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Evaluation of the butter flavoring chemical diacetyl and a fluorochemical paper additive for mutagenicity and toxicity using the mammalian cell gene mutation assay in L5178Y mouse lymphoma cells

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ABSTRACT

Diacetyl (2,3-butanedione) is a yellowish liquid that is usually mixed with other ingredients to produce butter flavor or other flavors in a variety of food products. Inhalation of butter flavoring vapors was first associated with clinical bronchiolitis obliterans among workers in microwave popcorn production. Recent findings have shown irreversible obstructive lung disease among workers not only in the microwave popcorn industry, but also in flavoring manufacture, and in chemical synthesis of diacetyl, a predominant chemical for butter flavoring. It has been reported that perfluorochemicals utilized in food packaging are migrating into foods and may be sources of oral exposure. Relatively small quantities of perfluorochemicals are used in the manufacturing of paper or paperboard that is in direct contact with food to repel oil or grease and water. Because of recent concerns about perfluorochemicals such as those found on microwave popcorn bags (e.g. Lodyne P-280E) and diacetyl in foods, we evaluated both compounds for mutagenicity using the mammalian cell gene mutation assay in L5178Y mouse lymphoma cells. Lodyne P-280E was less toxic than diacetyl and did not induce a mutagenic response. Diacetyl induced a highly mutagenic response in the L5178Y mouse lymphoma mutation assay in the presence of human liver S9 for activation. The increase in the frequency of small colonies in the assay with diacetyl indicates that diacetyl causes damage to multiple loci on chromosome 11 in addition to functional loss of the thymidine kinase locus.

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1. Introduction

Kreiss et al. (2002) were the first to report that inhalation of butter flavoring vapors is associated with obstructive lung disease resembling bronchiolitis obliterans among workers in microwave popcorn production. Subsequently, there were several additional reports on industrial exposure to diacetyl and its association with bronchiolitis obliterans, a severe respiratory illness producing fibrosis and obstruction of airways (Harber et al., 2006; Centers for Disease Control, 2007; Egilman et al., 2007; Kreiss, 2007; van Rooy et al., 2007). Diacetyl, which is chemically 2,3-butanedione, is a flavoring agent used to produce a buttery taste and has been widely used in the popcorn industry. Butter flavors used in microwave popcorn generally contain significantly more diacetyl than other types of flavors due to consumer preference. It is also used for butterscotch flavoring and for providing creaminess. Diacetyl occurs naturally in many foods such as butter, milk, cheese, fruit, wine, beer, coffee, and is a natural by-product of fermentation. It is an important flavor compound in dairy products, popcorn, wine, and beer (Harber et al., 2006). At low levels, it gives beer a slick mouth feel and a buttery aroma and adds complexity to the final sensory impact of wine.

Perfluorochemicals are used in food packaging as paper coatings for oil and moisture resistance. Begley et al. (2005) have reported on the migration of perfluorochemicals into foods and found that fluorochemical additives do migrate to food during package use. Begley et al. (2008) found that microwave popcorn contained 3.2 mg fluorochemical/kg popcorn after popping and that oil containing small amounts of an emulsifier can significantly enhance migration of a fluorochemical from paper.

Because of recent concerns about perfluorochemicals migrating into foods, especially microwave popcorn, and the use of diacetyl

Abbreviations: DMSO, dimethyl sulfoxide; EPA, US Environmental Protection Agency; FEMA, Flavoring and Extract Manufacturers Association; FDA, US Food and Drug Administration; DMBA, 7,12-dimethylbenzanthracene; GRAS, generally recognized as safe; OSHA, US Occupational Safety and Health Administration; NIOSH, US National Institute for Occupational Safety and Health; TK, thymidine kinase; TFT, trifluorothymidine.

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in butter flavoring of microwave popcorn, cellular toxicity and mutagenicity of diacetyl and Lodyne P-208E® were evaluated in L5178Y mouse lymphoma cells in the presence of pooled human liver S9.

The mouse lymphoma assay was chosen because it detects mutations known to be important in the etiology of cancer and other human genetically mediated illnesses. The assay detects gene mutations (point mutations), and chromosomal events such as deletions, translocations, mitotic recombination/gene conversion and aneuploidy (Food and Drug Administration, 2001, Update, 2006).

2. Materials and methods

2.1. Chemicals

The manufacturer/supplier, Chemical Abstracts Service number, formula, formula weight, and purity of each compound are listed in Table 1. The compounds were acquired by the US Food and Drug Administration (FDA) and supplied as coded samples to the contract laboratory (BioReliance, Rockville, MD). Stock solutions of each compound were prepared in the appropriate solvent immediately prior to use. The final concentration of solvent was 1% dimethyl sulfoxide (DMSO).

2.2. Human S9 preparation

Human liver S9 was purchased from In Vitro Technologies, Inc., Baltimore, MD. Human liver was pooled from 10 individuals of both genders and the S9 was prepared according to the methods described by Guengerich (1989), Easterbrook et al. (2001), and Nomeir et al. (2001). The S9 contains the subcellular fractions in which the drug metabolizing cytochrome P450 enzymes reside.

2.3. L5178Y $TK^{+/-}$ mouse lymphoma assay

L5178Y $TK^{+/-}$ 3.7.C mouse lymphoma cells were originally obtained from Ms. Patricia Poorman-Allen, Glaxo Wellcome Inc., Research Triangle Park, NC or American Type Culture Collection, Manassas, VA. The cells were grown in Fischer's medium for leukemic cells of mice (Irvine Scientific, Irvine, CA) supplemented with 10% horse serum (Gibco, Grand Island, NY) and 0.02% pluronic F-68 (BASF Wyandotte Corp., Wyandotte, MI). Cells were screened for the presence of mycoplasma before and after cryopreservation. New cultures were initiated from cells stored in liquid N_2 at approximately 3-month intervals.

The toxicity of each chemical was determined with pooled human liver S9. S9 mix was prepared following the procedure described by Clive et al. (1979). Cells at a concentration of 6×10^5/ml (6×10^6 cells total) were exposed for 4 h to a range of concentrations of each chemical. The cells were then washed, resuspended in growth medium and incubated at 37 ± 1 °C for 24 h. Cells in the cultures were then adjusted to 3×10^5 cells/ml and incubated at 37 ± 1 °C for an additional 24 h. The rate of cell growth was determined for each of the treated cultures and compared with the rate of growth of the solvent controls. The doses of each chemical selected for testing were within the range yielding approximately 0–90% cytotoxicity or up to the limit of solubility. For each assay there was a solvent control and a positive control of aflatoxin B-1 at 0.5 μg/ml. Methyl methanesulfonate at 15 μg/ml without activation was included as the positive control for colony size distribution.

The mutagenicity assay was performed as described (Clive and Spector, 1975). A total of 1.2×10^7 cells in duplicate cultures were exposed to the test chemical, positive control and solvent control for 4 h at 37 ± 1 °C, washed twice with growth medium and maintained at 37 ± 1 °C for 48 h in log-phase growth to allow recovery and mutant expression. Cells in the cultures were adjusted to 3×10^5 cells/ml at 24-h intervals. They were then cloned (1×10^6 cells/plate for mutant selection and 200 cells/plate for viable count determinations) in soft-agar medium containing Fischer's medium, 20% horse serum, 2 mM sodium pyruvate, 0.02% pluronic F-68 and 0.22% Noble agar (Becton Dickinson, Sparks, MD). Resistance to trifluorothymidine (TFT) was determined by adding TFT (final concentration, 3 μg/ml) to the cloning medium for mutant selection. The 100× stock solution of TFT in saline was stored at −70 °C and was thawed immediately before

Table 1
Chemical information and source for diacetyl and Lodyne P-208E®

Chemical name	CAS number	Formula	Formula weight	Purity %	Source
2,3-Butanedione (Diacetyl)	431-03-8	$C_4H_6O_2$	86.09	97	Sigma–Aldrich Co. (St. Louis, MO)
Lodyne P-208E®	–	5,5-bis[(γ,ω-perfluoroC_{4-20} alkylthio) methyl]-2-hydroxy-2-oxo-1,3,2-dioxaphosphorinane, ammonium salt	900–1200	95	Ciba (Basel, Switzerland)

Table 2
Mutagenicity and cytotoxicity of diacetyl in mouse lymphoma cells

	Dose (μg/ml)	Human S9	Absolute cloning efficiency[a]	Relative total growth (% of control)	Average number TFT^R colonies	Mutant frequency per 10^6 survivors[b,c]
Solvent control (DMSO)	0	+	1.18/0.93	100.0	99/78	84*
	100	+	0.96/0.87	84.5	77/75	84*
	150	+	0.97/1.07	80.5	75/92	82*
	180	+	0.95/1.04	76.0	111/94	104*
	200	+	0.96/0.35	38.0	129/82	183†
	250	+	0.83/0.69	31.0	261/275	357‡
Positive control (Aflatoxin B-1)	0.5	+	0.63	40.0	261	414

[a] Based on the average of three petri dishes each plated with 200 cells. The averages for each of the two cultures per dose are separated by a slash (/).
[b] 1×10^6 cells in a measured volume were plated in each of three plates/culture in the presence of trifluorothymidine (3 μg/ml) after 2 days of expression. The values are for duplicate cultures (two cultures/solvent control).
[c] Means for mutant frequencies not sharing a common symbol (*, †, ‡) are significantly different ($P < 0.05$) as determined by the Duncan method, which was applied only if significant differences were determined to exist by analysis of variance (ANOVA), P value = 0.002.

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use. Plates were incubated at 37 ± 1 °C in 5% CO_2 in air for 10–12 days and then counted with a ProtoCOL automated colony counter (Synoptics Ltd., Cambridge, UK). The results from this study were evaluated according to the guidelines in Moore et al. (2006). A data set was considered to show an indication of mutagenic activity if there was evidence of a dose response with at least one concentration giving an increase in mutant frequency of at least 90 mutants per 10^6 surviving cells above the concurrent solvent control value. Only colonies larger than approximately 0.1 mm in diameter were counted. Mutant frequencies were expressed as mutants per 10^6 surviving cells. Only doses yielding total growth values of $\geqslant$10% were used in the analysis of induced mutant frequency. The assay was performed only with S9 mix because it had previously been shown to be positive using *Salmonella typhimurium* strains TA100 and TA104 (Bjeldanes and Chew, 1979 and Marnett et al., 1985).

The size of mutant mouse lymphoma colonies was also determined using a ProtoCOL colony counter/sizer. The number and sizes of colonies were determined covering the range of approximately 0.2–1.1 mm.

2.4. Statistical analysis

Differences among mutant frequencies were assessed by analysis of variance (ANOVA) (Snedecor and Cochran, 1980). The Duncan multiple comparison method was used to differentiate among means, and was applied only if significant differences were found by ANOVA (Snedecor and Cochran, 1980).

3. Results

Diacetyl and Lodyne P-208E® were evaluated for toxicity and mutagenicity in L5178Y/$TK^{+/-}$ mouse lymphoma cells in the presence of pooled human liver S9. Dimethyl sulfoxide was selected as the solvent for both test compounds. Diacetyl was soluble in DMSO at 86 mg/ml and, in the preliminary toxicity assay; the maximum concentration of diacetyl tested was 860 μg/ml. In this assay, complete toxicity (that is, no cell suspension growth) was observed at concentrations of 500 and 860 μg/ml. Based on the results of this toxicity assay, the concentrations of diacetyl used in the mutagenicity assay ranged from 25 to 500 μg/ml. The concentrations chosen for cloning were 100, 150, 180, 200, and 250 μg/ml.

As shown in Table 2, the relative total growth for diacetyl was approximately 80% for the first three doses and for the two high doses (200 and 250 μg/ml) was reduced to 38 and 31% of the control. Diacetyl induced a dose related increase in the number of mutant colonies over the range of 150 to 250 μg/ml. The two highest concentrations had mutant frequencies of 183 and 357 mutants per 10^6 surviving cells, with greater than 90 mutants above the solvent control required for designation of a positive response.

The trifluorothymidine-resistant colonies for the diacetyl-treated cultures and for the positive and solvent control cultures from both assays were sized according to diameter over a range from 0.20 to 1.05 mm. Results on colony size distribution showed an increase in the frequency of small colonies when the diacetyl-treated cultures were compared to the solvent control cultures (Fig. 1). An increase in the frequency of small colonies is consistent with damage to multiple loci on chromosome 11 as well as functional loss of the thymidine kinase (TK) locus. Fig. 2 shows the typical colony size distribution obtained in this study for the positive control methyl methanesulfonate compared to the solvent control. Fig. 3 shows the colony size distribution with S9 activation obtained in this study for the positive control aflatoxin B-1 at 0.5 μg/ml compared to the solvent control.

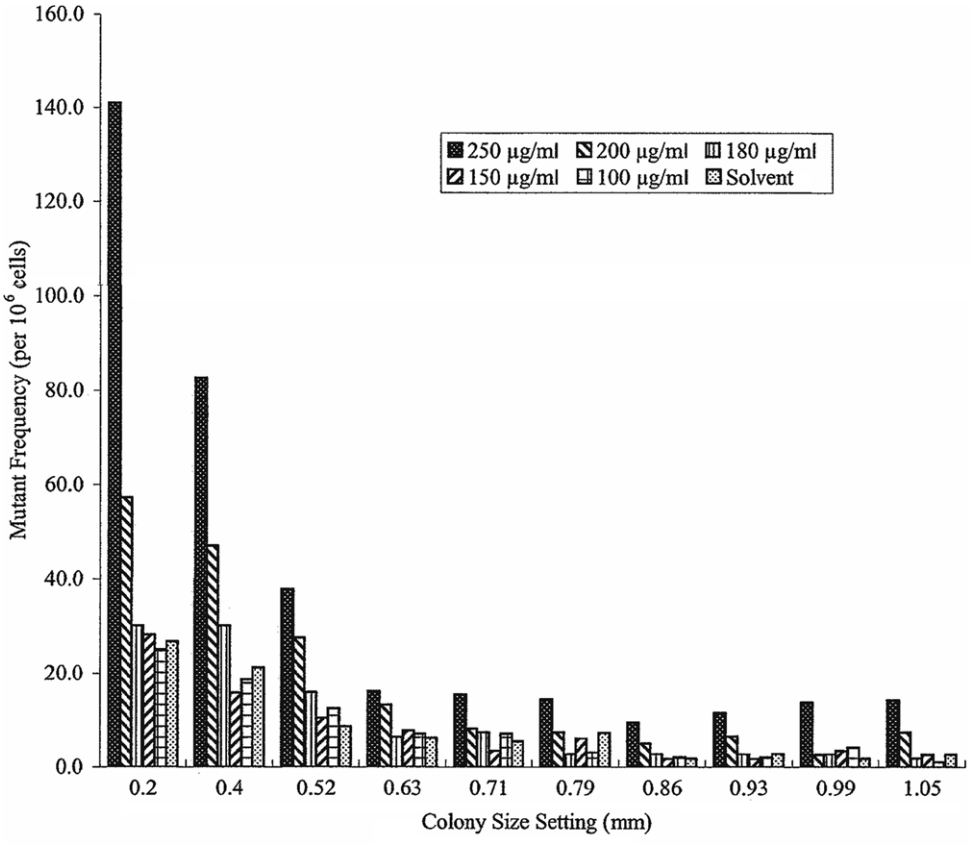

Fig. 1. Colony size distribution with S9 activation for diacetyl at 250, 200, 180, 150, 100 μg/ml compared to the solvent control.

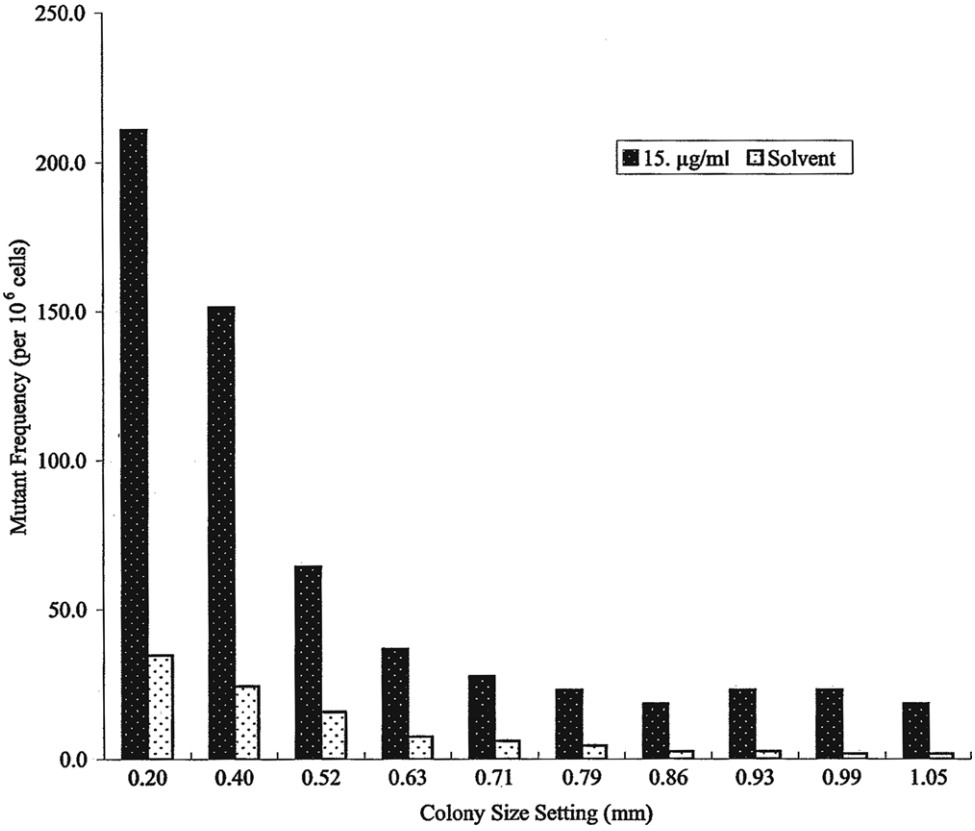

Fig. 2. Colony size distribution without metabolic activation for the positive control methyl methanesulfonate (MMS) at 15 µg/ml compared to the solvent control.

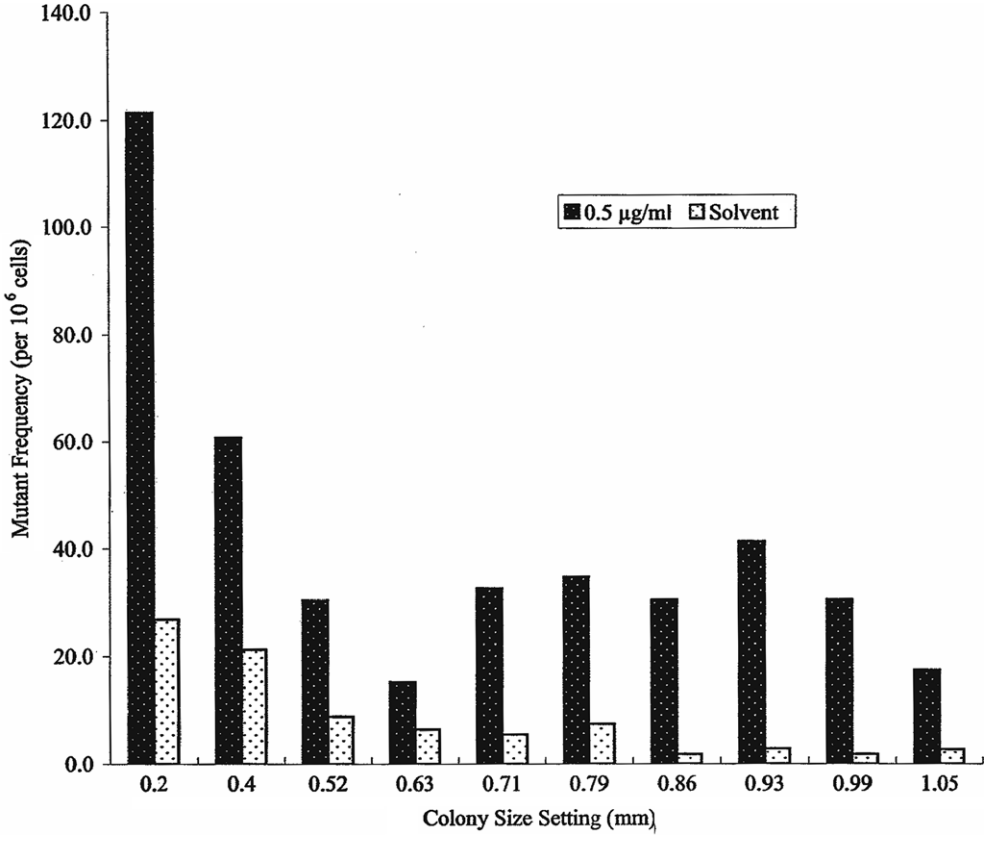

Fig. 3. Colony size distribution with S9 activation for the positive control aflatoxin B-1 at 0.5 µg/ml compared to the solvent control.

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Table 3
Mutagenicity and cytotoxicity of Lodyne P-208E® in mouse lymphoma cells

	Dose (μg/ml)	Human S9	Absolute cloning efficiency[a]	Relative total growth (% of control)	Average number TFT^R colonies	Mutant frequency per 106 survivors[b,c]
Solvent control	0	+	1.14/0.93	100.0	99/78	86
(DMSO)	300	+	0.93/1.06	96.5	97/87	94
	350	+	1.07/1.08	105.5	113/95	97
	400	+	1.19/1.06	103.0	98/85	82
	450	+	1.00/0.92	87.0	103/79	95
	500	+	1.03/1.11	95.5	116/78	93
Positive control (Aflatoxin B-1)	0.5	+	0.63	41.0	261	414

[a] Based on the average of three petri dishes each plated with 200 cells. The averages for each of the two cultures per dose are separated by a slash (/).
[b] 1×10^6 cells in a measured volume were plated in each of three plates/culture in the presence of trifluorothymidine (3 μg/ml) after 2 days of expression. The values are for duplicate cultures (two cultures/solvent control).
[c] Differences in mutant frequencies were assessed by analysis of variance (ANOVA). P value = 0.910.

Lodyne P-280E was soluble in DMSO at 43 mg/ml and, in the preliminary toxicity assay, the maximum concentration of Lodyne P-280E tested was 430 μg/ml. Based on the results of the preliminary toxicity assay, the concentrations of Lodyne P-280E used in the mutagenesis assay ranged from 50 to 500 μg/ml. The concentrations chosen for cloning were 300, 350, 400, 450, and 500 μg/ml. The relative total growth for Lodyne P-280E was similar to the solvent control at all dose concentrations and at the highest dose (500 μg/ml) was 95.5% (Table 3). None of the cloned cultures showed mutant frequencies $\geqslant$90 mutants per 10^6 cells over the solvent control (Table 3).

4. Discussion

In this study, diacetyl induced a dose related increase in the number of mutant colonies, with a reduction in relative total growth to 31% for the highest dose. Diacetyl was highly mutagenic in the L5178Y mouse lymphoma assay in the presence of human liver S9 for activation, with an increase in the number of small colonies. This increase is consistent with damage to multiple loci on chromosome 11 as well as functional loss of the TK locus. The purpose of testing in the L5178Y assay was to determine the genotoxic potential of diacetyl and Lodyne P-280E, and to indicate the need for further in vivo testing which will allow comparisons to be made to human occupational exposures.

In previous studies diacetyl has been shown to induce dose related mutagenic responses in *S. typhimurium* strains TA100 (Bjeldanes and Chew, 1979) and TA104 (Marnett et al., 1985). Scientists at the National Toxicology Program and Duke University Medical Center recently evaluated the respiratory toxicity of diacetyl at levels relevant to human health (Morgan et al., 2008). In the murine model used, concentrations of diacetyl and exposure times were comparable to conditions at some microwave popcorn packaging plants. Depending on the route and duration of exposure, diacetyl caused lymphocytic bronchiolitis an injury that replicates features of human obliterative bronchiolitis (Morgan et al., 2008). Animal studies will be required to interpret possible human risk. As reported in Morgan et al. (2008) the National Toxicology Program is planning additional safety studies for diacetyl for interpreting possible human risk from diacetyl.

Diacetyl is a substance that may be added directly to human food and is affirmed as generally recognized as safe (GRAS) (Code of Federal Regulations 21 Part 184.1278, 2007). The chemical is used as a flavoring agent and adjuvant and can be used in food at levels not to exceed current good manufacturing practice. In determining FDA GRAS status, the evaluation does not include an assessment of the safety of the chemicals when inhaled or when they come into contact with mucous membranes or skin.

The chronology for use of new butter flavorings containing higher concentrations of diacetyl, and a sudden increase in the number of cases of bronchiolitis obliterans has been described by Egilman et al. (2007). After their extensive review of documents which included internal correspondence, reports, programs, and presentations, as well as medical records of workers who died as a result of exposure to synthetic diacetyl, Egilman et al. (2007) recommended that the GRAS assessment by the Flavoring and Extract Manufacturers Association (FEMA) expert panel for FDA needs to be restructured to include inhalation and contact safety evaluation of GRAS flavoring chemicals based on conditions of intended use.

Since 1960, FEMA has had an expert panel for the safety evaluation of food flavoring for the flavor industry through its own GRAS assessment of flavoring substances. Since the flavoring industry refers to its food products as GRAS, workers assume the products are safe. However the FEMA GRAS process does not evaluate substances for inhalation or contact risks (Smith et al., 2003). FEMA has recently recommended that its members who manufacture butter flavors containing diacetyl for use in microwave popcorn consider reducing the diacetyl content of these flavors to the extent possible (Flavor and Extract Manufacturers Association, 2007).

Since carbonyl compounds, such as diacetyl, are widely distributed in foods, are by-products of cellular metabolism, and are present in body fluids, they may make a significant contribution to the risk of human cancer (Marnett et al., 1985).

Lodyne P-280E was less toxic than diacetyl and did not induce a mutagenic response. Relative total growth for Lodyne P-280E never fell below 80% as it did for diacetyl because it was not toxic to the cells. Begley et al. (2008) have shown that fluorochemicals migrate from packaging materials, and the presence of perfluorochemicals in human serum has been demonstrated in other studies (Calafat et al., 2007; D'eon and Mabury, 2007; Olsen et al., 2007). Calafat et al. (2007) reported a reduction in polyfluoroalkyl chemicals (10% for perfluorohexane sulfonic acid to 32% for perfluorooctane sulfonic acid) in the US population when comparing data from the 2003–2004 National Health and Nutrition Examination Survey to results from the 1999–2000 survey. Calafat et al. (2007) indicated that the reduced population exposures are likely due to recent efforts by industry and government to lower serum concentrations of polyfluoroalkyl chemicals. This decline may have resulted from the discontinued industrial production of perfluorooctane sulfonic acid and related compounds (Calafat et al., 2007). Since the half life for elimination of these perfluorochemicals from human serum is long (approximately 3–8 years), further studies are needed to determine other possible sources of human exposure and potential toxicity (Olsen et al., 2007; Tan et al., 2008).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either actual or implied endorsement of such products.

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