relative liver weights of all treated females were significantly ($p \leqslant 0.01$) greater than control animals. In males given 60 and 120 mg 2,4-hexadienal/kg bw/day, significant increased absolute ($p \leqslant 0.05$) and relative (60 mg/kg bw, $p \leqslant 0.05$; 120 mg/kg bw, $p \leqslant 0.01$) kidney (kidney wt./body wt.) weights were reported. In the forestomachs of females given 120 mg/kg bw/day, an increased incidence of minimal-to-mild epithelial hyperplasia was reported compared to the controls, but these effects were not associated with basal cell proliferation or appreciable inflammation. Minimal-to-mild olfactory epithelium necrosis was more frequently observed in both male and female mice exposed to 120 mg 2,4-hexadienal/kg bw/day than in control animals. Males given the same dose also showed a significant increase in the incidence of olfactory epithelial atrophy, but this was not observed in the females from this dose group. Based on these findings, a NOEL cannot be established in female B6C3F$_1$mice fed 2,4-hexadienal for 14 weeks due to the increased relative liver weights observed at all dose levels, but the NOEL in male B6C3F$_1$ mice fed 2,4-hexadienal daily for 14 weeks was 30 mg/kg bw/day (NTP, 2003).

In the main 2-year bioassay, 2,4-hexadienal (0, 30, 60, or 120 mg/kg bw/day) in corn oil was administered by gavage 5 days per week for 104 weeks to groups of B6C3F$_1$ mice (50/sex/dose) (NTP, 2003). Twice daily health and mortality observations were made, and body weights and clinical findings were recorded every 4 weeks throughout the study. No significant differences between treated and control animals were reported in survival rates or mean body weights. No treatment-related clinical findings were noted for any dose groups. In the forestomachs of males administered 120 mg/kg bw/day and females administered 60 or 120 mg/kg bw/day, a statistically significant increase in the incidence of squamous epithelial hyperplasia was reported (see Table 6). In these dose groups, incidences of squamous cell papillomas and

Table 6
Summary of the incidences of neoplasms and nonneoplastic lesions of the forestomach in male and female B6C3F$_1$ mice administered 2,4-hexadienal by gavage (NTP, 2003)

	Corn oil (control)	30 mg/kg	60 mg/kg	120 mg/kg
Male mice				
Squamous epithelium hyperplasia, incidence/Number animals necropsied (%)	14/50 (28%)	7/50 (14%)	9/50 (18%)	26/50 (52%)[a]
Squamous cell papilloma incidence[b]/Number animals necropsied (%)	2/50 (4%)	4/50 (8%)	5/50 (10%)	8/50 (16%)[c]
Combined squamous cell papilloma or carcinoma incidence[d]/Number animals necropsied (%)	2/50 (4%)	4/50 (8%)	5/50 (10%)	10/50 (20%)
Female mice				
Squamous epithelium hyperplasia, incidence/Number animals necropsied (%)	4/50 (8%)	8/49 (16%)	12/50 (24%)[e]	31/50 (62%)[a]
Squamous cell papilloma incidence[f]/Number animals necropsied (%)	2/50 (4%)	2/49 (4%)	11/50 (22%)[g]	13/50 (26%)[h]
Squamous cell carcinoma incidence[i]/Number animals necropsied (%)	0/50 (0%)	0/49 (0%)	0/50 (0%)	7/50 (14%)[a]
Combined squamous cell papilloma or carcinoma incidence[j]/Number animals necropsied (%)	2/50 (4%)	2/49 (4%)	11/50 (22%)	18/50 (36%)

[a] $p \leqslant 0.01$.
[b] Historical incidence for 2-year studies with controls given NTP-2000 diet (mean ± standard deviation): 10/659 (1.8 ± 1.9%), range, 0–6%; with corn oil vehicle controls given NIH-07 diet 19/464 (4.1 ± 1.7%), range 2–6%.
[c] $p = 0.035$.
[d] Historical incidence for NTP-2000: 11/659 (2.0 ± 2.0%), range, 0–6%; for NIH-07 diet: 22/464 (4.7 ± 2.0%), range 2–8%.
[e] Significantly different ($p \leqslant 0.05$) from the vehicle control group by the Poly-3 test.
[f] Historical incidence for NTP-2000: 9/659 (1.4 ± 2.0%), range, 0–6%; for NIH-07 diet: 19/463 (4.1 ± 3.5%), range 0–10%.
[g] $p = 0.006$.
[h] $p < 0.001$.
[i] Historical incidence for NTP-2000: 1/659 (0.2 ± 0.6%), range, 0–2%; for NIH-07 diet: 0/463.
[j] Historical incidence for NTP-2000: 9/659 (1.4 ± 2.0%), range, 0–6%; for NIH-07 diet: 19/463 (4.1 ± 3.5%), range 0–10%.

combined incidence of squamous cell papillomas and carcinomas of the forestomach also were significantly increased. Forestomach squamous cell carcinomas were reported to be significantly increased in females administered 120 mg/kg bw/day ($p \leqslant 0.01$) (NTP, 2003). No significant increases in the incidence of lesions or neoplasms were reported for other organs or tissues.

As a result of these findings, the NTP stated that "*there was clear evidence of carcinogenic activity of 2,4-hexadienal in male or female B6C3F$_1$ mice based on increased incidences of squamous cell neoplasms of the forestomach*" (NTP, 2003).

4.3.1.2. Rats. In a preliminary dose range-finding study, groups of F344/N rats (5/sex/group) were administered 2,4-hexadienal (0, 3, 9, 27, 80, or 240 mg/kg bw/day) in corn oil by gavage 5 days per week for a total of 12 doses over 16 days. Twice daily general health and mortality observations were made, and clinical findings were recorded at the start, on day 8, and at the end of the study. At the highest dose level, 60% mortality occurred in both sexes, accompanied by clinical signs of toxicity including diarrhea, ataxia, lethargy, and anal/eye discharge in males, and lethargy, paleness, and abnormal breathing in females. Additionally, male and female animals in the 240 mg/kg bw/day dose group had a significant reduction in weight gain ($p \leqslant 0.01$) in comparison to the controls. At all of the lower doses, no mortality or clinical signs of toxicity were reported, and no significant changes in weight gain were noted in comparison to the controls. Females given 240 mg 2,4-hexadienal/kg bw/day were reported to have increased liver weights relative to controls. During gross pathological evaluation, most of the animals given 240 mg/kg bw/day showed necrosis and forestomach ulceration. Mild-to-moderate forestomach epithelial hyperplasia was reported in rats in the 80 mg/kg bw/day dose group. Both male and female animals administered 27 mg/kg bw/day 2,4-hexadianal had no treatment-related effects (NTP, 2003).

In a follow-up dose range-finding study, groups of F344/N rats (10/sex/group) were administered 2,4-hexadienal (0, 7.5, 15, 30, 60, or 120 mg/kg bw/day) in corn oil by gavage 5 days per week, for a total of 70 doses over 14 weeks. Twice daily observations of general health and behavior were made, and clinical findings were recorded weekly. At termination of the study, animals were euthanized and,necropsies performed on all treated animals. Complete histopathology also was performed on the high-dose and control groups. No mortalities were reported. In male rats administered 30, 60, or 120 mg 2,4-hexadienal/kg bw/day, final mean body weights and weight gains were significantly reduced compared to controls. Increased salivation was reported in both males and females administered 30 or 120 mg/kg bw/day doses during week 4, and then only in the 120 mg/kg bw/day group thereafter. No other signs of clinical toxicity were observed in treated animals at any dose. Incidences of mild-to-moderate forestomach epithelial hyperplasia were reported to be increased in both males and females administered 120 mg/kg bw/day relative to controls. These effects were accompanied by forestomach-localized tissue degeneration and acute inflammation. Increased incidences of olfactory epithelial atrophy, osteofibrosis, and excessive exudate of the nose were reported in males given 120 mg/kg bw/day. No biologically significant changes in organ weights were observed at any dose level. Variations in hematological and clinical chemistry values were reported, and while these were statistically significant they were sporadic and not dose-dependent, and thus considered to be independent of the treatment. Based on the findings, a NOEL of 15 and 60 mg/kg bw/day for male and female rats, respectively, were determined (NTP, 2003).

In the main 2-year study, 2,4-hexadienal (0, 22.5, 45, or 90 mg/kg bw/day) in corn oil was administered to groups of F344/N rats (50/sex/group) by gavage 5 days per week for 104 weeks. Twice daily observations for general health and mortality were made. Every 4 weeks animal weights and clinical findings were recorded. Throughout the study, survival rates of treated animals at all doses were comparable to untreated animals. After week 27 of the study, male animals administered the highest dose of 2,4-hexadienal showed statistically significant lower mean body weights compared to the controls. No clinical findings were reported at any dose that could be attributed to test substance administration. At all dose levels and in both sexes, statistically significant ($p \leqslant 0.01$) increases in the incidences of mild-to-moderate epithelial hyperplasia were observed in the forestomach (see Table 7). Incidences of forestomach squamous cell papillomas were increased relative to the controls in the 45 and 90 mg/kg bw/day males and females. In males given 45 or 90 mg/kg bw/day, a statistically significant ($p \leqslant 0.01$) increase in the combined incidence of forestomach squamous cell papillomas or carcinomas was reported. At any other dose level, there was no statistically significant increase in the incidences of forestomach squamous cell carcinomas in either males or females (NTP, 2003). No other significant treatment-related tumors were observed in the treated animals.

Based on these findings, the National Toxicology Program (NTP) concluded "*there was clear evidence of carcinogenic activity of 2,4-hexadienal in male and female F344/N rats based on increased incidences of squamous cell neoplasms of the forestomach*" (NTP, 2003).

4.3.1.3. Forestomach effects in rodents. As has been described above, forestomach hyperplasia and squamous cell papillomas in rodents

Table 7
Data Incidences of neoplasms and nonneoplastic lesions of the forestomach in male and female F344/N rats administered 2,4-hexadienal by gavage (NTP, 2003)

	Corn oil (control)	22.5 mg/kg	45 mg/kg	90 mg/kg
Male rats				
Epithelium hyperplasia, incidence/Number animals necropsied (%)	3/50 (6%)	19/50 (38%)[a]	42/50 (84%)[a]	50/50 (100%)[a]
Squamous cell papilloma incidence[b]/Number animals necropsied (%)	0/50 (0%)	3/50 (6%)	10/50 (20%)[c]	29/50 (58%)[c]
Combined squamous cell papilloma or carcinoma incidence[b]/Number animals necropsied (%)	0/50 (0%)	3/50 (6%)	11/50 (22%)[a]	29/50 (58%)[a]
Female rats				
Epithelium hyperplasia, incidence/Number animals necropsied (%)	2/50 (4%)	16/50 (32%)[a]	37/50 (74%)[a]	41/50 (82%)[a]
Squamous cell papilloma incidence[d]/Number animals necropsied (%)	0/50 (0%)	1/50 (2%)	5/50 (10%)[e]	17/50 (34%)[c]

[a] $p \leqslant 0.01$.
[b] Historical incidence for 2-year studies with controls given NTP-2000 diet (mean ± standard deviation): 2/609 (0.3 ± 0.7%), range 0–2%; with corn oil vehicle controls given NIH-07 diet: 2/402 (0.5 ± 0.9%), range 0–2%.
[c] $p < 0.001$.
[d] Historical incidence for NTP-2000: 0/659; for NIH-07 diet: 2/401 (0.5 ± 0.9%), range 0–2%.
[e] $p = 0.031$.

were common occurrences in the 2-year NTP studies that utilize gavage administration of irritating test substances. Squamous cell papillomas are benign lesions on organs that are lined with squamous epithelium. Most squamous cell papillomas arise as a result of chronic irritation, or due to specific viral infections (Smith and Ford, 1993). Gavage administration in corn oil of aldehydes (i.e., malonaldehyde, furfural, benzaldehyde, and 2,4-hexadienal) and other irritating substances (i.e., ethyl acrylate, dihydrocoumarin, and coumarin) in high concentrations are consistently associated with these papillomas (NTP, 1986a, 1988, 1990, 1992, 1993, 2003). The bolus dosing of high concentrations of irritating aldehydes into the mouse forestomach may result, over the typical rodent lifetime, in progression to malignant neoplasms.

In the NTP studies described above, the papillomas that were reported in the rodent forestomach were likely due to a combination of high concentrations of irritating aldehydes, use of corn oil (which is a mild irritant and mitogen) as the vehicle, and daily introduction of a gavage tube into the forestomach. Gavage administration of a bolus dose can stress the epithelium of the forestomach. Daily repetition of this distress in chronic studies would likely lead to chronic inflammation and regenerative hyperplasia. Conversely, dietary administration to rodents produces lower maximum short-term concentrations in circulation, although ultimately the same total doses are achieved. As a result, the forestomach effects observed for 2,4-hexadienal that are a consequence of gavage installation of high bolus doses of would not likely occur if dietary administration routes were utilized. This hypothesis is supported by the disparity between the increased incidences of squamous cell papillomas and forestomach hyperplasia in gavage administration in corn oil of benzyl acetate for 2 years (NTP, 1986b) that do not occur when the same substance is provided in the diet at similar exposure levels (NTP, 1993). In recent 2-year studies, aromatic and aliphatic aldehydes [*trans*-cinnamaldehyde and 3,7-dimethyl-2,6-octadienal (citral)] were microencapsulated and given in the diet at higher concentration than those used in the gavage studies mentioned above. No evidence of forestomach hyperplasia, papillomas or carcinomas was found. (NTP, 2001, 2002).

In a 2-year NTP gavage study, ethyl acrylate produced almost identical forestomach neoplasms, and was therefore listed as "*reasonably anticipated to be a human carcinogen*". The opinion in the study report concluded that ethyl acrylate was carcinogenic due to the observation of dose-related benign and malignant forestomach neoplastic responses in rats and mice (NTP, 1986a). In 2000, the ethyl acrylate data was re-evaluated, and as a result, it was delisted as a human carcinogen. The opinion stated that " (1) *the forestomach tumors induced in animal studies were seen only when the chemical was administered by gavage at high concentrations that induced marked local irritation and cellular proliferation; (2) animal studies by other routes of administration including inhalation were negative; and (3) significant chronic human oral exposure to high concentrations of ethyl acrylate monomer is unlikely*" (NTP, 2000).

The development of forestomach tumors in rodents in these studies, and their relevance to the potential for human carcinogenicity, has been the subject of much consideration (Grice, 1988; Wester and Kroes, 1988; Clayson et al., 1990). The mucosa of the rodent forestomach is not similar to that of the human esophagus; it is partially comprised of a keratinizing squamous epithelial layer. The rodent forestomach can store food and is constantly exposed to strongly acidic gastric juice. Conversely, the human distal esophagus is not involved in storage and does not have constant contact to the highly acidic gastric medium. The human esophagus contains a non-keratinizing squamous epithelium that is adversely affected by strongly acidic medium.

Therefore, the incidences of forestomach lesions in 2-year rodent studies on *trans*,*trans*-2,4-hexadienal where high concentrations of test material were administered by gavage are not relevant to humans, since these lesions occur at the contact site (forestomach) and arise from the irritating effect of a bolus dose of the aldehyde given by gavage. These forestomach lesions are not due to the effects of high concentrations that are achieved in the whole animal. Human exposure to 2,4-hexadienal occurs through dietary consumption, and intake is low. The concentrations of 2,4-hexadienal used in the NTP study that resulted in forestomach papillomas and carcinomas in mice and rats are approximately 8 orders of magnitude higher than the amount ingested by human eaters of this flavor (Gavin et al., 2008; Waddell, 2002, 2004).

trans-2-Hexenal and *trans*,*trans*-2,4-hexadienal are structurally similar and when administered to rats by gavage, they have produced almost identical biological and toxicological responses. Ethyl acrylate is also an α,β-unsaturated compound that produces similar responses when administered in the same manner. These observations suggest that these substances would have a common mode of carcinogenicity, and would therefore be similarly toxic and have similar carcinogenic potency. While such studies have not been conducted, it is reasonable to hypothesize that lifetime gavage administration of high concentrations of *trans*-2-hexenal to rats would result in carcinogenicity centered in the forestomach, similar to that observed for 2,4-hexadienal. It is also likely that the ulcerative and necrotizing lesions and consequent regenerative cell proliferation in the forestomach produced under these unique conditions would be associated with increased DNA adducts, as was observed in the 2-hexenal adduct study (Stout et al., 2008). Given the similarity of these two aldehydes to the response observed with ethyl acrylate, which does not generate DNA adducts, the role of DNA adducts in the forestomach carcinogenic activity is unclear, especially given the lack of hepatic tumors even in the presence of DNA adducts. The loss of biochemical processes that would normally rapidly metabolize α,β-unsaturated aldehydes and the disruption of cell membranes and functions that occur under these extreme conditions increase the probability of a direct reaction between the aldehyde with DNA nucleotides.

4.3.2. (*E*,*E*)-2,4-Hexadienoic acid (No. 35)

4.3.2.1. Mice. (*E*,*E*)-2,4-Hexadienoic acid (sorbic acid) was administered to groups of ASH/CS1mice (48 males and 50 females) at 0%, 1%, 5%, or 10% of the diet for a period of 80 weeks (Hendy et al., 1976). These dietary percentages correspond to approximate daily intakes of 0, 1400, 7500, or 15,000 mg/kg bw, respectively (FDA, 1993). Animal body weights were recorded at study commencement and then at varying unspecified intervals up to week 74. At the end of the exposure period all surviving animals were euthanized and necropsied. Macroscopic examinations of major organs were conducted and tissue samples were collected. No treatment-related effects on mortality were observed.. In males fed 5% or 10% diets and in females fed 10% diets, weight gains were significantly reduced compared to controls. No hematological differences of statistical significance between treated and untreated mice were reported. Animals fed the 5% or 10% diets displayed increased relative kidney weights but the authors did not conclude that this was treatment-related, since histological examinations showed that incidences of kidney lesions were significantly greater in control animals than in treated animals. Treated mice had increased absolute liver weights relative to controls but the authors concluded that this was not indicative of toxicity and was because of increased metabolic demand. This was supported by the observation that the livers of treated mice had fewer incidences of chronic inflammation, hyperplastic nodules, and early degenerative change when compared to control groups. These results indicate that (*E*,*E*)-2,4-hexadienoic acid is not carcinogenic in mice provided at dietary levels of up to 10% in the diet for a period of

80 weeks. When administered at 1% in the diet, (*E*,*E*)-2,4-hexadienoic acid produced no treatment-related effects in mice (Hendy et al., 1976).

4.3.2.2. Rats. In a 2-year study, groups of Wistar rats (48/sex/group) were fed diets containing 0, 1.5, or 10% (*E*,*E*)-2,4-hexadienoic acid. These dietary levels correspond to approximate intakes of 0, 750, or 5000 mg/kg bw/day, respectively (FDA, 1993). Body weight, food consumption and water consumption were recorded,at study initiation, after 1 month of treatment, and at 3-month intervals thereafter. Daily observations of general health and behavior were made. No significant differences in survival rates between treated and control animals were reported. In animals of both sexes fed (*E*,*E*)-2,4-hexadienoic acid as 10% of the diet, slight decreases in body weight gain was reported. Treated and untreated rats had no consistent differences in food consumption. No treatment-related effects were observed in the hematological examinations or clinical chemistry evaluations. Measurement of renal function and histopathological examinations showed no significant differences between control and test groups. Animals of both sexes fed (*E*,*E*)-2,4-hexadienoic acid at 10% of the diet had increased relative liver weights (liver wt./body wt.) and females at this dose levels had increased relative kidney weights (kidney wt./body wt.). Increased liver weights were not accompanied by any histological findings, and the authors concluded that the high levels of fatty acid administered produced increased metabolic demand. Tumors were reported in animals fed 1.5% (*E*,*E*)-2,4-hexadienoic acid, but were not observed in animals receiving higher dietary concentrations. Therefore, the authors concluded that tumor induction in treated animals at the lower dose level was unrelated to treatment with (*E*,*E*)-2,4-hexadienoic acid. Overall, these results indicate that no carcinogenic effects are associated with the administration of a diet consisting of up to 10% (approximately 5,000 mg/kg bw/day) of (*E*,*E*)-2,4-hexadienoic acid to rats, which is higher than the FDA recommended maximum standard of 5%. Based on the findings of this study, the NOEL was established at 1.5% in rats, or approximately 750 mg/kg bw/day (Gaunt et al., 1975).

The potassium salt of (*E*,*E*)-2,4-hexadienoic acid (potassium sorbate) was administered to two groups of six male rats at 0.1% in the diet or 0.3% in drinking water for 60 weeks. These exposure levels correspond to daily intakes of approximately 50 or 300 mg/kg bw, respectively (FDA, 1993). A control group of animals received no potassium sorbate. The general health of animals was monitored throughout the study, and no treatment-related changes were reported. Some of the rats exposed to potassium sorbate via drinking water were reported to have small white nodules scattered on the surface of the liver, but diagnostic laparotomies on all surviving rats at the end of the experiment revealed no liver tumors or other treatment-related effects. The non-neoplastic nature of the observed lesions was confirmed by histological examination. Animals were necropsied as they died, and all surviving animals were sacrificed at week 100. Necropsy revealed no treatment-related tumors in either group of rats. The authors concluded that potassium sorbate administered continuously at levels of 0.1% in the diet (approximately 50 mg/kg bw/day) or 0.3% in drinking water (approximately 300 mg/kg bw/day) for a period of 60 weeks did not induce any toxic or carcinogenic effect in male rats (Dickens et al., 1968).

In the majority of long-term studies with (*E*,*E*)-2,4-hexadienoic acid, it has been given at levels greater than 5% of the diet (Deuel et al., 1954; Gaunt et al., 1975; Hendy et al., 1976). National Toxicology Program (NTP) protocols have generally used 5% as the maximum level for any dietary addition, since levels >5% have been shown to cause dietary alterations that can impact the chronic toxicity and carcinogenicity evaluation of the test material.

4.4. Genotoxicity

4.4.1. In vitro

In vitro genotoxicity assays have been performed on five homologous mono-α,β-unsaturated aldehydes (2-pentenal to 2-nonenal), 2 α,β-alkynyl esters (methyl octynoate and methyl nonynoate), 4 α,β-unsaturated dienals (2,4-hexadienal, 2,4-nonadienal, 2,6-nonadienal, and 2,4-decadienal), ethyl 2,4,7-decatrienoate, and 2,4-hexadienoic acid (see Table 8). Standard Ames assays indicated no genotoxic effects, however, substantial cytotoxicity was observed in these assays, which limits the value of these results. Non-standard genotoxicity assays (i.e., using high-sensitivity tester strains or adopting alterations in standard test protocols, such as increased cell densities) have been undertaken in an attempt to understand the possible *in vitro* mutagenicity of α,β-unsaturated aldehydes.

Methyl 2-nonynoate (No. 65) and methyl 2-octynoate (No. 54) were not mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 when tested at concentrations up to 3600 μg/plate, with and without metabolic activation (Wild et al., 1983).

The potential mutagenicity of (*E*)-2-butenoic acid (No. 1) was evaluated in an Ames assay using *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 in the presence or absence of S-9 fraction. At concentrations up to 1000 μg/plate, no mutagenicity was observed when the plate incorporation method was used (Lijinsky and Andrews, 1980). However, using a liquid preincubation method, (*E*)-2-butenoic acid gave positive results in *S. typhimurium* strain TA100 with or without metabolic activation (Lijinsky and Andrews, 1980). Without metabolic activation, (*E*)-2-butenoic acid concentrations as low as 10 μg/plate gave positive results, but in the presence of metabolic activation, significant mutagenic activity was not observed until concentrations reached 250 μg of the test material. According to the authors, the addition of the S-9 mix partially detoxifies the compound, producing a mutagen different from that detected without the added S-9. In a similar assay in strain TA100, no evidence of mutagenicity was observed for (*E*)-2-butenoic acid at concentrations ranging from 0.1 to 1000 μg/plate (Rapson et al., 1980).

Ames assays with or without S-9 metabolic activation in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 were conducted for 2-hexenal (No. 21). No evidence of mutagenicity was seen at concentrations up to 3 μmol/plate (294 μg/plate) (Florin et al., 1980). Similarly, up to 55 μg 2,4-nonadienal/plate, up to 344 μg nona-2-*trans*-6-*cis*-dienal/plate (Eder et al., 1992), up to 333 μg 2-*trans*,4-*trans*-decadienal/plate (NTP, 1997), and up to 5000 μg ethyl 2,4,7-decatrienoate/plate (Thompson, 1996) produced negative results in Ames assays with *S. typhimurium* strains TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, and TA1538 (Marnett et al., 1985), with or without metabolic activation.

At concentrations up to 1500 μg/plate *trans,trans*-2,4-hexadienal was not mutagenic in the *S. typhimurium* strains TA102 (Marnett et al., 1985), TA98, TA1535 (Florin et al., 1980; NTP, 2003), TA100, and TA1537 (Florin et al., 1980; Marnett et al., 1985; NTP, 2003), with or without metabolic activation.

S. typhimurium strain TA104 is more sensitive than other standard Ames assay strains in identifying mutagenic effects of α,β-unsaturated aldehydes, This strain contains a nonsense mutation (–TAA–) at the site of reversion and is much more sensitive to carbonyl mutagenesis than standard *Salmonella* strains due to the deletion of the uvrB gene, which encodes for an error-free DNA excision repair. Additionally, incorporation of the pKM101 plasmid, which encodes for an error-prone DNA polymerase involved in bypass replication of lesions, contributes to the increased sensitivity (Marnett et al., 1985).

Table 8
In vitro genotoxicity studies on α,β-unsaturated aldehydes and related substances

#	Substance name	Test system *in vitro*	Test object	Maximum concentration of substance	Result	Reference
1	(*E*)-2-Butenoic acid	Reverse mutation	*Salmonella typhimurium* TA100	0.1–1000 μg/plate	Negative	Rapson et al. (1980)
1	(*E*)-2-Butenoic acid	Reverse mutation	*Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	Up to 1000 μg/plate	Negative[a]	Lijinsky and Andrews (1980)
1	(*E*)-2-Butenoic acid	Reverse mutation	*Salmonella typhimurium* TA100	Up to 1000 μg/plate	Positive[a,b,c]	Lijinsky and Andrews (1980)
1	(*E*)-2-Butenoic acid	Sister chromatid exchange	Human lymphocytes	2.5, 5.0, or 10.0 mM (215.2, 430.4, or 860.9 μg/ml)[d]	Positive[e]	Sipi et al. (1992)
6	2-Pentenal	Reverse mutation	*Salmonella typhimurium* TA100	0.01–0.75 μl	Positive[a,b,f]	Eder et al. (1992)
6	2-Pentenal	SOS chromotest	*Escherichia coli* PQ37 and PQ243	60–435 nmol/plate (5.0–36.7 μg/plate)[g]	Negative[h]	Eder et al. (1992)
6	2-Pentenal	Mutation induction	V79 Chinese hamster cells	0.03, 0.10, or 0.30 mM (2.5, 8.4, or 25.2 μg/ml)[g]	Positive[i,j,k] Negative[l,k]	Canonero et al. (1990)
6	2-Pentenal	DNA single strand break	L1210 mouse leukemia cells	400, 600, or 800 μmol (33,648, 50,4725, or 67,2963 mg)[g]	Positive	Eder et al. (1993)
21	2-Hexenal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 2 μmol/plate[m] (196.3 μg/plate)[n]	Positive[i,b]	Marnett et al. (1985)
21	2-Hexenal	Reverse mutation	*Salmonella typhimurium* TA104	5 μmol/plate (>490.7 μg/plate)[n]	Positive[i,b,o]	Marnett et al. (1985)
21	2-Hexenal	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, and TA1537	3 μmol/plate (294.4 μg/plate)[n]	Negative[a]	Florin et al. (1980)
21	2-Hexenal	Reverse mutation	*Salmonella typhimurium* TA100	0.01–0.50 μl/plate	Negative[a,p] Positive[q,t,r]	Eder et al. (1992)
21	2-Hexenal	SOS chromotest	*Escherichia coli* PQ37 and PQ243	70–435 nmol/plate (6.9–42.7 μg/plate)[n]	Negative[h]	Eder et al. (1992)
21	2-Hexenal	Mutation induction	Chinese hamster V79 cells	0.03, 0.10, or 0.30 mM (2.9, 9.8, or 29.4 μg/ml)[n]	Positive[i,j] Negative[i,s]	Canonero et al. (1990)
21	2-Hexenal	Micronucleus induction	Human blood lymphocytes	5–250 μM (0.5–24.5 μg/ml)[n]	Positive	Dittberner et al. (1995)
21	2-Hexenal	Micronucleus induction	Lymphoblastoid Namalva cells	5–250 μM (0.5–24.5 μg/ml)[n]	Positive[t]	Dittberner et al. (1995)
21	2-Hexenal	Chromosomal aberration	Human blood lymphocytes	5–250 μM (0.5–24.5 μg/ml)[n]	Negative	Dittberner et al. (1995)
21	2-Hexenal	Chromosomal aberration	Lymphoblastoid Namalva cells	5–150 μM (0.5–14.7 μg/ml)[n]	Positive[u]	Dittberner et al. (1995)
21	2-Hexenal	Sister chromatid exchange	Human blood lymphocytes	5–250 μM (0.5–24.5 μg/ml)[n]	Positive	Dittberner et al. (1995)
21	2-Hexenal	Sister chromatid exchange	Lymphoblastoid Namalva cells	5–200 μM (0.5–19.6 μg/ml)[n]	Positive	Dittberner et al. (1995)
21	2-Hexenal	DNA single strand break	L1210 mouse leukemia cells	100, 250, or 500 μmol (9814, 24,535, or 49,070 μg)[n]	Positive[h]	Eder et al. (1993)
21	2-Hexenal	DNA repair	Rat hepatocytes	60–600 nmol (5.9–58.9 μmol)[n]	Positive	Griffin and Segall (1986)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, and TA1537	3 μmol/plate (288 μg/plate)[v]	Negative[w,a]	Florin et al. (1980)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 1 μmol/plate (96 μg/plate)[v]	Positive[b,i]	Marnett et al. (1985)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA104	>5 μmol/plate (>481 μg/plate)[v]	Positive[b,i,o]	Marnett et al. (1985)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA100	0.01–0.4 μl/plate (8.95–358 μg/plate)[x]	Positive[i,f,y]	Eder et al. (1992)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA100	0.01–0.75 μl/plate (8.95–671.3 μg/plate)[x]	Positive[f,y,s]	Eder et al. (1992)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA100	0.005–0.4 μl/plate (4.48–358 μg/plate)[x]	Positive[s,f,z]	Eder et al. (1992)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA100	0.005–0.4 μl/plate (4.48–358 μg/plate)[x]	Positive[s,f,z]	Eder et al. (1992)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA1535, TA98	Up to 1500 μg/plate	Negative[s]	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA98	Up to 150 μg/plate	Negative[i]	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA1535	Up to 166 μg/plate	Negative[i]	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA100	Up to 333 μg/plate	Positive[i,aa]	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal	Reverse mutation	*Salmonella typhimurium* TA100	Up to 1,500 μg/plate	Positive[s]	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal	SOS chromotest	*Escherichia coli* PQ37 and PQ243	Up to 590 nmol (57 μg/plate)[v]	Negative	Eder et al. (1992)
30	*trans*,*trans*-2,4-Hexadienal	SOS chromotest	*Escherichia coli* PQ37	Not reported	Positive[bb]	Eder et al. (1993)

(*continued on next page*)

Table 8 (*continued*)

#	Substance name	Test system *in vitro*	Test object	Maximum concentration of substance	Result	Reference
30	*trans*,*trans*-2,4-Hexadienal	DNA strand breaks	L1210 mouse leukemia cells	20 μmol/ml (1923 μg/ml)[v] 300, 500 μmol/ml (28,839, 48,065 μg/ml)[v]	Negative Positive[cc]	Eder et al. (1993)
35	(*E*,*E*)-2,4-Hexadienoic acid potassium salt	Cell cycle alterations	V79 Chinese hamster cells	Up to 2500 μg/ml	Positive[dd]	Schlatter et al. (1992)
35	(*E*,*E*)-2,4-Hexadienoic acid sodium salt	Cell cycle alterations	V79 Chinese hamster cells	Up to 2500 μg/ml	Positive[dd,ee]	Schlatter et al. (1992)
40	2-Heptenal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 0.9 μmol/plate[m] (101 μg/plate)[ff]	Negative[i,b]	Marnett et al. (1985)
40	2-Heptenal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 4.4 μmol/plate[m] (493.5 μg/plate)[ff]	Negative[i,b,o]	Marnett et al. (1985)
40	2-Heptenal	Reverse mutation	*Salmonella typhimurium* TA100	0.005–0.400 μl/plate	Negative[a,gg]	Eder et al. (1992)
40	2-Heptenal	SOS chromotest	*Escherichia coli*PQ37 and PQ243	35–270 nmol/plate (3.9–30.3 μg/plate)[ff]	Negative[h]	Eder et al. (1992)
40	2-Heptenal	Mutation induction	Chinese hamster V79 cells	0.01, 0.03, or 0.10 mM (1.1, 3.4, 11.2 μg/ml)[ff]	Positive[i,hh]	Canonero et al. (1990)
40	2-Heptenal	DNA single strand break	L1210 mouse leukemia cells	200, 400, or 500 μmol (22,434, 44,868, or 56,085 mg)[ff]	Positive	Eder et al. (1993)
47	2-Octenal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 0.8 μmol/plate[m] (101.0 μg/plate)[ii]	Negative[i,b]	Marnett et al. (1985)
47	2-Octenal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 4 μmol/plate[m] (504.8 μg/plate)[ii]	Negative[i,b,o]	Marnett et al. (1985)
47	2-Octenal	Mutation induction	Chinese hamster V79 cells	0.01, 0.03, or 0.10 mM (1.3, 3.8, or 12.6 μg/ml)[ii]	Positive[i] Negative[ij]	Canonero et al. (1990)
47	2-Octenal	DNA single strand break	L1210 mouse leukemia cells	350 μmol (44,170 μg/plate)	Positive	Eder et al. (1993)
54	Methyl 2-octynoate	Reverse mutation	*Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	Up to 3.6 mg/plate (3600 μg/plate)	Negative[a]	Wild et al. (1983)
57	2-Nonenal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 0.007 μmol/plate[m] (1.0 μg/plate)[kk]	Negative[i,b]	Marnett et al. (1985)
57	2-Nonenal	Mutation induction	Chinese hamster V79 cells	0.003 or 0.01 mM (0.4 or 1.4 μg/ml)[kk]	Positive[i,j] Negative[ij]	Canonero et al. (1990)
57	2-Nonenal	Micronucleus induction	Rat hepatocytes	0.1, 1, 10, or 100 μM (0.01, 0.1, 1.4, or 14.0 μg/ml)[kk]	Positive	Esterbauer et al. (1990)
57	2-Nonenal	Micronucleus induction	Rat hepatocytes	0.1, 10, or 100 μM (0.01, 1.4, or 14.0 μg/ml)[kk]	Negative	Eckl et al. (1993)
57	2-Nonenal	Chromosomal aberration	Rat hepatocytes	0.1, 1, 10, or 100 μM (0.01, 0.1, 1.4, or 14.0 μg/ml)[kk]	Negative	Esterbauer et al. (1990)
57	2-Nonenal	Chromosomal aberration	Rat hepatocytes	0.1, 10, or 100 μM (0.01, 1.4, or 14.0 μg/ml)[kk]	Negative	Eckl et al. (1993)
57	2-Nonenal	Sister chromatid exchange	Rat hepatocytes	0.1, 10, or 100 μM (0.01, 1.4, or 14.0 μg/ml)[kk]	Positive	Eckl et al. (1993)
57	2-Nonenal	DNA repair	Rat hepatocytes	60–600 nmol (8.4–84.1 μg/plate)[kk]	Positive	Griffin and Segall (1986)
59	2,4-Nonadienal	Reverse mutation	*Salmonella typhimurium* TA104	Up to 0.4 μmol/plate (up to 55 μg/plate)[ll]	Negative[b,i]	Marnett et al. (1985)
59	2,4-Nonadienal	SOS chromotest	*Escherichia coli* PQ37	Not reported	Negative	Eder et al. (1993)
59	2,4-Nonadienal	DNA strand breaks	L1210 mouse leukemia cells	400 μmol/ml (55,284 μg/ml)[ll] 500 μmol/ml (69,105 μg/ml)[ll]	Negative[cc] Positive	Eder et al. (1993)
62	Nona-2-*trans*-6-*cis*-dienal	Reverse mutation	*Salmonella typhimurium* TA100	0.01–0.1 μl/plate (8.6 to 86 μg/plate)[mm]	Negative[i,f,y]	Eder et al. (1992)
62	Nona-2-*trans*-6-*cis*-dienal	Reverse mutation	*Salmonella typhimurium* TA100	0.005–0.4 μl/plate (4.3–344 μg/plate)[mm]	Negative[i,f,z]	Eder et al. (1992)
62	Nona-2-*trans*-6-*cis*-dienal	Reverse mutation	*Salmonella typhimurium* TA100	0.005–0.25 μl/plate (4.3 to 344 μg/plate)[mm]	Negative[i,f,z]	Eder et al. (1992)
62	Nona-2-*trans*-6-*cis*-dienal	SOS chromotest	*Escherichia coli* PQ37 and PQ243	Up to 80 nmol (11 μg/plate)[ll]	Negative	Eder et al. (1992)
62	Nona-2-*trans*-6-*cis*-dienal	Sister chromatid exchange	Human lymphoblastoid Namalva cell line	0–10 μM (0–1.38 μg/ml)[ll] 20–40 μM (2.8–5.5 μg/ml)[ll]	Negative Positive	Dittberner et al. (1995)
62	Nona-2-*trans*-6-*cis*-dienal	Sister chromatid exchange	Primary human blood lymphocytes	0–10 μM (0–1.38 μg/ml)[ll] 20–50 μM (2.8–6.9 μg/ml)[ll]	Negative Positive	Dittberner et al. (1995)
62	Nona-2-*trans*-6-*cis*-dienal	Structural chromosomal aberration test	Human lymphoblastoid Namalva cell line	5–40 μM (0.69–5.5 μg/ml)[ll]	Positive	Dittberner et al. (1995)
62	Nona-2-*trans*-6-*cis*-dienal	Structural chromosomal aberration test	Primary human blood lymphocytes	0–40 μM (0–5.5 μg/ml)[ll]	Negative	Dittberner et al. (1995)
62	Nona-2-*trans*-6-*cis*-dienal	Numerical chromosomal aberration test	Primary human blood lymphocytes	0–20 μM (0–2.76 μg/ml)[ll] 40 μM (5.5 μg/ml)[ll]	Negative Positive	Dittberner et al. (1995)
62	Nona-2-*trans*-6-*cis*-dienal	Micronucleus induction	Primary human blood lymphocytes	0–10 μM (0–1.38 μg/ml)[ll]	Negative	Dittberner et al. (1995)

Table 8 (*continued*)

#	Substance name	Test system *in vitro*	Test object	Maximum concentration of substance	Result	Reference
20–50 μM (2.76–6.9 μg/ml)[ll]	Positive					
62	Nona-2-*trans*-6-*cis*-dienal	Micronucleus induction	Human lymphoblastoid Namalva cell line	0–20 μM (0–2.76 μg/ml)[ll] 40–50 μM (5.5–6.9 μg/ml)[ll]	Negative Positive	Dittberner et al. (1995)
62	Nona-2-*trans*-6-*cis*-dienal	Reverse mutation	*Salmonella typhimurium* TA97, TA98, TA100, TA102, TA104, and TA1535	Up to 333 μg/plate	Negative[a]	NTP (1997)
65	Methyl 2-nonynoate	Reverse mutation	*Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	Up to 3.6 mg/plate (3,600 μg/plate)	Negative[a]	Wild et al. (1983)
81	Ethyl 2,4,7-decatrienoate	Reverse mutation	*Salmonella typhimurium* TA100, TA1535, TA1538, TA98, and TA1537	1.5–5000 μg/plate	Negative[a]	Thompson (1996)
21	2-Hexenal	Micronucleus induction	Human buccal mucosa cells	10 ppm	Positive[nn]	Dittberner et al. (1997)
30	*trans*,*trans*-2,4-Hexadienal	Micronucleus induction	Mouse	40, 80, 120, or 160 mg/kg	Inconclusive[q]	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal	Micronucleus induction	Mouse	7.5, 15, 30, 60, or 120 mg/kg	Negative[j]	NTP (2001)
30	*trans*,*trans*-2,4-Hexadienal	Micronucleus induction	Rat	50, 100, 150, or 200 mg/kg	Inconclusive[s]	NTP (2001)
35	(*E*,*E*)-2,4-Hexadienoic acid	Chromosome aberration	Mouse	15 mg/kg bw	Positive[u,ff]	Banerjee and Giri (1986)
35	(*E*,*E*)-2,4-Hexadienoic acid	Micronucleus induction	Mouse	2.5, 20 mg/kg bw 150 mg/kg bw	Negative[s] Positive[v]	Mukherjee et al. (1988)
35	(*E*,*E*)-2,4-Hexadienoic acid	Sister chromatid exchange	Mouse	25 to 50 mg/kg bw 75, 100, or 150 mg/kg bw	Negative[s] Positive[s]	Mukherjee et al. (1988)
35	2,4-Hexadienoic acid, potassium salt	Somatic mutation and recombination	*Drosophila melanogaster*	3.75 mg/ml	Negative	Schlatter et al. (1992)
35	2,4-Hexadienoic acid, sodium salt	Somatic mutation and recombination	*Drosophila melanogaster*	3.35 mg/ml	Negative	Schlatter et al. (1992)
54	Methyl 2-octynoate	Sex-linked recessive lethal mutation	*Drosophila melanogaster*	1 mM (154.2 μg/ml)[oo]	Negative	Wild et al. (1983)
54	Methyl 2-octynoate	Micronucleus induction	NMRI Mouse bone marrow	154, 231, 308 mg/kg[pp]	Negative	Wild et al. (1983)
65	Methyl 2-nonynoate	Sex-linked recessive lethal mutation	*Drosophila melanogaster*	2.5 mM (420.6 μg/ml)[qq]	Negative	Wild et al. (1983)
65	Methyl 2-nonynoate	Micronucleus induction	NMRI Mouse bone marrow	168, 336, or 505 mg/kg[pp]	Negative	Wild et al. (1983)
73	2-*trans*,4-*trans*-Decadienal	Micronucleus induction	Mouse	50, 100, 200, 400, or 800 mg/kg	Negative[rr]	NTP (1997)

[aa] Positive at 1 of 2 testing centres.
[a] With and without metabolic activation.
[bb] With ethanol as solvent instead of dimethylsulfoxide (DMSO).
[b] Liquid pre-incubation procedure.
[cc] Results demonstrated in the presence of cytotoxicty.
[c] Positive results were first observed at doses as low as 10 μg/plate in the absence of metabolic activation, and as high as 250 μg/plate in the presence of metabolic activation. Cytotoxicity was reported at doses greater than 50 and 500 μg/plate in the absence and presence of metabolic activation, respectively.
[dd] Pattern of positive effects is suggestive of weak aneugenic activity.
[d] Calculated using the molecular weight of (*E*)-2-butenoic acid = 86.09.
[ee] Positive effects observed only with stored solutions (28 days old).
[e] A slight dose-dependent increase in the induction of sister chromatid exchange was observed; however, a significant increase relative to controls was noted only at the highest dose tested (10 mM). At the highest dose, the pH of the medium was decreased by 0.4–0.68 pH units relative to that of controls.
[ff] No significant increase in the number of ouabain mutants was observed relative to controls.
[f] In standard and three-fold bacterial cell density assays.
[gg] Dose-dependent increases in mutation frequency were noted in standard and three-fold bacterial cell density assays; however, these increases were never two-fold higher than the spontaneous mutation frequency.
[g] Calculated using the molecular weight of 2-pentenal = 84.12.
[hh] Dose-dependent increases in the number of 6-thioguanine and ouabain mutants were observed; however, these increases were significantly different from controls only at the highest dose tested (0.10 mM).
[h] Cytotoxicity was observed at the highest dose tested.
[ii] Calculated using the molecular weight of 2-octenal = 126.20.
[i] Without metabolic activation.
[jj] No significant increase relative to controls was observed in the number of ouabain mutants.
[j] A dose-dependent increase in the number of 6-thioguanine mutants was observed. However, a significant increase in mutation frequency relative to controls was noted only at the highest dose tested.
[kk] Calculated using the molecular weight of 2-nonenal = 140.22.
[k] Relative cell viabilities were reduced from 0.92 to 0.20 and from 0.83 to 0.17, respectively, in the 6-thioguanine and ouabain mutation experiments.
[ll] Calculated using molecular weight = 138.21.
[l] No significant increase relative to controls was observed in the number of ouabain mutants.
[mm] Calculated using specific gravity = 0.850–0.870 g/ml (FCC, 1996).
[m] Maximum non-toxic dose.
[nn] Statistically significant increases in micronuclei were observed on days 6 and 7 post-administration.

Table 8 (*continued*)

[oo] Calculated using the molecular weight of methyl 2-octynoate = 154.21.
[o] Addition of 10 mM glutathione.
[pp] Administered intraperitoneally.
[p] Without metabolic activation in a three-fold bacterial cell density assay.
[qq] Calculated using the molecular weight of methyl 2-nonynoate = 168.24.
[q] With metabolic activation.
[rr] Administered *via* injection.
[r] Conducted in a three-fold bacterial cell density assay.
[s] No significant increase in the number of ouabain mutants was observed relative to controls.
[t] Significantly increased relative to controls only at doses $\geqslant$150 μM.
[u] Significantly increased relative to controls only at doses $\geqslant$100 μM.
[v] Calculated using molecular weight = 96.13.
[w] Spot test method.
[x] Calculated using density = 0.896 g/ml (Sigma–Aldrich).
[y] 30-Minute preincubation.
[z] With metabolic activation.

A series of α,β-unsaturated aldehydes were incubated with TA104 in a modified Ames assay that used liquid pre-incubation protocols (i.e., addition of a GSH chase at the end of a 20-minute incubation period in strain TA104). In these assays, 2-hexenal (No. 21) and 2,4-hexadienal produced significant increases in reverse mutations in the absence of metabolic activation at concentrations >196 μg/plate and >481 μg/plate, respectively (Marnett et al., 1985). 2-Heptenal (No. 40) (up to 101 μg/plate), 2-octenal (No. 47) (up to 101 μg/plate), and 2-nonenal (No. 57) (up to 1 μg/plate) gave no evidence of mutagenicity when incubated with strain TA104 without metabolic activation, but were too cytotoxic to test at higher concentrations. To reduce cytotoxicity, GSH was incorporated into the Ames assay. The addition of GSH at the end of the pre-incubation period is hypothesized to reduce toxicity by preventing excess aldehyde from reacting with protein sulfhydryl groups. After the addition of 10 mM reduced GSH at the end of the pre-incubation period, the maximum non-cytotoxic doses of 2-hexenal and 2,4-hexadienal tested increased from 196 to >491 μg/plate and 96 to >481 μg/plate, respectively, but their mutagenic potential remained unaltered. Higher concentrations of 2-heptenal (No. 40) (up to 494 μg/plate) or 2-octenal (No. 44) (up to 505 μg/plate) were also tested when 10 mM GSH was added, and no mutagenic effects were observed. In analogous experiments with *S. typhimurium* strain TA102, no evidence of mutagenicity was reported for six 2-alkenals and *trans,trans*-2,4-hexandienal (Marnett et al., 1985). *S. typhimurium* strain TA102 contains the uvrB gene that encodes for an error-free DNA excision repair (Marnett et al., 1985).

Modification of the standard Ames assay protocol has been adopted to allow for mutagenicity evaluation in the presence of significant cytotoxicity. When Ames assays using *S. typhimurium* strain TA100 were conducted for α,β-unsaturated aldehydes using a 30-minute pre-incubation period and standard cell densities, simple linear aldehydes (e.g., butenal, pentenal, hexenal, and heptenal) displayed high cytotoxicity that limited the detection of mutagenic responses. When a higher cell density (three times standard) was used along with a 90-minute pre-incubation period, however, butenal, pentenal, hexenal, or 2,4-hexadienal produced at least two times the spontaneous reversion frequency compared to standard conditions, either with or without S-9 metabolic activation (Eder et al., 1992, 1993). These results with strain TA100 are consistent with the reported mutagenicity of α,β-unsaturated aldehyde in strain TA104 in the presence of GSH (Marnett et al., 1985). From a chemical perspective, aldehydes that were more lipophilic displayed increased cytotoxicity and mutagenicity relative to their less lipophilic counterparts. In general, S-9 metabolic activation leading to aldehyde detoxication resulted in higher non-cytotoxic dose levels and higher peak revertant frequencies.

No mutagenicity was reported in the SOS chromotest with *Escherichia coli* strains PQ37 and PQ243 incubated in the presence of 2-pentenal (No. 6), 2-hexenal (No. 21), 2-heptenal (No. 40), *trans*,-*trans*-2,4-hexadienal, and nona-2-*trans*-6-*cis*-dienal at concentrations up to 37, 43, 30, 57, and 11 μg/plate, respectively (Eder et al., 1992). High cytotoxicity was observed, and it was noted that this interfered with the performance of the test. In a related experiment, *trans,trans*-2,4-hexadienal gave positive results, but only when ethanol was used as the solvent (Eder et al., 1993).

(*E*)-2-Butenoic acid was reported to induce a small, dose-dependent increase in sister chromatid exchanges (SCEs) *in vitro* in human lymphocytes, at concentrations ranging from 2.5 to 10.0 mM (215–861 μg/ml) (Sipi et al., 1992). However, this increase was only significant relative to controls at the highest concentration tested (10 mM), and at this high concentration the pH also dropped by 0.4–0.68 pH units relative to controls.

The ability of α,β-unsaturated aldehydes to induce SCE, numerical and structural chromosomal aberrations (ABS), and micronuclei (MN) has been evaluated in cell lines that are low in GSH and detoxication enzymes (i.e., human blood lymphocytes and Namalva cell lines) (Dittberner et al., 1995). *trans*-2-Butenal (5–250 μM), 2-hexenal (5–250 μM), and *trans*-2-*cis*-6-nonadienal (5–50 μM) were separately incubated with human lymphocyte and Namalva cells. In lymphocytes at concentrations of 10 μM (0.7 μg/ml), 40 μM (3.9 μg/ml) and 20 μM (2.8 μg/ml) for 2-butenal, 2-hexenal, and *trans*-2-*cis*-6-nonadienal, respectively, the number of SCE increased significantly ($p < 0.05$). In Namalva cells, the number of SCE increased significantly at 20 μM for 2-butenal (1.4 μg/ml) and 2-hexenal (2.0 μg/ml), and at 10 μM for *trans*-2-*cis*-6-nonadienal (1.4 μg/ml). Structural ABS significantly increased in human blood lymphocytes only for 2-butenal, and then only at concentrations $\geqslant$10 μM. In Namalva cells concentrations of 100 μM (7.0 μg/ml) for 2-butenal, 100 μM (9.8 μg/ml) for 2-hexenal (No. 21), and 5 μM (0.7 μg/ml)[10] for *trans*-2-*cis*-6-nonadienal resulted in increased ABS. Significant increases in MN occurred at 50 μM for 2-butenal, 50 μM for 2-hexenal, and at 20 μM for *trans*-2-*cis*-6-nonadienal in blood lymphocytes, and in Namalva cells at 40 μM for 2-butenal, 150 μM for 2-hexenal, and at 40 μM for *trans*-2-*cis*-6-nonadienal. Severe cytotoxicity was observed for *trans*-2-*cis*-6-nonadienal at concentrations >50 μM. Based on these findings, the authors concluded that 2-butenal was clastogenic under the conditions of the experiment. Since chromosome breaks were not significantly increased, and because MN were positive for centromere-specific DNA, 2-hexenal and *trans*-2-*cis*-6-nonadienal were classified as aneugens. However, no attempts were made in the SCE and ABS experiments to identify what concentrations of test materials produced lysosomal breakdown. Increases in the incidence of SCE and ABS near or at observable levels of cytotoxicity may be due to secondary effects resulting from apoptosis, lysosome breakdown and release of DNAse (Zajac-Kaye and Ts'o, 1984; Bradley et al., 1987).

A number of α,β-unsaturated aldehyde lipid peroxidation products [2-pentenal (No. 6), 2-hexenal (No. 21), 2-heptenal (No. 40),

2-octenal (No. 47), and 2-nonenal (No. 57)] were tested for possible mutagenic effects in Chinese hamster V79 cells at concentrations ranging from 0.003 mM to 0.3 mM (Canonero et al., 1990). Dose-dependent increases in the frequency of 6-thioguanine-resistant mutants were reported for all aldehydes tested. Increasing potency of the mutagenic effect correlated with increasing carbon chain length. Only 2-heptenal treatment resulted in an increase in the number of mutations to ouabain resistance, but this was statistically significant relative to controls only at the highest dose tested (0.10 mM) (Canonero et al., 1990).

Incubation of *trans*-2-nonenal (0.1, 1.0, 10, or 100 μM) for 3 h with freshly prepared rat hepatocytes resulted in significant increases in MN at 10 and 100 μM, but not at 0.1 or 1.0 μM (Esterbauer et al., 1990). No statistically significant increases in the incidences of ABS were found for any of the concentrations tested. Similarly, SCEs were reported to significantly increase in rat hepatocytes when treated with *trans*-2-nonenal at concentrations of 0.1, 10, and 100 μM. No significant induction of ABS or MN could be demonstrated (Eckl et al., 1993).

Freshly prepared rat hepatocytes were incubated with *trans*-2-hexenal or *trans*-2-nonenal (60–600 nmol/10^6 cells) for 20 h (Griffin and Segall, 1986) and unscheduled DNA synthesis (UDS) was assessed. Extracellular release of lactate dehydrogenase was measured to gauge cytotoxicity. UDS, was quantified by measuring net grain counts (nuclear-cytoplasmic grain counts). Dose-dependent increases in UDS were reported beginning at 120 nmol/10^6 cells for *trans*-2-hexenal and at 60 nmol/10^6 cells for *trans*-2-nonenal. In both cases, UDS increases were accompanied by increased LDH release. The authors concluded that there was a correlation between cytotoxicity and genotoxicity, and that these likely occurred through a common pathway or mechanism (Griffin and Segall, 1986).

An alkaline elution assay using L1210 mouse leukemia cells was used to assess the ability of high concentrations of a series of α,β-unsaturated aldehyde to induce single strand breaks (Eder et al., 1993). Strand breaks were reported at 600-800 μmol for 2-pentenal (No. 6), 250–500 μmol for 2-hexenal (No. 21), 400–500 μmol for 2-heptenal (No. 40), 350 μmol for 2-octenal (No. 47), 300 μmol for 2,4-hexadienal, and at 500 μmol 2,4-nonadienal. However, the majority of concentrations that induced strand breaks also produced cytotoxicity. The exceptions were *trans*-2-pentenal at 600 μmol, *trans*-2-hexenal at 250 μmol, and *trans*-2-heptenal at 400–500 μmol. When the ability of the 2-alkenals to produce DNA adducts was investigated, it was reported that *trans*-2-pentenal, *trans*-2-hexenal, and 2,4-hexadienal were capable of forming a 1,2-cyclic deoxyguanosine adduct. Additionally, 2,4-hexadienal produced a small amount of 7,8-cyclic guanosine adducts. No evidence for cross-linked adducts was found (Eder et al., 1993).

Sodium and potassium sorbate (salts of (*E*,*E*)-2,4-hexadienoic acid) are both used as food preservatives and have demonstrated antifungal and antibacterial activities. The potential mutagenicity of these food preservatives and their parent acid was investigated using V79 Chinese hamster cells. No genotoxic activity was observed for 2,4-hexadienoic acid or its potassium salt. Decreased cell viability and cell cycle alterations were observed only at the highest concentration of fresh sodium sorbate (2500 μg/ml) examined. Solutions of sodium sorbate stored for periods of up to 208 days exhibited effects similar to those observed with the fresh preparations. Alternatively, potassium sorbate tested at concentrations of up to 2500 μg/ml induced toxic effects following a 28-day storage period. Specific, cellular effects mediated by either test substances included decreased number of cells in the S-phase, an increase in G_2/M cells, and an increase in cellular protein content in both G_1 and G_2 cells (Schlatter et al., 1992). When freshly prepared solutions of the sodium salt of 2,4-hexadienoic acid were used, no genotoxic or cell-transforming activities were detected. However, if the sodium salt was stored as a solution which previously had been heated and sonicated to facilitate solubilization, a positive response was produced in both test systems. The authors note that it is most likely oxidation products of the sodium salt of 2,4-hexadienoic acid that form in the heating, sonication and/or storage process that are responsible for the positive genotoxic response (Schiffmann and Schlatter, 1992).

4.4.2. In vivo

Methyl 2-nonynoate and methyl 2-octynoate were evaluated in the Basc test for their ability to induce sex-linked recessive lethal mutations in adult *Drosophila melanogaster*. When flies were fed 2.5 (421 μg/ml) and 1.0 mM (154 μg/ml) solutions of methyl 2-nonynoate and methyl 2-octynoate, respectively for 3 days, no changes were reported in the frequency of mutations (Wild et al., 1983).

Sodium and potassium sorbate (3.35 and 3.75 mg/ml, respectively) gave negative results for genotoxicity when tested in the *D. melanogaster* somatic cells in a 48-hour chronic feeding study (Schlatter et al., 1992).

Methyl 2-nonynoate and methyl 2-octynoate were evaluated in a micronucleus assay. Groups of male and female NMRI mice (4/group) were administered single intraperitoneal doses of methyl 2-nonynoate (168, 336, or 505 mg/kg bw) or methyl 2-octynoate (154, 231, and 308 mg/kg bw). At 30 h post-administration, no increase in micronucleated erythrocytes in bone marrow samples was observed for either substance (Wild et al., 1983).

Human volunteer subjects rinsed their mouths with *trans*-2-hexenal (10 ppm) (No. 21) on three consecutive days. Exfoliated buccal mucosa cells were collected, and the induction of MN was assessed. Levels of MN increased in a statistically significant manner on days 6 and 7 after administration, but no increases were observed on prior days (Dittberner et al., 1997).

trans,trans-2,4-Hexadienal was evaluated in micronucleus assays in bone marrow polychromatic erythrocytes (PCEs) for its ability to induce chromosomal damage in mice and rats. *trans,-trans*-2,4-Hexadienal was given intreaperitoneally to animals at doses ranging from 40 to 160 mg/kg bw for mice and 50 to 200 mg/kg bw for rats. Trend analyses indicated that the numbers of micronucleated PCEs per 1000 PCEs were significant (mouse, $p = 0.024$; rat $p = 0.017$), but the study was judged to be inconclusive as the mean values obtained for all treated groups were larger than the control mean values (NTP, 2003). Negative results were reported for micronucleus assays of peripheral blood normochromatic erythrocytes of mice exposed to *trans,trans*-2,4-hexadienal (7.5 to 120 mg/kg bw) (NTP, 2003) and 2-*trans*,4-*trans*-decadienal (50 to 800 mg/kg bw) *via* gavage for 14 weeks (NTP, 1997).

In a repeat-dose mouse ABS test, the preservative and antifungal agent sorbic acid ((*E*,*E*)-2,4-hexadienoic acid) was investigated for its genotoxic potential. Sorbic acid (15 mg/kg bw/day) was administered by gavage daily for 30 days to a group of 10 Swiss albino male mice. A control group also was maintained. An increase in mitotic index was observed in the treated animals relative to controls, but this was not accompanied by a significant increase in structural ABS in treated versus untreated animals (Banerjee and Giri, 1986). In a later study, sorbic acid (0, 25, 50, 75, 100, or 150 mg/kg bw) was administered as a single dose to groups (8/dose) of male Swiss albino via intraperitoneal injection. Treated animals that received 75, 100, or 150 mg/kg bw sorbic acid had a statistically significant increase in SCEs ($p < 0.05$) compared to controls. Concurrently, mice were administered a single intraperitoneal dose of sorbic acid (0, 2.5, 20, or 150 mg/kg bw). Four animals from each dose group were euthanized at 24 and at 48 h, and the number of micronucleated cells per 500 PCEs was determined. Significant increases in micronucleated PCEs ($p < 0.05$) were reported only at the highest does (150 mg/kg bw)

(Mukherjee et al., 1988). While intraperitoneal administration in these experiments has produced positive *in vivo* findings, this method of test material installation into animals is not relevant to human consumption of flavors. No genotoxic activity was found in studies that utilized gavage dosing.

4.4.3. Discussion of genotoxicity data

No evidence of mutagenicity has been found when α,β-unsaturated aldehydes have been tested in standardized Ames assays in a variety of *S. typhimurium* strains (TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, and TA1538) (Florin et al., 1980; NTP, 2003, 1997). The cytotoxicity of these substances has made it difficult to evaluate their genotoxic potential and therefore alternative protocols have been developed. In modified Ames assays, positive results were reported when pre-incubation conditions were used that were conducive to depletion of metabolic detoxication pathways (Eder et al., 1992, 1993). Positive evidence of genotoxicity was also reported in other assays (SCE, ABS, and MN) if cell lines that have low detoxication capacity (Namalva cells and human lymphocytes) were used (Dittberner et al., 1995). In these studies, α,β-unsaturated aldehydes at high concentrations (20–40 μM) in cell lines poor in detoxication capacity provided opportunity for either direct interaction of α,β-unsaturated aldehydes with DNA or indirect formation of DNA adducts due to oxidative stress, leading to single DNA strand breaks but no cross-linking of DNA. The depletion of GSH by high concentrations of α,β-unsaturated aldehydes is known to lead to oxidative stress and to the release of nucleocytolytic enzymes, causing DNA fragmentation, cellular damage and apoptosis (see discussion in metabolism section). In normal human consumption, however, the low concentrations of α,β-unsaturated aldehydes are rapidly metabolized in the high-capacity β-oxidation pathway

4.5. Reproductive toxicity

4.5.1. (*E*,*E*)-2,4-Hexadienoic acid (sorbic acid; No. 35)

A diet of Purina Laboratory Chow containing 10% (*E*,*E*)-2,4-hexadienoic acid was fed for 60 days to groups of 90-day old Sprague–Dawley rats that were subsequently mated. The offspring of the parent generation were fed the same diet for 70 days and then mated at 90 days of age. Individual animals from the parent generation were weighed at the beginning of the study and at 30, 60, 90, and 120 days of feeding. Animals from the first generation were weighed at 40, 70, and 120 days of feeding. General appearance, behavior, and food intake were not affected by the diet. All groups except for treated first-generation females displayed increased ratios of liver weight to body weight compared to the control groups. No treatment-related reproductive effects were reported. Livers from randomly selected control and treated rats were removed at the conclusion of the feeding study to evaluate the metabolism in liver homogenates. Oxygen consumption in liver homogenates from treated animals and control animals in the parent generation were not significantly different. Liver homogenates from treated animals from the first generation did show differences in oxygen consumption versus first generation controls at the 95% level, with female control animal liver homogenates displaying the highest rate of oxygen consumption and those from male controls the lowest. As female rats are known to have a higher metabolic rate than males, these results were not considered by the authors to be of toxicological significance, and the rates of oxygen consumption for treated male and female liver homogenates from the first generation were almost identical. The authors concluded from these findings that at 10% of the diet, (*E*,*E*)-2,4-hexadienoic acid did not produce any significant treatment-related reproductive effects in rats (Demaree et al., 1955).

5. Recognition of GRASr status

The group of aliphatic, linear α,β-unsaturated, mono-, di- and trienals and related alcohols, acids and esters discussed here was determined to be generally recognized as safe (GRAS) under conditions of intended use as flavor ingredients by the Flavor and Extract Manufacturers Association (FEMA) Expert Panel in 1965 or in subsequent years. In 1978, the Panel evaluated the available data and affirmed the GRAS status of these flavor ingredients (GRASa). In 1993, the Panel initiated a comprehensive program to reevaluate the status of all FEMA GRAS flavor ingredients concurrent with a systematic revision of the FEMA Scientific Literature Reviews (SLRs). The group of aliphatic, linear α,β-unsaturated, mono-, di- and trienals and related alcohols, acids and esters was reaffirmed as GRAS (GRASr) based, in part, on their rapid absorption, metabolic detoxication, and excretion in humans and other animals; their low level of flavor use; the wide margins of safety between the conservative estimates of intake and the no-observed-adverse effect levels determined from subchronic and chronic studies, and the lack of significant genotoxic and mutagenic potential when tested at non-cytotoxic concentrations.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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