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Food and Chemical Toxicology 43 (2005) 1521–1539
www.elsevier.com/locate/foodchemtox

Toxicological evaluation of glycerin as a cigarette ingredient

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Received 11 November 2004; accepted 30 April 2005

Abstract

Glycerin is applied to cigarette tobacco at levels in the range of about 1–5% to improve moisture holding characteristics of tobacco and act as a surface active agent for flavor application. Neat material pyrolysis studies, smoke chemistry and biological activity studies (bacterial mutagenicity, cytotoxicity, in vivo micronucleus, and sub-chronic rodent inhalation) with mainstream smoke, or mainstream smoke preparations from cigarettes containing various target levels (5%, 10%, and 15%) of the glycerin were performed to provide data for an assessment of the use of glycerin as a cigarette tobacco ingredient. The actual levels of glycerin in the respective test cigarettes were determined to be 3.2%, 6.2% and 8.4% after cigarette production. At simulated tobacco burning temperatures up to 900 °C, neat glycerin did not pyrolyze extensively suggesting that glycerin would transfer intact to mainstream smoke (smoke was not analyzed for glycerin in this study). On a tar basis, nicotine in smoke was significantly decreased at 10% and 15% glycerin while water was increased at all addition levels. Addition of 10% or 15% glycerin also resulted in a statistically significant increase in acrolein (9%) and a decrease in acetaldehyde, propionaldehyde, aromatic amines, nitrogen oxides, tobacco specific nitrosamines, and phenols. Addition of 5% glycerin produced the same decrease in smoke constituents as the 10% and 15% groups but there was no concomitant increase in acrolein. Biological tests indicated no relevant differences in the genotoxic or cytotoxic potential of either mainstream smoke (or smoke preparations) from cigarettes with added glycerin compared to control cigarettes. Cigarette smoke atmosphere dilution, coupled with the lower nicotine delivery in the test cigarettes containing glycerin resulted in a lower nicotine delivery to the glycerin cigarette smoke exposed rats of the 90-day inhalation study. Smoke atmosphere acrolein was also reduced in a concentration-related manner. Incorporation of glycerin at target levels up to 15% did not produce any adverse effects in rats exposed for 90-days. The major observation in the study was a reduced biological activity of the smoke as indicated by a reduction in the severity and/or incidence of focal macrophage accumulation in the lungs and goblet cell hyperplasia/hypertrophy in the nose (level 1), and goblet cell staining depletion in the nose (level 1). The results of these studies with glycerin applied to cigarette tobacco suggest that adding glycerin to cigarette tobacco at typical use levels does not adversely alter the smoke chemistry or biological effects normally associated with exposure to mainstream cigarette smoke.

Abbreviations: °C, degrees centigrade; CAS, chemical abstract service; Cig, cigarette; CFR, Code of Federal Regulations; CPSC, US Consumer Product Safety Commission; CO, carbon monoxide; EC_{50}, concentration that reduces the number of viable cells by 50% compared to the vehicle control; DMSO, dimethyl sulfoxide; DMBA, 7,12-dimethylbenz(*a*)anthracene; FTC, Federal Trade Commission; GC, gas chromatograph; GRAS, generally recognized as safe; GSD, geometric standard deviation; HPLC, high performance liquid chromatography; IARC, International Agency for Research on Research; ISO, International Organization for Standardization; K, degrees Kelvin; MMAD, mass median aerodynamic diameter; MS, mass spectrometer; NBUA, *N*-nitrosodi-*n*-butylamine; NDMA, *N*-nitrosodimethylamine; NDEA, *N*-nitrosodiethylamine; NIST, National Institute of Standards and Technology; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NPI, *N*-nitrosopiperidine; NPRA, *N*-nitrosodi-*n*-propylamine; NPY, *N*-nitrosopyrrolidine; NRU, neutral red uptake; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocytes; SCOGS, Select Committee on GRAS substances; Tar, a calculated number determined by subtracting the weight of water and nicotine from the weight of TPM; TPM, total particulate matter (smoke mater collected on a filter pad); WBC, white blood cells.

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doi:10.1016/j.fct.2005.04.010

Keywords: Glycerin; Cigarette smoke; Inhalation; Mutagenicity; Smoke chemistry; Glycerol

1. Introduction

Glycerin (CAS # 56-81-5) is a trihydric alcohol. It is present naturally in foods, predominantly as the tri-acyl backbone of fats. There are four recognized names for glycerin, the other three being glycerol, 1,2,3-propanetriol and trihydroxypropane. The two most common names are glycerol and glycerin. Glycerin is widely used by the food, cosmetic and pharmaceutical industries, because it can serve many functions such as a humectant (moisture absorbing), plasticizer (softening), bodying agent, flavoring, denaturant, emollient (soothing), antimicrobial, thickener and solvent.

Glycerin is considered to have low acute oral toxicity. Studies have reported glycerin's oral LD_{50} values to be about 25 g/kg in rats (Kudo and Ito, 1972; Gerarde, 1959; Smyth et al., 1941). The most common non-lethal acute toxic effects of high level glycerin exposure include restlessness, tremors and hyperemia in the lung, kidney and small intestine of rat, mice, guinea pig and rabbit (Deichmann, 1941; Hine et al., 1953). These effects are elicited when glycerin is administered orally, subcutaneously, intraperitoneally or intravenously. Other acute toxic effects of high levels of glycerin observed in laboratory animals range from diarrhea and vomiting to ataxia, benign muscle weakness and inactivity, to hemoglobinuria. Hemoglobinuria only occurs when glycerin is administered intravenously (Deichmann, 1940). Short-term oral exposure to glycerin in the dose range of 10 g/kg results in hypoactivity and mild kidney effects. No overt sign of toxicity was observed in rats administered orally 0.75 g/kg for 3 days (Staples et al., 1967), 7 g/kg for 100 days (Haag and Ambrose, 1937), or 20 g/kg for 25 weeks (Johnson et al., 1933). Overt signs of toxicity were also absent in mice when administered 10 g/kg for 25 weeks in drinking water (Inayama et al., 1986; Kitamura et al., 1987). Growth was unaffected in rats fed 15 g/kg for 20 weeks (Whitlock et al., 1944).

The potential toxicity of inhaled aerosolized glycerin has been reported in three studies. Greenspan (1988) exposed rats to 1, 2 or 4 mg/l for 2 weeks. The major findings were reduced body weight gain in rats receiving 1 mg/l or more. Blood, food consumption and major organs (liver, kidney, heart, etc.) were unaffected by glycerin exposure. Renne (1992) studied the toxicity in 2-week and 13-week nose-only inhalation studies in Sprague-Dawley rats. In the 2 week study rats were exposed to aerosol concentrations of 0, 1.0, 1.9, and 3.9 mg/l of glycerin in air for 6 h/day, 5 days/week. Rats exposed to all three exposure concentrations had minimal to mild squamous metaplasia of the epithelial lining at the base of the epiglottis. In the 13-week study, rats were exposed to 0, 0.033, 0.167, and 0.662 mg glycerin/l of air for 6 h/day, 5 days/week. Minimal to mild squamous metaplasia of the epithelial lining at the base of the epiglottis was noted in the high exposure concentration group. The squamous metaplasia observed in a majority of rats from both studies indicated that exposure to aerosolized glycerin produced a mild irritant effect upon the laryngeal epithelium.

Glycerin is reported to be not mutagenic (Doolittle et al., 1988; Litton Bionetics, 1975), carcinogenic (Hine et al., 1953; Anonymous, 1969) nor produce adverse reproductive effects (Anonymous, 1982; Guerrant et al., 1947).

Glycerin has been given the status as a multipurpose "generally recognized as safe" (GRAS) food substance by the United States Food and Drug Administration (21 CFR 182.1320). There are no restrictions on functionalities or food category uses. It is also approved for the manufacture of various direct and indirect food additives (21 CFR 172.811, §172.830, §172.834, §172.850, §172.852, §172.861, and §172.866.) It was evaluated by the Select Committee on GRAS Substances who found no evidence of hazard from food use (SCOGS, 1975.) The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed glycerin as a food additive and concluded that food use does not represent a health hazard (Anonymous, 1987). In addition to food, the FDA has approved the use of glycerin in drug products. Specifically, the FDA has approved the use of glycerin, in topical over-the-counter drug products (§344.10); oral wound healing agents (§310.534); prevention of swimmer's ear, drying of water-clogged ears (§310.545); anorectal over-the-counter drug products (§346.14); ophthalmic over-the-counter drug products (§349.12), and anticaries over-the-counter drug products (§355.10 and §355.3).

Glycerin has been identified as a natural component of oriental tobacco (0.34–0.48%), flue-cured tobacccos (0.07–0.12%), and burley (0.23–0.31%) (Rodgman, 2002). It is used as a processing aid on tobacco as a humectant to prevent the tobacco leaves from becoming friable and thus crumbling during processing and may also be applied directly to the cured tobacco during cigarette manufacturing as a flavoring material or vehicle. In the cigarette, glycerin helps to retain moisture and prevents drying out of the tobacco. Glycerin is typically applied to tobacco at levels of 1–5%.

Because cigarette ingredients added to tobacco are potentially combusted during the smoking process, it is not possible to justify the use of cigarette ingredients based solely upon their approved use in foods or drugs. While there are no regulatory requirements for testing cigarette ingredients, in 1997, the United Kingdom

tobacco industry and the UK Department of Health reached a voluntary agreement on a testing approach for the approval and use of new ingredients in tobacco products (Secretary of State for Health, 1997). The approach suggested an evaluation of "potentially noxious components" (analysis of the constituents of smoke) and the use of biological studies such as genotoxicity and animal inhalation studies. We have developed an ingredient evaluation process that is based in part on the FDA Redbook (FDA, 2004) and guidance from the CPSC proposed testing program for low ignition propensity cigarettes (CPSC, 1993). The degree of testing is based upon the application level and the tests selected upon the sensitivity of the methods when applied to cigarette smoke, the ability of the methods to measure endpoints which are related to the health effects for which smoking is a risk factor, the likelihood of the methods to detect known adverse effects of cigarettes and recommended regulatory guidelines.

Previous studies have addressed various ingredients and mixtures of ingredients added to cigarettes (Baker et al., 2004a,b; Carmines, 2002; Gaworski et al., 1997, 1998, 1999; Heck et al., 2002; Stavanja et al., 2003). While some of these studies have indicated slight changes in the composition of the smoke from cigarettes containing ingredients, they have not suggested any relevant increases in the established biological activity of the smoke. As part of our continuing effort to assess cigarette ingredient use, we have evaluated certain ingredients on an individual basis. By testing individual ingredients we are able to use higher levels in the test cigarette than can be accomplished when mixtures of ingredients are tested thus increasing the probability that effects of the ingredient itself can be detected. The test cigarettes were designed to encompass representative use levels, as well as higher levels. While the use of multiple test levels provides the opportunity to potentially generate a dose–response in the effects, the maximum inclusion level is limited by the physical capability to make a cigarette which burns in a manner similar to the control cigarette (that is, the number of puffs and the amount of tar being approximately equivalent). This limitation restricts the highest level that can be tested. Routinely, one would like to test the potential application level and some multiples of the level to generate a dose–response and provide information for margins of exposure calculations. Since the glycerin was being tested as part of a toxic matrix (smoke), it is not possible to test at extreme inclusion levels of the glycerin without diluting the smoke and thus reducing the overall apparent toxicity. None-the-less, the use of exaggerated application levels does provide an opportunity to detect any new or different effects of the ingredients that might not be apparent at the lower, typical use levels.

The series of tests reported here were conducted to evaluate the potential effects of glycerin as a single ingredient in a test cigarette on the composition of smoke, to examine the potential genotoxic and cytotoxic effects of the smoke, and to evaluate the inhalation toxicity of the smoke in an animal model.

2. Materials and methods

2.1. Glycerin

Glycerin was purchased from a commercial supplier and was of food grade or greater purity.

2.2. Cigarette construction

Studies were conducted with research cigarettes prepared with components (cellulose acetate filters, papers and adhesives) and construction processes consistent with commercial American cigarette manufacturing. Tobacco blends comprised bright (35%), Burley (23%), oriental (15%) and reconstituted tobacco sheet (27%). No other ingredients were added to the tobacco of the control or test cigarettes with the exception of water. Cigarettes were 84 mm in length (57 mm tobacco rod, 27 mm filter) and 25 mm in circumference. The cellulose acetate filter contained 8% triacetin, with 30% ventilation. The cigarette paper was 100% flax and contained 0.6% potassium citrate. Adhesives were ethylene vinyl acetate based materials.

Test cigarettes contained target levels of 5%, 10%, or 15% glycerin added to the tobacco during processing. The low level of glycerin (5%) represents an application level typical of commercial cigarettes. The exaggerated higher levels (10% and 15%) were selected to maximize the potential to reveal a dose–response. Following dissolution of the glycerin in water, the solution was applied to the total tobacco blend which was subsequently conditioned, cut and processed through a rotary dryer to achieve the specified moisture level. The total cut blend was made into finished cigarettes to a specified tobacco weight and ventilation target on a standard cigarette-making machine. The tobacco used for the control cigarette was treated with an equal amount of the water. The average tobacco rod weights ($n = 3$) were 0.709 g, 0.728 g, 0.721 g and 0.726 g for the control, low, medium, and high glycerin test cigarettes, respectively. A University of Kentucky reference cigarette, 1R4F, was utilized in the studies as an internal reference to monitor study consistency (Diana and Vaught, 1997). This cigarette was designed to contain 2.8% glycerin.

2.3. Glycerin in tobacco analysis

The glycerin application solutions and the treated tobacco were analyzed for glycerin. Ethylene glycol was added to the glycerin solutions as an internal standard.

The solutions were diluted to volume with dry *n*-propanol. Samples were analyzed on a Hewlett Packard model 5890 gas chromatograph using a thermal conductivity detector. The tobacco was crushed and extracted with methanol and a sample of the extract injected on a Hewlett Packard model 6890 gas chromatograph with a flame ionization detector (Agilent Technologies, Palo Alto, CA).

2.4. Neat ingredient pyrolysis studies

Pyrolysis studies followed an approach similar to that of Baker and Bishop (2004) Neat samples of glycerin were pyrolyzed in air, using a Pyroprobe 2000 pyrolysis unit (CDS Analytical, Inc. Oxford, PA). The heating rate was programmed to raise the temperature from ambient up to about 900 °C in three stages: 400 °C for 10 s, 700 °C for 10 s, and 1000 °C for 10 s. The products of pyrolysis were swept out of the heating zone and condensed at 77 °K before injection into a Hewlett Packard 5890 Gas Chromatograph (DB-1701 column; Chrom Tech, Inc. Apple Valley, MN) with a Hewlett Packard 5973 mass selective Detector detector (Agilent Technologies, Palo Alto, CA). Pyrolysis products were identified based on their retention times and mass-spectral library comparisons (NIST 98 and Wiley mass spectral databases, Agilent Technologies, Palo Alto, CA).

2.5. Smoke composition

Analyses of smoke composition were conducted on preparations of mainstream smoke, gas vapor phase or particulate fractions collected during machine smoking to determine the potential effects of glycerin on toxicologically important constituents of smoke. The methods utilized have been previously described by Rustemeier et al. (2002). Briefly, cigarettes were conditioned and smoked in basic conformity with ISO standards (ISO 3308, ISO 3402). The mainstream smoke was generated using a 20-port Borgwaldt smoking machine (Borgwaldt-KC, Richmond, VA). Total particulate matter (TPM) or volatile gas phase components were collected using glass fiber filters or selective trapping/solvent systems. A total of 38 analytes (including the FTC analytes: TPM, nicotine, water and carbon monoxide) were determined in the smoke. The analytes (minus the FTC analytes) were selected based on two source documents: a proposal that specifically focused on smoke chemistry testing from the US Consumer Product Safety Commission (1993) and a monograph from the International Agency for Research on Cancer (1986). FTC analytes were determined according to ISO standards with only slight modifications (ISO 10362-2, ISO 8454, ISO 4387, ISO 10315). Mainstream smoke was not analyzed for glycerin as no validated method existed in the testing laboratory.

2.6. Bacterial mutagenicity

The bacterial mutagenic potential of cigarette smoke condensate preparations was evaluated using an assay method based on the microbial reverse mutation assay described by Maron and Ames (1983). The methods and statistical evaluation utilized have been previously described by Roemer et al. (2002). Briefly, mainstream smoke condensate was collected using an 30-port smoking machine (Borgwaldt-KC, Richmond, VA) equipped with a specially designed glass impaction device. Following collection, the particulate matter was diluted in DMSO and stored at −70 °C until use. Preparations were evaluated both with and without metabolic activation (Aroclor-induced rat liver S9). Five *Salmonella typhimurium* tester strains were used in the assay: TA98, TA100, TA1535, TA1537, and TA102 (OECD, 1997a). Within the assay, the genotype of the tester strains was confirmed, spontaneous revertants were measured, and response to positive controls was measured. The specific mutagenicity of the cigarette smoke condensate preparations was measured at a minimum of three non-toxic dose levels. The slopes of the regression lines were compared to determine the effect of the test glycerin on the mutagenic response of mainstream smoke condensate. Assays were conducted twice using two separate condensate collections (batches). Differences between mutagenic response, and the consistency of the response between the two assays were used as evaluation criteria.

2.7. In vitro cytotoxicity

The potential cytotoxic effects of smoke fractions were evaluated using the neutral red uptake assay (NRU) with mouse embryo BALB/c 3T3 cells (Borenfreund and Puerner, 1985). The methods and statistical evaluation utilized have been previously described by Roemer et al. (2002). Briefly, the TPM and the water-soluble fraction of the gas/vapor phase of mainstream smoke were collected using a 30-port smoking machine (Borgwaldt-KC, Richmond, VA) equipped with a glass fiber filter to collect the particulate phase and a glass bottle containing ice-cold phosphate buffered saline to collect the gas vapor phase passing through the filter. A reference cigarette, 1R4F, was included as an internal control. Following collection, the filters were extracted by shaking with dimethyl sulfoxide. Preparations were added to the in vitro cell cultures within 50 min (particulate) or 20 min (gas vapor phase). The cells were exposed for 24 h to the smoke fractions suspended/dissolved in culture medium. At the end of exposure, the culture medium containing the smoke fraction was replaced with culture medium containing neutral red. Following a 3-hour incubation period, the neutral red that was taken up by viable cells was extracted and

the optical density of the neutral red was determined photometrically at 540 nm. Three separate batches of particulate or gas vapor phase fractions were collected and assayed independently. For each assay, the unit of cytotoxicity ($1/EC_{50}$: the reciprocal concentration that reduces the number of viable cell by 50% compared to the vehicle control) was calculated.

2.8. Micronucleus genotoxicity

The potential clastogenic effect of diluted mainstream cigarette smoke was evaluated as part of the 90-day nose-only inhalation studies. The protocol followed the OECD guidelines for the mammalian erythrocyte micronucleus test (OECD, 1997b). Peripheral blood (retro-orbital sinus) was collected during exposure weeks 1, 5, 9 and 13 and evaluated using a flow cytometric approach (Litron Laboratories, Rochester, NY). At least 20,000 reticulocytes and up to 1,000,000 erythrocytes were analyzed for the presence of micronuclei. Bone marrow samples were collected from the femur at the terminal necropsy. Three smears per animal were prepared on glass slides and stained with acridine orange. Approximately 2000 polychromatic erythrocytes were scored for each animal. Cyclophosphamide (Sigma-Aldrich, St. Louis MO) was used as a positive control.

2.9. 90-Day inhalation

The biological response of inhaled mainstream smoke was evaluated in a 90-day subchronic inhalation study in rats. The general methods and statistical evaluations utilized have been previously described by Vanscheeuwijck et al. (2002) and followed OECD guidelines (OECD, 1981). Briefly, groups of 10 male and 10 female Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were exposed via nose-only inhalation for 6 h/day, 7 days/week, for 13 weeks to 150 mg TPM/m^3 mainstream smoke from cigarettes containing glycerin treated tobacco. For comparison, two control groups of rats were maintained; one exposed to smoke from a control cigarette prepared without glycerin, with another group sham exposed to air. A 1R4F cigarette smoke exposure group was included as an internal control. The smoke was produced on a 30-port automatic smoking machine (Borgwaldt-KC, Richmond, VA) set to deliver a 2 s 35 ml puff (FTC/ISO conditions). The sham air control, control cigarette and high level glycerin groups also contained 10 male and 10 female rats which were observed for 42 days following exposure to evaluate recovery. Groups of 6 rats/sex were also included in each exposure group for the periodic collection of blood for measurement of carboxyhemoglobin, nicotine and cotinine. The rats were randomized to each group using a body weight constraint process. During quarantine and non-exposure periods, the animals were double housed in polycarbonate "shoe box" cages (Lab Products, Inc., Maywood, NJ) with adsorbent hardwood chip beeding. Certified Rodent Diet 5002 (PMI Nutrition International, Inc., Brentwood, MO) was provided ad libitum except during inhalation exposures and scheduled fasting periods. During the exposure phase of the study, smoke was analyzed for TPM, CO, nicotine and aldehyde concentrations. Clinical observations, body weights, food consumption and pulmonary function (respiratory frequency and volume were determined by whole body plethysmography) were measured. At scheduled necropsies, blood was collected for clinical pathology measurements, major organs were weighed and tissues were collected for histopathological evaluation. Histological sections were prepared for the nose according to Young (Young, 1981), and for the larynx according to Lewis (Lewis, 1981).

2.10. Data presentation and statistical evaluation

Smoking machines are used to create a consistent cigarette smoke for research purposes. The FTC and ISO have set forth conditions under which smoking machines should be operated. These conditions include the volume of the puff (35 ml), the duration of the puff (2 s) and the puff interval (once per minute). These machine methods were not intended to reflect what and how smokers actually inhale. In 1967 when the FTC announced the completion of its trial tests of the current method, it stated that "[n]o test can precisely duplicate conditions of actual human smoking and, within fairly wide limits, no one method can be said to be either 'right' or 'wrong' ... the purpose of testing is not to determine the amount of tar and nicotine inhaled by any human smoker, but rather to determine the amount of tar and nicotine generated when a cigarette is smoked by machine in accordance with the prescribed method (FTC, 1967)."

The general unit of exposure for the biological assays reported here with mainstream cigarette smoke is TPM. Smoke chemistry is generally measured on a per cigarette basis. However, since the addition of glycerin altered the yield of the test cigarettes by increasing the amount of water in the smoke (see Table 2), the smoke chemistry and in vitro studies have been normalized on a tar[1] basis to show the effect without the change in water content. For each ingredient group, the results of the control, low, medium, and the high ingredient level were compared. For continuous data, the one-way analysis of variance was used for the overall comparison followed by a post hoc pair wise comparison test. Results were evaluated for statistical significance at $p \leqslant 0.05$ without

[1] Tar is determined mathematically by subtracting the amount of water and nicotine from the amount of TPM.

adjustment for multiple testing. No statistical analysis was performed on the histopathology data. A difference from control of one severity unit was used as a guide in the evaluation of the histopathological data.

3. Results

3.1. Glycerin analysis

Table 1 shows the target and measured amounts of the glycerin evaluated at each step in the production process. Each group of analyses was performed separately. In the first step, solutions of glycerin were made up and analyzed to assure that the proper amount of glycerin would be applied to the tobacco. The solutions were made up at different concentrations so that a constant volume of solution would be applied to each lot of tobacco. The solution analysis indicated that the solutions were made properly. In the second step of the manufacturing process, the blended tobacco was sprayed with the glycerin solution. Approximately 500 kg of tobacco were treated in a continuous process and then dried to a constant moisture content. This was done under normal manufacturing conditions. The analytical results indicated that there were some normal losses of material during this step. The tobacco was then processed further, cut into shreds and made into cigarettes. Analysis of the tobacco after the cigarettes had been made also indicated that there had been some loss of glycerin. The test cigarettes were reanalyzed after completion of the studies to assure that there was no substantial loss of glycerin during the testing program. The analytical results indicated the glycerin levels were stable over the testing period. Although there were differences between target and measured results during the production process, the results were considered to be due to normal manufacturing variations and losses and to be representative of normal manufacturing losses.

3.2. Pyrolysis studies

Combustion of tobacco has been reported to yield approximately 4000 chemicals (Hoffmann and Hoffmann, 1997). Neat glycerin was pyrolyzed in air to determine any potential combustion products. Fig. 1 shows the total ion chromatogram and the identities of the chemicals detected. Under the conditions of this test, glycerin did not pyrolyze extensively, suggesting that glycerin would be expected to transfer intact to the smoke. Acrolein and glycolaldehyde appeared to be minor pyrolysis products produced under these test conditions.

3.3. Smoke composition

The individual results of smoke composition studies are listed in Table 2 (cigarette basis) and in the radar chart in Fig. 2 (percentage in tar relative to the control cigarette). On a cigarette basis, addition of glycerin significantly increased the amount of tar and water in the smoke while decreasing the amount of nicotine. Glycerin is a humectant and is expected to add water to the tobacco and smoke and it also transfers to the smoke itself. The net affect of addition of glycerin is therefore to dilute the smoke with water and glycerin. As a result, most of the smoke constituents decrease when measured on a cigarette basis. The data in Fig. 2 were normalized on a tar basis to account for the effect of dilution by water. On a tar basis, nicotine was significantly decreased at 10% and 15% glycerin while water was increased at all addition levels. Since the cigarettes were manufactured to an approximate constant tobacco weight, as glycerin was added, the amount of tobacco was decreased. The reduction in nicotine in the smoke on a tar basis is therefore thought to be simply due to the reduction in the amount of tobacco in the cigarette. Addition of 10% or 15% glycerin resulted in a statistically significant increase in acrolein and a decrease in acetaldehyde, propionaldehyde, aromatic amines, nitrogen oxides, tobacco specific nitrosamines, and phenols. Addition of glycerin at the 5% level also reduced many smoke constituent yields, but there was no increase in acrolein.

3.4. Bacterial mutagenicity

In general, cigarette smoke condensate is mutagenic in certain Salmonella strains. A clear mutagenic

Table 1
Glycerin content in prepared test samples

Glycerin group	Glycerin application solution		Glycerin levels (μg/g tobacco)[a]			
	Target levels (g/ml)	Measured levels (g/ml)[a]	Target (%)	In tobacco before cigarette production	In cigarettes after cigarette production	In cigarettes after study completion
Control	0	ND[b]	0 (0)	<2500	<2500	<2500
Low	0.394	0.430	50,000 (5)	43,400	32,200	31,500
Medium	0.825	0.892	100,000 (10)	84,100	62,200	63,800
High	1.294	1.184	150,000 (15)	113,700	84,200	88,000

[a] Measured values represent the mean of two analyses.
[b] Not determined.

Table 2
Mainstream smoke constituent concentrations (per cig) in control and test cigarettes containing glycerin

	Control	Low	Medium	High	1R4F
FTC parameters					
TPM (mg/cig)	8.97 ± 0.14[a]	9.67 ± 0.25*	10.08 ± 0.20*	10.48 ± 0.26*	10.73 ± 0.22
Tar (mg/cig)	7.48 ± 0.16	7.84 ± 0.19*	8.16 ± 0.17*	8.39 ± 0.18*	8.73 ± 0.15
Nicotine (mg/cig)	0.780 ± 0.013	0.782 ± 0.021	0.694 ± 0.051*	0.665 ± 0.019*	0.808 ± 0.022
Water (mg/cig)	0.71 ± 0.07	1.04 ± 0.047*	1.23 ± 0.084*	1.43 ± 0.067*	1.19 ± 0.07
Carbon monoxide (mg/cig)	11.1 ± 0.4	11.7 ± 0.7	11.5 ± 0.4	11.3 ± 1.2	12.8 ± 1.0
Aliphatic hydrocarbons					
1,3-Butadiene (μg/cig)	34.2 ± 2.5	35.7 ± 5.4	37.4 ± 0.8	35.5 ± 2.6	45.0 ± 1.6
Isoprene (μg/cig)	312 ± 16	323 ± 41.3	343 ± 10	318 ± 19	384 ± 16
Aldehydes					
Formaldehyde (μg/cig)	21.2 ± 0.8	19.7 ± 0.9*	19.6 ± 0.1*	18.7 ± 0.2*	27.0 ± 1.3
Acetaldehyde (μg/cig)	572 ± 22	546 ± 16	554 ± 6.4	541 ± 14.2	701 ± 24
Acrolein (μg/cig)	56.1 ± 2.8	59.9 ± 2.9	66.6 ± 1.0*	68.8 ± 1.8*	70.8 ± 2.3
Propionaldehyde (μg/cig)	53.4 ± 2.3	50.0 ± 2.4	51.5 ± 0.7	50.5 ± 1.5	64.8 ± 2.9
Aliphatic nitrogen compounds					
Acrylonitrile (μg/cig)	7.88 ± 0.38	8.95 ± 1.39	8.61 ± 1.09	7.39 ± 0.76	10.70 ± 0.47
Hydrogen cyanide (μg/cig)	86.2 ± 3.9	93.7 ± 6.3	94.9 ± 6.5	99.7 ± 7.5	121 ± 9.4
2-Nitropropane (ng/cig)	18.8 ± 1.1	13.5 ± 1.0*	10.0 ± 1.1*	12.2 ± 0.7*	24.0 ± 1.3
Aromatic amines					
o-Toluidine (ng/cig)	31.7 ± 2.2	33.8 ± 2.0	25.4 ± 2.7*	24.6 ± 1.2*	29.8 ± 0.8
2-Naphthylamine (ng/cig)	5.31 ± 0.22	4.82 ± 0.10	4.01 ± 0.28*	3.84 ± 0.40*	4.07 ± 0.16
4-Aminobiphenyl (ng/cig)	1.15 ± 0.066	1.06 ± 0.055	0.970 ± 0.10	0.929 ± 0.13*	0.986 ± 0.075
o-Anisidine (ng/cig)	1.66 ± 0.18	1.63 ± 0.19	1.18 ± 0.31*	1.25 ± 0.13*	1.56 ± 0.26
Inorganic compounds					
Nitrogen oxides (mg/cig)	0.273 ± 0.018	0.217 ± 0.02*	0.185 ± 0.01*	0.230 ± 0.02*	0.349 ± 0.0087
Monocyclic aromatic compounds					
Benzene (μg/cig)	36.3 ± 1.5	40.1 ± 5.6	40.7 ± 3.4	38.0 ± 2.5	44.1 ± 1.1
Toluene (μg/cig)	61.1 ± 1.5	75.9 ± 9.5	73.0 ± 10.2	71.1 ± 7.2	76.0 ± 2.3
N-nitrosamines					
NDMA (ng/cig)	2.91 ± 0.30	2.56 ± 0.38	<2.4	<2.4	4.02 ± 0.43
NDEA (ng/cig)	<1.45[b]	<1.45	<1.45	<1.45	<1.45
NPRA (ng/cig)	<3.84	<3.84	<3.84	<3.84	<3.84
NBUA (ng/cig)	<1.49	<1.49	<1.49	<1.49	<1.49
NPY (ng/cig)	9.42 ± 0.91	6.94 ± 0.66*	5.13 ± 0.35*	5.01 ± 0.32*	12.35 ± 1.08
NPI (ng/cig)	<1.88	<1.88	<1.88	<1.88	<1.88
NNN (ng/cig)	99.8 ± 5.2	87.1 ± 2.7*	71.4 ± 2.9*	68.7 ± 0.9*	80.1 ± 2.7
NNK (ng/cig)	86.5 ± 4.0	83.3 ± 4.2	69.3 ± 1.6*	67.1 ± 2.9*	90.1 ± 3.7
Phenols					
Phenol (μg/cig)	16.08 ± 1.87	7.66 ± 0.57*	8.22 ± 0.50*	4.36 ± 0.32*	12.03 ± 0.31
Catechol (μg/cig)	45.33 ± 3.35	36.28 ± 3.29*	32.83 ± 1.55*	26.95 ± 0.21*	42.48 ± 0.43
Polycyclic aza-arenes					
Dibenz(*a*,*j*)acridine (ng/cig)	<5.0	<5.0	<5.0	<5.0	<5.0
Polycyclic aromatic hydrocarbons					
Benzo(*a*)anthracene (ng/cig)	9.22 ± 0.46	9.46 ± 0.09	9.34 ± 0.76	9.34 ± 0.77	8.57 ± 0.37
Benzo(*b*) fluoranthene (ng/cig)	7.28 ± 0.46	7.02 ± 0.15	7.42 ± 1.07	7.57 ± 0.89	6.63 ± 0.37
Benzo(*a*)pyrene (ng/cig)	5.17 ± 0.32	4.99 ± 0.05	5.06 ± 0.74	5.07 ± 0.55	4.81 ± 0.29
Indeno(1,2,3-*cd*)pyrene (ng/cig)	3.20 ± 0.07	2.63 ± 0.11*	2.72 ± 0.39	3.28 ± 0.40	2.99 ± 0.20
Dibenz(*a*,*h*)anthracene (ng/cig)	<5.0	<5.0	<5.0	<5.0	<5.0
5-Methylchrysene (ng/cig)	<10.0	<10.0	<10.0	<10.0	<10.0
Puff count (puffs/cig)	8.6 ± 0.13	8.5 ± 0.13	8.7 ± 0.18	8.4 ± 0.17	9.2 ± 0.08

Target glycerin levels were: low = 5%, medium = 10% and high = 15%.
* Significantly different from the respective control cigarette $p \leqslant 0.05$.
[a] Mean ± standard deviation, $n = 4$ determinations per analyte with 4–20 cigarettes being smoked per determination.
[b] Values listed with a < were below the level of quantification.

response was obtained with S9 in strains TA98, TA100, and TA1537 for all control and test cigarettes types (Table 3). No (or only borderline) responses were seen in strains TA102 and TA1535. In the bacterial strains

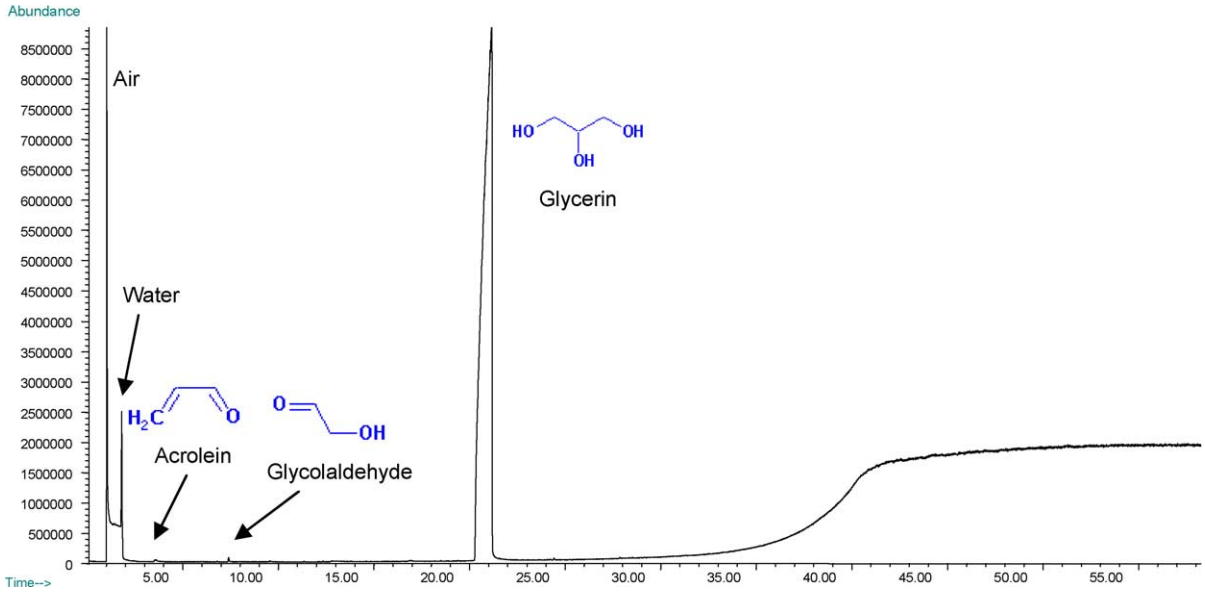

Fig. 1. Total ion chromatogram after pyrolysis of glycerin in air up to 900 °C.

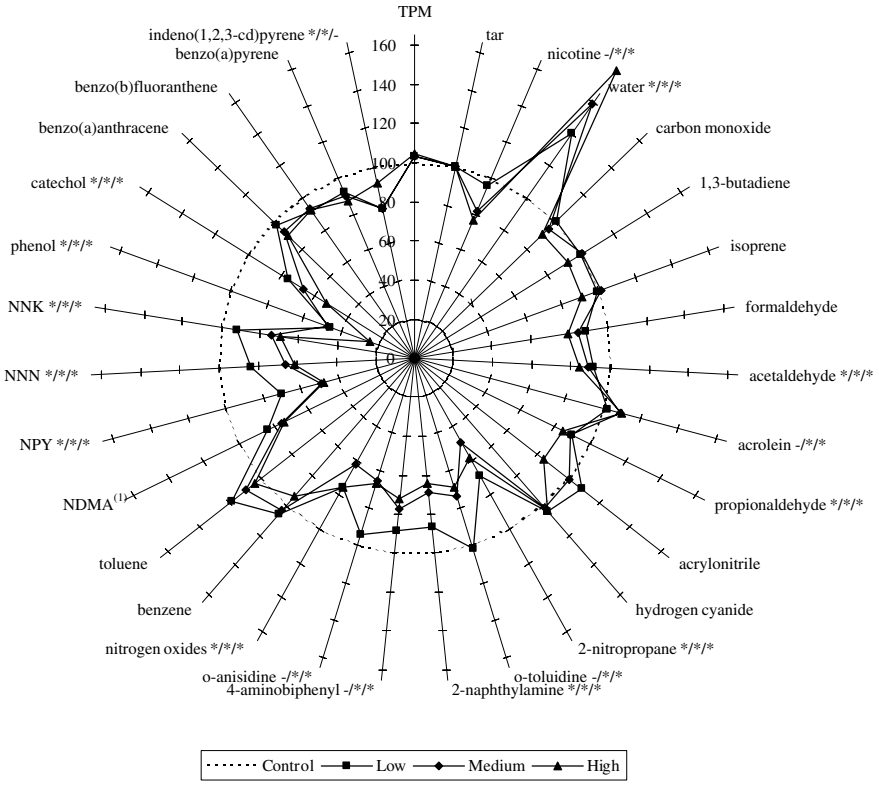

Fig. 2. Relative changes in smoke chemical constituents of glycerin test cigarettes. Data were calculated and compared on an equal tar basis relative to the control cigarette. Target glycerin levels were: low = 5%, medium = 10% and high = 15%. Symbols for the test cigarettes represent mean increases or decreases compared to control samples. For clarity, sample variations have not been included, but are shown in the individual chemistry report. * = Significantly different from control ($p \leqslant 0.5$) are indicated for low/medium/high levels. (1) Medium and high cigarette results below LOQ, plotted as LOQ.

tested, the specific mutagenicity of the smoke condensate obtained from cigarettes prepared with glycerin added to tobacco was not significantly increased from that of the respective control cigarettes.

Table 3
Mutagenic activity of mainstream smoke condensate from control and test cigarettes containing glycerin

S9	Group (target % glycerin)	Bacterial strain specific mutagenicity (per mg Tar)[a]				
		TA98	TA100	TA102	TA1535	TA1537
Yes	Control	4466 ± 680.2	1134 ± 334.5	153 ± 36.1	1 ± 5.7	221 ± 234.1
	Low (5%)	4539 ± 411.5	972 ± 564.3	48 ± 263.8	−1 ± 2.8	437 ± 75.0
	Medium (10%)	4372 ± 1062.1	1456 ± 1120.8	94 ± 97.6	−5 ± 1.4	342 ± 2.8
	High (15%)	3986 ± 182.4	949 ± 101.8	55 ± 92.6	−3 ± 2.8	425 ± 123.0
	1R4F	4538 ± 413.0	1514 ± 364.2	125 ± 1.4	2 ± 2.1	322 ± 149.9
No	Control	17 ± 5.7	135 ± 70.0	41 ± 34.6	3 ± 3.5	9 ± 0.0
	Low (5%)	8 ± 7.1	85 ± 0.7	20 ± 33.9	5 ± 0.7	4 ± 2.1
	Medium (10%)	11 ± 0.7	85 ± 16.3	42 ± 68.6	8 ± 2.1	6 ± 7.1
	High (15%)	25 ± 6.3	66 ± 119.5	8 ± 2.1	5 ± 9.2	6 ± 2.8
	1R4F	30 ± 12.7	279 ± 191.6	6 ± 7.1	1 ± 4.2	14 ± 2.1

[a] Specific mutagenicity (regression coefficient) calculated from approximately linear part of the dose–response curve using Poisson weights, mean ± SD; $n = 2$ independent assays with three plates at three different doses of condensate.

Table 4
Cytotoxicity of mainstream smoke from control cigarettes or cigarettes containing glycerin

Group (target % glycerin)	$(1/EC_{50})$/mg Tar	
	Particulate phase	Gas vapor phase
Control	4.35 ± 0.15[a]	6.16 ± 0.55
Low (5%)	4.92 ± 0.29	5.21 ± 0.19
Medium (10%)	4.30 ± 0.08	5.19 ± 2.28
High (15%)	3.67 ± 0.14	4.00 ± 0.22
1R4F	3.37 ± 0.02	4.76 ± 0.34

[a] Mean ± SE, $n = 2$ independent batch collections.

3.5. In vitro cytotoxicity

In the cytotoxicity assay, a dose-related decrease in the number viable cells was seen for all smoke fractions (i.e., cigarette smoke fractions are cytotoxic) (Table 4). No statistically significant differences were observed between the test and control cigarettes regardless of the mainstream smoke fraction tested.

3.6. Micronucleus genotoxicity

Mainstream smoke from cigarettes made with glycerin did not produce micronuclei in circulating or bone immature erythrocytes of rats during or after 90 days of inhalation exposure (Table 5). Cyclophosphamide, the positive control, elicited a positive response at all evaluation time points.

3.7. Subchronic smoke inhalation

Table 6 summarizes the exposure atmosphere conditions for the inhalation study. The overall mean TPM concentrations for the glycerin test cigarette

Table 5
Micronuclei frequency in rats exposed to air (Sham) or 150 mg/m^3 mainstream smoke from reference, control, or test cigarettes containing glycerin

Group (target % glycerin)	Sex	% Micronucleated erythrocytes[a]				% Micronucleated PCE[a]
		Week 1	Week 5	Week 9	Week 13	Terminal
Control	M	0.11 ± 0.041	0.14 ± 0.053	0.12 ± 0.047	0.13 ± 0.051	0.15 ± 0.089
Low (5%)	M	0.09 ± 0.039	0.16 ± 0.045	0.11 ± 0.034	0.08 ± 0.024	0.26 ± 0.129
Medium (10%)	M	0.10 ± 0.037	0.16 ± 0.048	0.12 ± 0.030	0.12 ± 0.031	0.21 ± 0.111
High (15%)	M	0.10 ± 0.026	0.20 ± 0.054	0.12 ± 0.035	0.13 ± 0.051	0.23 ± 0.052
1R4F	M	0.11 ± 0.040	0.15 ± 0.031	0.16 ± 0.047	0.16 ± 0.049	0.17 ± 0.068
Sham	M	0.10 ± 0.022	0.12 ± 0.040	0.11 ± 0.042	0.14 ± 0.016	0.33 ± 0.189
Positive Control	M	1.52 ± 0.491[b]	–[c] ± –	2.76 ± 0.679[b]	2.83 ± 0.610[b]	2.18 ± 0.473[d]
Control	F	0.10 ± 0.027	0.12 ± 0.026	0.14 ± 0.046	0.17 ± 0.054	0.21 ± 0.136
Low (5%)	F	0.13 ± 0.026*	0.20 ± 0.054*	0.15 ± 0.045	0.17 ± 0.052	0.23 ± 0.103
Medium (10%)	F	0.12 ± 0.028	0.19 ± 0.028	0.12 ± 0.023	0.16 ± 0.061	0.28 ± 0.147
High (15%)	F	0.09 ± 0.016	0.14 ± 0.059	0.14 ± 0.037	0.14 ± 0.043	0.32 ± 0.125
1R4F	F	0.13 ± 0.032	0.12 ± 0.022	0.13 ± 0.014	0.16 ± 0.041	0.31 ± 0.107
Sham	F	0.11 ± 0.033	0.09 ± 0.038	0.10 ± 0.022	0.12 ± 0.033	0.14 ± 0.086

* Statistically significant different from control ($p \leqslant 0.05$).
[a] Mean ± SD; $n = 5$ or 6 rats/sample.
[b] Animals were injected intraperitoneally with cyclophosphamide (15 mg/kg) 24 h prior to blood collection.
[c] % Micronucleated reticulocytes not calculated due to severe stem cell toxicity.
[d] Animals were injected intravenously with cyclophosphamide (30 mg/kg) 24 h prior to necropsy.

Table 6
Smoke atmosphere characteristics measured in the 90-day nose-only inhalation study

Group (target % glycerin)	TPM (mg/m^3) (n = 94)	CO (ppm) (n = 94)	Nicotine (mg/m^3) (n = 49–50)	Acrolein (mg/m^3) (n = 11–13)	MMAD (μm) (n = 2)	GSD (n = 2)
Control	144 ± 6.4[a]	159 ± 10.1	10.4 ± 0.95	1.13 ± 0.174	0.29	1.93
Low (5%)	149 ± 7.2	159 ± 9.5	10.4 ± 1.45	1.20 ± 0.332	0.30	2.06
Medium (10%)	153 ± 8.5	156 ± 10.5	8.2 ± 1.28	1.24 ± 0.108	0.28	1.98
High (15%)	155 ± 8.5	146 ± 10.3	8.1 ± 1.08	0.92 ± 0.326	0.27	1.98
1R4F	147 ± 8.9	176 ± 11.1	8.9 ± 1.93	1.11 ± 0.344	0.28	2.25

[a] Mean ± SD.

atmospheres were within ~3% of the target concentration of 150 mg TPM/m^3. The overall mean nicotine concentrations for the test cigarette atmospheres were 8.1–10.4 mg/m^3, while the overall mean CO concentrations were 146–159 ppm. The particle size distribution was within the rat respirable range with mean MMADs of 0.27–0.30 μm and mean GSDs of 1.93–2.06. The study design was to expose rats to a constant target concentration of 150 mg TPM/m^3. The smoke generation machines used in this study produce more smoke than is required to perform rat inhalation studies at a constant 150 mg TPM/m^3. Excess smoke is diverted to the inhalation chamber exhaust system. The remaining smoke is diluted to the desired exposure concentration. Since the glycerin cigarettes produced more TPM than the control cigarettes (8%, 12% and 17% in the low, medium, and high glycerin groups, respectively), it was necessary to dilute the smoke with air at slightly different ratios to maintain the constant TPM delivery. This necessary dilution of the exposure atmospheres, coupled with the lower nicotine delivery in the medium and high glycerin test cigarettes (see Table 2), resulted in a lower

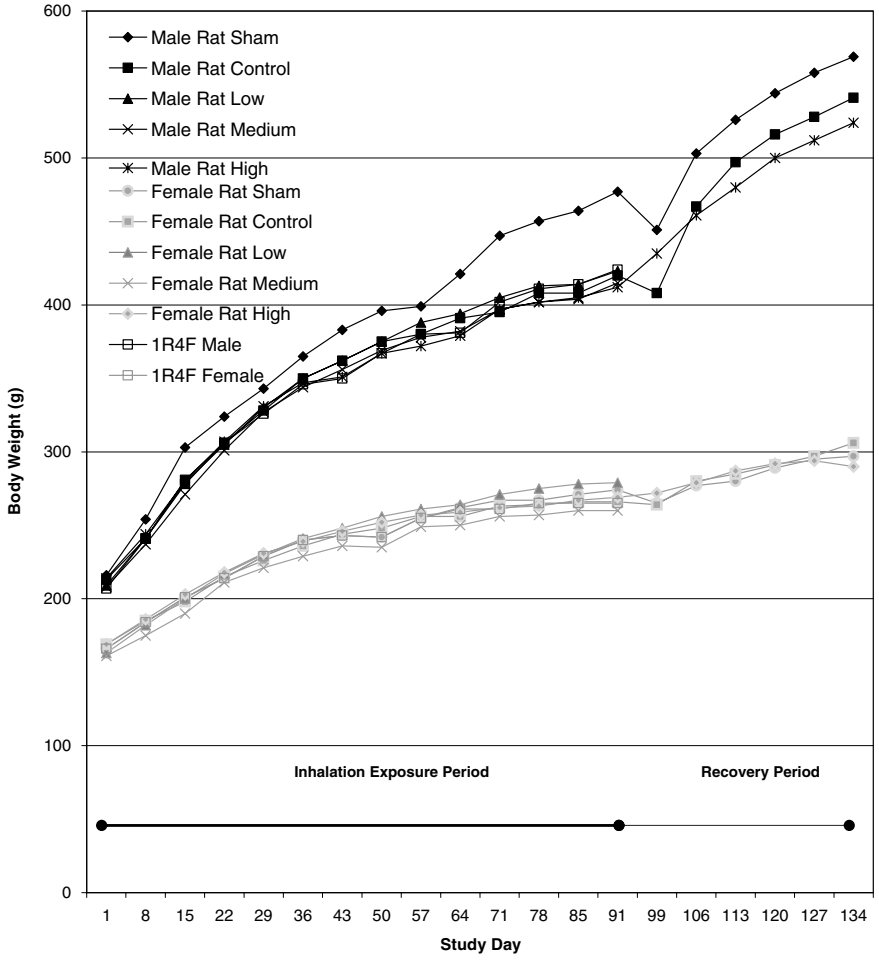

Fig. 3. Mean weekly body weights for glycerin cigarette smoke exposed rats.

nicotine delivery to the glycerin cigarette smoke exposed animals. Acrolein delivery was also reduced.

Exposure to the control or test cigarettes did not result in any smoke-related mortality during the study, nor were there any clinical observations related to glycerin smoke exposure (data not shown). Consistent with previous cigarette smoke inhalation studies (Vanscheeuwijck et al., 2002), male rats in all smoke exposed groups exhibited decreases in mean weekly body weight gains during the exposure (Fig. 3) when compared to the sham control. However, no glycerin-related body weight differences were seen between any of the test cigarette groups and the control cigarette group for either sex. Mean weekly food consumption values were generally comparable among the cigarette smoke exposed groups (Fig. 4). Consistent with the increased body weight gain, the male sham treated rats consumed more food than the smoke exposed animals. Serum nicotine and cotinine levels were determined periodically during the study (Table 7). There was a clear pattern of reduced serum levels of both biomarkers in the medium and high glycerin groups when compared to the control cigarette exposed animals through out the study. This is consistent with the reduced delivery of nicotine in the exposure atmospheres of these groups (Table 6). Steady state carboxyhemoglobin concentration in the blood was not affected by inclusion of glycerin when compared to the control group (Table 8). This is consistent with the relatively constant CO concentrations in the exposure chambers (Table 6). Pulmonary function measurements did not reveal any differences between the test cigarette and control cigarette groups (Table 8).

Clinical pathology results obtained either at the end of the smoke exposure period or at the end of the recovery period were unremarkable, with the possible exception of a statistically significant increase in white blood cell and lymphocyte counts in male rats from all glycerin cigarette groups at the end of exposure (Table 9). In a previous study (Vanscheeuwijck et al., 2002) we also found a non-statistically significant increase in leukocyte and lymphocyte count in male rats when cigarettes containing glycerin (along with other ingredients) were tested. This effect was considered to be of little biological relevance since it was only apparent in males, did not appear to be in a dose-related fashion related to glycerin concentration, was not apparent at the end of the recovery period and did not appear to have a histological correlate. Organ weights were measured at the end of the 90 day exposure (Table 10) and the recovery period (data not shown). Exposure to smoke (comparing all of the smoke exposed animals to the sham animals) produced an increase in adrenal weights, lung weight, ovary weights, and a decrease in thymus weights. Inclusion of 15% glycerin produced an increase in the absolute

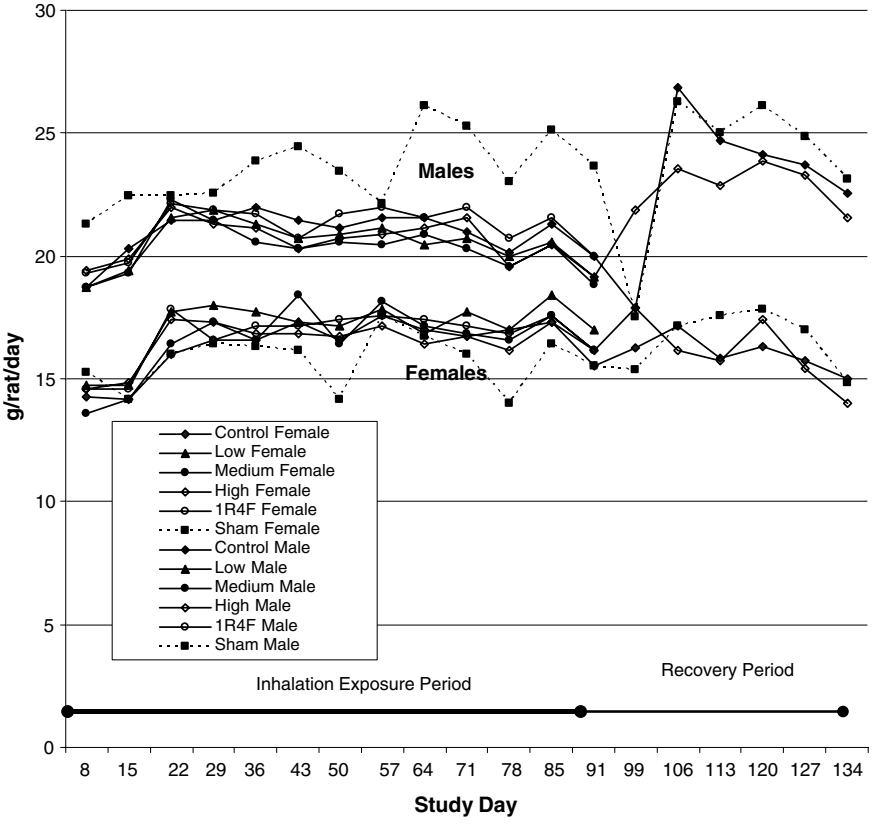

Fig. 4. Food consumption for cigarette glycerin smoke exposed rats.

Table 7
Biomarkers of exposure from rats exposed to air (sham) or 150 mg/m^3 mainstream smoke from reference, control, or test cigarettes containing glycerin

Group (target % glycerin)	Sex	Analyte	Serum levels (ng/ml)			
			Week 1	Week 5	Week 9	Week 13
Control	M	Nicotine	129.3 ± 24.18[a]	190.5 ± 23.83	193.9 ± 18.51	180.3 ± 97.65
		Cotinine	475.2 ± 77.58	510.1 ± 90.26	620.6 ± 116.39	464.9 ± 54.10
Low (5%)	M	Nicotine	133.0 ± 37.39	159.0 ± 9.78	221.8 ± 59.47	144.2 ± 23.72
		Cotinine	380.8 ± 35.42*	362.8 ± 37.46*	491.5 ± 78.8	394.8 ± 35.71
Medium (10%)	M	Nicotine	89.0 ± 29.92	143.4 ± 38.20*	169.8 ± 41.79	95.4 ± 21.20
		Cotinine	343.3 ± 57.50*	378.6 ± 56.26*	474.3 ± 75.25*	331.8 ± 45.54*
High (15%)	M	Nicotine	92.0 ± 19.92	126.5 ± 15.33*	128.4 ± 38.17*	133.3 ± 25.51
		Cotinine	355.1 ± 30.21*	330.2 ± 33.47*	397.9 ± 50.56*	384.3 ± 64.26*
1R4F	M	Nicotine	121.6 ± 27.83	182.2 ± 52.44	190.9 ± 67.02	163.3 ± 45.4
		Cotinine	441.4 ± 60.07	439.6 ± 72.83	509.0 ± 90.11	451.8 ± 85.49
Sham	M	Nicotine	0.1 ± 0.24	1.2 ± 0.40	0.6 ± 0.53	0.3 ± 0.63
		Cotinine	1.8 ± 0.59	5.3 ± 0.93	2.0 ± 0.40	2.1 ± 0.57
Control	F	Nicotine	180.3 ± 23.51	291.2 ± 74.85	311.8 ± 90.98	186.1 ± 44.36
		Cotinine	550.4 ± 42.26	525.8 ± 87.75	526.9 ± 59.75	492.0 ± 55.53
Low (5%)	F	Nicotine	138.6 ± 33.09*	258.3 ± 82.97	306.3 ± 63.26	216.3 ± 48.79
		Cotinine	451.2 ± 58.69*	452.0 ± 80.99	439.3 ± 58.57*	469.8 ± 117.87
Medium (10%)	F	Nicotine	131.0 ± 16.92*	202.1 ± 43.01	243.8 ± 39.25	174.5 ± 52.74
		Cotinine	442.3 ± 40.50*	462.3 ± 92.01	530.9 ± 42.61	473.3 ± 67.95
High (15%)	F	Nicotine	128.2 ± 28.02*	143.0 ± 39.19*	166.3 ± 33.76*	194.6 ± 64.89
		Cotinine	439.5 ± 48.62*	422.5 ± 77.18	412.1 ± 61.06*	468.5 ± 96.62
1R4F	F	Nicotine	145.6 ± 41.00	215.1 ± 39.51	253.3 ± 27.02	159.2 ± 7.35
		Cotinine	405.0 ± 65.47	443.9 ± 40.86	478.7 ± 60.53	469.2 ± 34.15
Sham	F	Nicotine	0.9 ± 1.26	3.4 ± 3.56	2.5 ± 2.24	0.4 ± 0.71
		Cotinine	2.5 ± 0.41	5.5 ± 0.58	2.0 ± 0.21	2.2 ± 0.23

* Statistically significant different from control ($p \leqslant 0.05$).
[a] Mean ± SD; $n = 5$–6.

Table 8
Respiratory physiology and carboxyhemoglobin after 5 weeks of exposure to air (sham) or 150 mg/m^3 mainstream smoke from reference, control, or test cigarettes containing glycerin

Group (target % glycerin)	Sex	COHb (%)[a]	Respiratory rate (breaths/min)[b]	Tidal volume (ml/breath)	Minute volume (ml/min)
Control	M	15.3 ± 1.30	180 ± 22.3	1.4 ± 0.62	264 ± 131.2
Low (5%)	M	15.8 ± 0.81	174 ± 28.1	1.0 ± 0.52	178 ± 67.9
Medium (10%)	M	16.0 ± 0.44	191 ± 17.3	1.1 ± 0.25	225 ± 38.7
High (15%)	M	15.1 ± 0.51	191 ± 11.2	1.2 ± 0.29	238 ± 51.1
1R4F	M	18.1 ± 1.52	189 ± 18.8	1.1 ± 0.52	206 ± 79.9
Sham	M	0.4 ± 0.43	201 ± 10.6	1.5 ± 0.45	315 ± 74.0
Control	F	15.4 ± 1.21	179 ± 10.5	1.2 ± 0.36	226 ± 67.9
Low (5%)	F	15.0 ± 1.70	184 ± 32.0	1.5 ± 0.49	283 ± 113.6
Medium (10%)	F	14.8 ± 0.74	186 ± 16.6	1.1 ± 0.37	217 ± 63.6
High (15%)	F	14.9 ± 1.35	163 ± 26.6	1.2 ± 0.33	213 ± 77.8
1R4F	F	18.0 ± 1.12	160 ± 26.6	1.1 ± 0.28	163 ± 47.8
Sham	F	0.3 ± 0.23	167 ± 13.3	1.4 ± 0.63	234 ± 98.2

[a] Mean ± SD; $n = 5$–6.
[b] Mean ± SD; $n = 4$–5.

thymus weight of the female rats when compared to controls. Glycerin appeared to reduce the thymus atrophy and move the organ weight in the direction of the sham control. Exposure to the glycerin test cigarette smoke

Table 9
Significant hematological data from rats exposed to air (sham) or 150 mg/m^3 mainstream smoke from reference, control, or test cigarettes containing glycerin

Group (target % glycerin)	Sex	Period	Hematological data	
			WBC (thsn/cmm)	Lymphocytes (thsn/cmm)
Control	M	Terminal	11.1 ± 2.14[a]	8.6 ± 2.08
		Recovery	13.7 ± 2.55	10.8 ± 1.83
Low (5%)	M	Terminal	13.3 ± 1.58*	10.6 ± 1.79*
Medium (10%)	M	Terminal	14.1 ± 2.19*	11.4 ± 1.57*
High (15%)	M	Terminal	13.2 ± 1.49*	11.1 ± 1.13*
		Recovery	11.4 ± 2.68	8.8 ± 2.65
1R4F	M	Terminal	13.4 ± 2.76	10.7 ± 2.80
Sham	M	Terminal	11.5 ± 2.71	9.4 ± 2.62
		Recovery	15.3 ± 4.78	10.5 ± 3.72
Control	F	Terminal	11.1 ± 2.19	8.9 ± 2.14
		Recovery	9.9 ± 3.11	8.3 ± 3.13
Low (5%)	F	Terminal	12.3 ± 3.21	10.1 ± 2.78
Medium (10%)	F	Terminal	9.9 ± 2.28	8.4 ± 2.38
High (15%)	F	Terminal	10.9 ± 2.86	9.1 ± 3.14
		Recovery	10.0 ± 3.62	8.4 ± 3.43
1R4F	F	Terminal	10.4 ± 2.42	8.5 ± 2.68
Sham	F	Nicotine	10.4 ± 2.89	9.0 ± 3.15
		Recovery	9.6 ± 1.86	8.0 ± 1.36

* Statistically significant different from control ($p \leqslant 0.05$).
[a] Mean ± SD; $n = 10$.

did not result in any other smoke-related changes in organ weights or gross necropsy observations.

Microscopic examination of the tissues of rats exposed to smoke indicated exposure-related changes limited to the respiratory tract. There were no smoke related histopathologic affects in any other organs. All findings in the respiratory tract were comparable in spectrum to those seen in previous inhalation studies with cigarette smoke conducted by the testing laboratory at comparable TPM concentrations, and have also been reported in the literature for subchronic cigarette smoke inhalation studies (Coggins et al., 1989). Selected major respiratory tract histopathological results from the rats exposed to glycerin smoke are shown in Table 11. Daily exposure to mainstream smoke from cigarettes containing glycerin at a target concentration of 15% decreased the incidence and/or severity (different by a score of at least 1.00) compared to the control cigarette group of focal macrophage accumulation in the left and right lung in males, goblet cell hyperplasia/hypertrophy in the nose (level 1) in males, and olfactory epithelium atrophy in the nose (levels 2 and 3) in females compared to the respective control cigarette group. Exposure to smoke from cigarettes containing glycerin at a target concentration of 10% increased goblet cell hyperplasia/hypertrophy in the nose (level 1) in females but decreased focal macrophage accumulation in the right lung and goblet cell hyperplasia/hypertrophy in the nose (level 1) in males. In addition, there was a statistically significant increase in the number of goblet cells in the bronchial epithelium in the left lung of the medium dose females compared to the smoke-exposed control females. Smoke from cigarettes containing glycerin at a target concentration of 5% decreased focal macrophage accumulation in the left and right lung in males, goblet cell hyperplasia/hypertrophy in the nose (level 1) in males, and goblet cell staining depletion in the nose (level 1) in males.

After the 42-day recovery period, the incidence and/or severity of squamous cell hyperplasia-metaplasia in the larynx (lower medial region arytenoid) and lateral wall hyperplasia in the larynx (ventral arytenoid) in females were increased and goblet cell hyperplasia/hypertrophy in the nose (level 1) in males was decreased compared to the control cigarette group. The increased incidence and severity of the changes in the larynx in the 15% glycerin group females compared to the control cigarette group females at the end of the recovery period were interpreted as incomplete resolution of expected histopathological changes observed following exposure to cigarette smoke (changes seen with similar incidence and severity in control and high cigarette groups at the end of the 13-week exposure period), rather than delayed effects of glycerin exposure. Thus, the most prevalent effect noted in the smoke inhalation study with glycerin added to tobacco was a general decrease in the incidence and/or severity of those smoke-related lesions which are typically seen in rat cigarette smoke inhalation studies. Microscopic changes seen at an increased incidence or severity compared to the control group occurred only at the medium concentration level or in the recovery animals, and were not considered related to glycerin smoke exposure.

4. Discussion

Commercial cigarettes are prepared by blending various types of tobacco leaf (bright, Burley, and oriental) and processed tobacco (expanded, reconstituted, and stems). During the blending and processing of tobacco, humectants such as glycerin may be added to increase the moisture holding capacity of the tobacco and to aid in processing. While analysis of the cigarette smoke for glycerin was not conducted as part of this study, glycerin would be expected to transfer essentially intact to smoke without undergoing significant pyrolysis. Transfer studies support this conclusion. Laurene et al. (1965) analyzed 70 mm non-filtered cigarettes containing 4.39% glycerin added to tobacco. They reported 8.9% of the glycerin transferred in the mainstream

Table 10
Absolute organ weights from rats exposed to air (sham) or 150 mg/m^3 mainstream smoke from reference, control, or test cigarettes containing glycerin

Group (target % glycerin)	Sex	Left adrenal	Right adrenal	Brain	Heart	Left kidney	Right kidney	Liver	Lung	Left testis/ovary	Right testis/ovary	Spleen	Thymus
Control	M	0.034 ± 0.008[a]	0.032 ± 0.006	2.13 ± 0.114	1.37 ± 0.151	1.30 ± 0.131	1.31 ± 0.110	9.04 ± 1.197	2.06 ± 0.234	1.66 ± 0.179	1.67 ± 0.152	0.56 ± 0.057	0.23 ± 0.066
Low (5%)	M	0.031 ± 0.006	0.028 ± 0.006	2.11 ± 0.049	1.32 ± 0.080	1.25 ± 0.110	1.28 ± 0.115	8.97 ± 0.381	1.94 ± 0.156	1.63 ± 0.107	1.65 ± 0.083	0.62 ± 0.089	0.22 ± 0.062
Medium (10%)	M	0.032 ± 0.003	0.030 ± 0.003	2.07 ± 0.075	1.35 ± 0.156	1.24 ± 0.195	1.23 ± 0.135	8.74 ± 1.185	1.90 ± 0.154	1.65 ± 0.119	1.65 ± 0.099	0.56 ± 0.064	0.21 ± 0.064
High (15%)	M	0.035 ± 0.006	0.033 ± 0.006	2.12 ± 0.111	1.38 ± 0.173	1.27 ± 0.151	1.28 ± 0.137	9.13 ± 1.165	1.98 ± 0.199	1.63 ± 0.104	1.64 ± 0.113	0.58 ± 0.071	0.21 ± 0.067
1R4F	M	0.032 ± 0.005	0.029 ± 0.008	2.11 ± 0.081	1.37 ± 0.193	1.30 ± 0.099	1.37 ± 0.184	8.99 ± 0.614	1.95 ± 0.145	1.67 ± 0.143	1.59 ± 0.307	0.57 ± 0.114	0.22 ± 0.044
Sham	M	0.025 ± 0.005	0.025 ± 0.006	2.17 ± 0.086	1.37 ± 0.133	1.46 ± 0.170	1.42 ± 0.161	10.71 ± 0.566	1.89 ± 0.102	1.70 ± 0.128	1.73 ± 0.179	0.66 ± 0.089	0.25 ± 0.077
Control	F	0.043 ± 0.010	0.040 ± 0.009	1.97 ± 0.119	0.97 ± 0.138	0.86 ± 0.099	0.89 ± 0.137	6.96 ± 0.649	1.58 ± 0.141	0.065 ± 0.019	0.067 ± 0.024	0.41 ± 0.066	0.16 ± 0.039
Low (5%)	F	0.039 ± 0.008	0.036 ± 0.006	1.93 ± 0.077	1.00 ± 0.097	0.90 ± 0.105	0.92 ± 0.129	6.88 ± 0.793	1.61 ± 0.178	0.059 ± 0.018	0.053 ± 0.016	0.49 ± 0.098	0.19 ± 0.037
Medium (10%)	F	0.038 ± 0.006	0.037 ± 0.003	1.91 ± 0.067	0.93 ± 0.096	0.84 ± 0.075	0.87 ± 0.084	6.34 ± 0.709	1.51 ± 0.318	0.062 ± 0.014	0.064 ± 0.019	0.40 ± 0.084	0.16 ± 0.059
High (15%)	F	0.038 ± 0.006	0.036 ± 0.009	1.98 ± 0.085	0.93 ± 0.078	0.86 ± 0.081	0.88 ± 0.076	6.60 ± 0.663	1.60 ± 0.208	0.065 ± 0.017	0.066 ± 0.020	0.46 ± 0.071	0.22 ± 0.048[*]
1R4F	F	0.040 ± 0.005	0.038 ± 0.005	1.91 ± 0.093	0.94 ± 0.064	0.85 ± 0.047	0.88 ± 0.054	6.54 ± 0.358	1.53 ± 0.154	0.053 ± 0.017	0.058 ± 0.016	0.39 ± 0.071	0.20 ± 0.057
Sham	F	0.032 ± 0.004	0.030 ± 0.005	1.97 ± 0.073	0.90 ± 0.070	0.91 ± 0.053	0.92 ± 0.049	7.21 ± 0.831	1.39 ± 0.089	0.049 ± 0.018	0.047 ± 0.010	0.49 ± 0.068	0.27 ± 0.046

[*] Statistically significant different from control ($p \leqslant 0.05$).
[a] Mean ± SD.

smoke, while 15.4% remained in the butt. Using filtered cigarettes, they found from 0.4% to 8.1% transferred to the smoke, while 20.4–29.4% remained in the butt. Kobashi et al. (1965) found that mainstream smoke from filter and non-filter cigarettes contained 14% and 12%, respectively of the original added glycerin. Best (1987) found 10% transfer to the mainstream smoke using a conventional filtered cigarette and ^{14}C-labeled glycerin. More recently Liu (2004) evaluated the transfer of glycerin over different glycerin addition levels and cigarette designs. He found that the maximum glycerin contribution to tar was 36% after an 11.4% addition to the tobacco. The glycerin transfer was generally found to be proportional to the tobacco glycerin level.

The generation of acrolein from glycerin has also been shown from pyrolysis studies of glycerin in steam conducted in a laminar flow reactor at 650–700 °C (Stein et al., 1983). The initial products of decomposition were carbon monoxide, acetaldehyde, and acrolein. Acetaldehyde and acrolein decomposed further to ethylene, methane and hydrogen. Baker and Bishop (2004) pyrolyzed glycerin under a set of conditions designed to more realistically represent the dynamic conditions present during tobacco burning (applying the sample to a matrix of quartz wool, heating it to 900 °C at a rate of 30 °C/s under a flow of 9% oxygen in nitrogen). Glycerin was the major constituent (99.8%) with two unidentified products observed. While we did not quantify the products of our pyrolysis experiment, these results are consistent with ours where glycerin was the principle material identified with extremely small amounts of acrolein and glycolaldehyde observed.

When glycerin is added to tobacco, the relationship between glycerin and acrolein formation becomes less clear, since the quantitative determination of a product-precursor relationship for glycerin to acrolein is difficult to isolate in smoke because acrolein is a thermal degradation product of many naturally occurring tobacco leaf constituents. The thermal breakdown of glycerin to acrolein is an incomplete reaction (complete combustion of glycerin would produce water and carbon dioxide.) Glycerin is naturally present in smoke as is acrolein. Although the degradation reaction would produce one mole of acrolein for each mole of glycerin, apparently there are many physical and chemical factors of the pyrolysis of tobacco that negate this relationship. Some of these factors are reported to include the type of tobacco, the temperature of pyrolysis and rate of temperature increase, static burning rate, puff number, weight of tobacco, proportion of non-smoking materials, smoke delivery, and smoke chemistry (John, 1981). Studies were conducted with cigarettes containing ^{14}C-labeled glycerin smoked under FTC conditions (Gager et al., 1960). About 0.1% of the original glycerin ^{14}C activity was found as acrolein. The total contribution from glycerin was about 8.5% of the total acrolein in

Table 11
Major histopathological changes in rats exposed to air (sham) or 150 mg/m^3 mainstream smoke from control cigarettes or test cigarettes containing glycerin

Organ—Lesion	Sex	Group					
		Sham	Control	Low	Medium	High	1R4F
Terminal necropsy							
Nose, level 1							
Hyperplasia/hypertrophy, goblet cell	M	4/10 (0.50)[a]	10/10 (3.40)	10/10 (2.10)[c]	8/10 (1.60)[c]	10/10 (2.30)[c]	10/10 (2.60)
	F	0/10	7/10 (1.40)	8/10 (1.20)	10/10 (2.40)[c]	6/10 (1.00)	8/10 (1.50)
Depletion, goblet cell staining	M	1/10 (0.10)	9/10 (1.40)	2/10 (0.30)[c]	4/10 (0.90)	4/10 (0.50)	4/10 (0.60)
	F	0/10	1/10 (0.20)	9/10 (1.10)	2/10 (0.30)	2/10 (0.30)	6/10 (1.20)
Nose, level 2							
Atrophy, olfactory epithelium	M	1/10 (0.10)	1/10 (0.20)	4/10 (0.70)	2/10 (0.40)	2/10 (0.40)	3/10 (0.60)
	F	0/10	8/10 (2.10)	8/10 (2.10)	6/10 (1.50)	3/10 (0.60)[c]	7/10 (2.00)
Nose, level 3							
Atrophy, olfactory epithelium	M	0/10	0/10	0/10	0/10	1/10 (0.20)	0/10
	F	0/10	4/10 (1.30)	2/10 (0.60)	4/10 (1.00)	1/10 (0.10)[c]	2/10 (0.60)
Larynx, arytenoid, ventral hyperplasia,	M	0/9	9/9 (3.00)	9/9 (3.11)	10/10 (2.90)	10/10 (2.90)	9/9 (3.56)
lateral wall	F	0/10	10/10 (2.9)	10/10 (3.10)	10/10 (3.00)	10/10 (3.00)	10/10 (2.90)
Larynx, arytenoid, lower medial region	M	0/9	9/10 (2.40)	9/9 (3.11)	9/10 (2.80)	10/10 (3.00)	8/9 (2.67)
squamous cell hyperplasia-metaplasia	F	0/10	10/10 (2.50)	10/10 (3.20)	10/10 (3.30)	10/10 (3.10)	10/10 (2.90)
Lung, left accumulation, macrophage, focal	M	1/10 (0.10)	7/10 (2.40)	7/10 (1.40)[c]	7/10 (1.60)	1/10 (0.30)[c]	7/10 (1.70)
	F	1/10 (0.10)	8/10 (2.00)	9/10 (1.70)	8/10 (1.90)	7/10 (1.40)	6/10 (1.10)
Lung, right accumulation, macrophage, focal	M	1/10 (0.20)	10/10 (3.20)	7/10 (1.70)[c]	8/10 (1.90)[c]	3/10 (0.50)[c]	7/10 (1.70)
	F	2/10 (0.20)	9/10 (2.10)	9/10 (2.20)	8/10 (2.40)	6/10 (1.40)	6/10 (1.50)
Goblet cell count left lung	M	10 ± 8	36 ± 20	139 ± 162	135 ± 78	111 ± 78	106 ± 69
	F	29 ± 31	87 ± 88	63 ± 43	183 ± 119*	66 ± 44	88 ± 55
Recovery necropsy							
Nose, level 1							
Hyperplasia/hypertrophy, goblet cell	M	8/10 (1.40)	9/10 (2.50)	NE[b]	NE	9/10 (1.50)[c]	NE
	F	0/10	7/10 (0.90)	NE	NE	3/10 (0.60)	NE
Larynx, arytenoid, ventral hyperplasia,	M	1/10 (0.10)	2/10 (0.20)	NE	NE	3/10 (0.70)	NE
lateral wall	F	1/10 (0.20)	3/10 (0.80)	NE	NE	8/10 (2.00)[c]	NE
Larynx, arytenoid, lower medial region	M	2/10 (0.20)	2/10 (0.30)	NE	NE	5/10 (1.10)	NE
squamous cell hyperplasia–metaplasia	F	1/10 (0.10)	5/10 (1.00)	NE	NE	9/10 (2.00)[c]	NE

* Statistically significant difference from Control, $p \leqslant 0.05$.
[a] Incidence (mean group severity score graded on a 4 point grading scale of 1 = minimal and 4 = severe).
[b] NE = Not Evaluated.
[c] Group severity score different by at least 1.00 from Control group; interpreted as a finding of probable biological significance.

smoke. The study suggested that glycerin is a precursor yet minor contributor of acrolein in smoke.

Smoke inhalation studies in rats and smoke condensate mouse skin painting studies conducted with cigarettes having glycerin added to the tobacco as a component of an ingredient mixture have previously been reported (Carmines, 2002; Gaworski et al., 1998, 1999). Heck et al. (2002) reported on the inhalation toxicity of glycerin added to tobacco at 5.1% in combination with propylene glycol and concluded that these materials did not discernibly alter the toxicity of smoke. In the present study, glycerin was added to tobacco as a single ingredient up to 15% and evaluated as in a series of studies designed to detect if the ingredient altered the smoke composition or biological effects associated with cigarette smoke.

Chemical analysis of the smoke from cigarettes containing glycerin at a target application level of 5% did not indicate any relevant increases in the amounts of the measured smoke constituents other than TPM, water and tar, either on a per cigarette or per mg tar basis. As the amount of glycerin was increased (up to 15%) a pattern of decreased acetaldehyde, propionaldehyde, aromatic amines, nitrogen oxides, tobacco specific nitrosamines, and phenols appeared. In most cases the decrease was greater than would have been predicted from simple dilution of the tobacco. There was a 9% (tar basis) increase in acrolein at both of the exaggerated glycerin application levels (not glycerin concentration related). Glycerin was evaluated as part of the NCI's "Less Hazardous" Cigarette Program (Gori, 1977). In

this study, cigarettes with 2.95% glycerin produced an 11% increase in acrolein on a cigarette basis but a 4% reduction on a TPM basis. Baker et al. (2004c) evaluated the effect of a mixture of cigarette ingredients on smoke chemistry similar to our previous studies (Rustemeier et al., 2002). In their cigarettes containing 7% glycerin, there was an increase (cigarette basis) in the TPM, acetaldehyde, acrolein, and proprionaldehyde and decrease in nicotine, NNN, NNK, phenols, aromatic amines, nitrogen oxides. While these cigarettes contained other ingredients, the effects on smoke chemistry are remarkably similar to ours reported here. Baker et al. (2004c) did not conclude that glycerin addition resulted in increased acrolein levels in the smoke.

It has been suggested (Vleeming et al., 2004) that it is more appropriate to evaluate smoke chemistry results on a tobacco basis rather than relative to the cigarette, tar or TPM. The test cigarettes used in these studies were manufactured to a constant weight with the glycerin displacing tobacco. If the glycerin were inert and did not combust, the particulate smoke constituents would be expected to be reduced. This is essentially what was observed when the data were evaluated on a cigarette or tar basis (see Table 2 and Fig. 2). Since glycerin has the humectant effect of increasing the water in smoke, presentation of the data on a tar basis is more relevant. Presuming that water has no toxicity in the smoke, removing it from the calculations allows one to see the true effect of glycerin on the relative deliveries of the various smoke constituents. In Fig. 5 the data have been calculated on a tobacco basis. The net effect of this presentation is the apparent substantial increase in the water. This is essentially the function of glycerin in cigarettes. The relative amounts of the other smoke constituents are not appreciably different when compared to the calculation on a tar basis (compare Figs. 2 and 5). Based on these results calculated in this manner, there does not appear to be any additional effect of adding glycerin that is not observed when the data are calculated on a cigarette or tar basis. Calculation in this manner does not appear to underestimate the amount of the smoke constituents as suggested by Vleeming et al. (2004).

Genotoxicity and in vitro cytotoxicity studies did not reveal any increases in the biological activity of various fractions of smoke from the glycerin (up to target levels

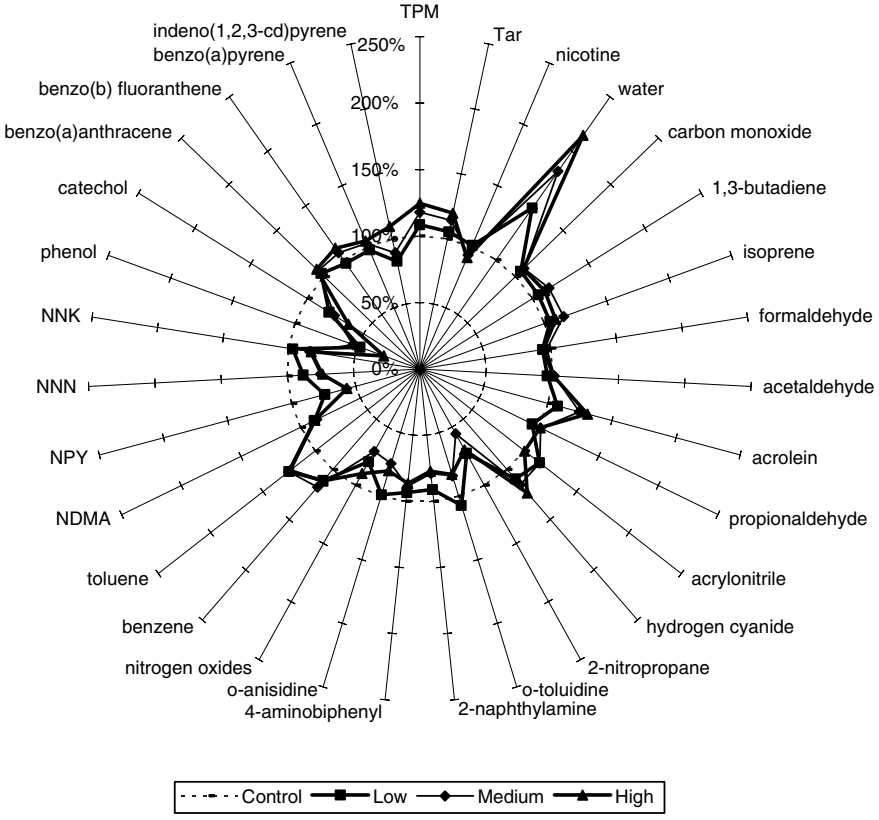

Fig. 5. Relative changes in smoke constituents of glycerin treated cigarettes calculated on a tobacco basis. This radar plot was constructed by first dividing the measured cigarette yield by the average weight of tobacco in the cigarette excluding the weight of the glycerin. The values for the control cigarette were set to 100% and each glycerin analyte divided by the control cigarette value. Target glycerin levels were: low 5%, medium = 10%, and high = 15%.

of 15%) containing cigarettes. This is in agreement with our previous work with groups of cigarette ingredients (including glycerin up to 4.2%) (Roemer et al., 2002) where no effects were observed. Baker et al. (2004c) recently published an ingredient mixture study using 7% glycerin as one of the ingredients and also did not find any increases in mutagenicity or in vitro cytotoxicity. Additional non-published internal studies available on the internet (Tewes, 1987; Oey, 1990) have been conducted to investigate the potential effects of glycerin on the cytotoxicity and mutagenicity of mainstream and sidestream smoke. In the mutagenicity study, strains TA 98 and TA100 with metabolic activation were exposed to smoke condensate collected from cigarettes prepared with glycerin at 3.2%, 6.0% or 9.4%. None of the treatments resulted in any increase in the mutagenicity of the mainstream or sidestream smoke. There appeared to be a reduction in the specific mutagenicity of the mainstream smoke of the 9.4% glycerin cigarettes in strains TA 98 and TA 100. There was also an apparent reduction in the 6.0% and 9.4% glycerin cigarette sidestream smoke in strain TA 100. In the cytotoxicity study, Chinese hamster embryo V79 cells were exposed to whole smoke from cigarettes containing 2.0, 3.4 or 5.4% glycerin. In contrast to the particulate phase and gas vapor phase studies reported in the present study, whole smoke was used in a unique exposure system designed to exposed in vitro cell cultures in cell culture flasks to airborne materials. For purposes of the exposure, mainstream smoke was diluted to a concentration similar to that of the sidestream smoke. The cytotoxicity of the mainstream smoke obtained from cigarettes treated with glycerin was not significantly different from the control cigarette. In sidestream, the authors noted that the cytotoxicity tended to decrease with increasing glycerin concentration.

In the 90-day rat inhalation study, the smoke from cigarettes with target levels of up to 15% glycerin failed to increase the overall toxicity of the smoke. Despite the fact that the exposures were conducted at equivalent particulate levels, there was a reduction in the amount of serum nicotine and cotinine in animals as the glycerin level increased in tobacco, suggesting that glycerin displaced tobacco. There were no glycerin concentration related adverse findings in any of other the parameters evaluated. The exaggerated application levels of glycerin also appeared to reduce the overall toxicity of the smoke as measured by the histopathological lesions in the rat upper respiratory tract. The lack of an effect at the 5% glycerin application level is consistent with the findings of Baker et al. (2004c) who tested a mixture of ingredients including 7% glycerin in a 90-day inhalation study and our previous findings where a mixture of ingredients including glycerin up to 4.2% was also evaluated (Vanscheeuwijck et al., 2002). Heck et al. (2002) also did not find an effect of glycerin on the inhalation toxicity of rats up to 5.1%.

While skin painting studies were not part of this investigation, a number of evaluations have been performed on condensate from cigarettes containing glycerin. In a National Cancer Institute (Gori, 1977) cigarette smoke condensate skin painting study, ICR Swiss female mice were painted six days/week with solutions containing up to 25 mg dry smoke condensate. The duration of the study was 18 months. Comparisons among condensates with and without various additives indicated that glycerin (2.8%) alone had little or no effect on condensate tumorigenicity. Glycerin was also tested in combination with invert sugar. At the lower condensate dose of 12.5 mg condensate/mouse, invert sugar (5.8% on tobacco) in combination with glycerin (2.8% on tobacco) had little effect on condensate tumorigenicity, but at the higher condensate application dose of 25 mg condenstate/mouse the report stated that the combination "may contribute to condensate tumorigenicity". Gaworski et al. (1999) evaluated glycerin cigarettes as a part of four comparative two-stage SENCAR mouse skin painting bioassays. Groups of 30–50 female SENCAR mice each were initiated topically with 50 μg of 7,12-dimethylbenz(*a*)anthracene (DMBA), and promoted three times a week for 26 weeks with either 10 or 20 mg of cigarette smoke condensate from test cigarettes containing a mixture of flavor ingredients including glycerin at a target of 2.4%. For comparison, separate groups of mice received concurrent treatment with cigarette smoke condensate from reference cigarettes prepared without added ingredients. While tumor incidence, latency, and multiplicity data occasionally differed between test and comparative reference cigarette smoke condensate groups, all effects appeared to be within normal variation for the model system. The authors concluded that none of the changes appeared to be substantial enough to conclude that the tumor promotion capacity of cigarette smoke condensate obtained from cigarettes containing tobacco with ingredients was discernibly different from the cigarette smoke condensate obtained from reference cigarettes containing tobacco processed without ingredients. In summary, these studies do not suggest that addition of glycerin to cigarettes will increase the tumorigenicity as measured by the skin painting assay.

The results of the studies presented here as well as those in the literature suggest that addition of glycerin to cigarette tobacco at levels which represent our current manufacturing practices do not adversely alter the profile of major toxic constituents of smoke, nor lead to an increase in the biological activity of smoke as measured by a series of in vitro and in vivo assays. The smoke composition data suggests that the use of higher levels of glycerin might reduce some of the toxic constituents of smoke. Additional studies would be needed to understand the mechanism and the impact of these reductions.

Acknowledgements

The authors thank the The Burdock Group for reviewing the published literature on glycerin. We also thank Karen Edwards and Linda Wettle for their help in preparing the glycerin test cigarettes, the Philip Morris USA Analytical Methods and Applications group for analysis of the test cigarettes for glycerin and for the pyrolysis study. We acknowledge the study directors at Illinois Institute of Technology and Research Institute for their excellent technical assistance in conducting the studies: B. Gingras (cytotoxicity); M. Cwik (smoke chemistry); M. Nagabhushan (mutagenicity); and W. Johnson (inhalation and micronucleus).

References

Anonymous, 1969. Glycerol: a two-year feeding study in rats. Atlas Chemical Industries. Wilmington, Delaware.

Anonymous, 1982. Toxicological principles for the safety assessment of direct food additives and color additives used in food. US Food and Drug Administration, Washington, DC.

Anonymous, 1987. Environmental Health Criteria 70: Principles for the Safety Assessment of Food Additives and Contaminants in Food. Joint FAO/WHO Expert Committee on Food Additives, Geneva.

Baker, R.R., Bishop, L.J., 2004. The pyrolysis of tobacco ingredients. Journal of Analytical and Applied Pyrolysis, 1–91.

Baker, R.R., Pereira Jr., Da Silva, Smith, G., 2004a. The effect of tobacco ingredients on smoke chemistry. Part I: Flavourings and additives. Food and Chemical Toxicology 42 (Suppl.), 3–37.

Baker, R.R., Pereira Jr., Da Silva, Smith, G., 2004b. The effect of tobacco ingredients on smoke chemistry. Part II: Casing ingredients. Food and Chemical Toxicology 42 (Suppl.), 39–52.

Baker, R.R., Massey, E.D., Smith, G., 2004c. An overview of the effects of tobacco ingredients on smoke chemistry and toxicity. Food and Chemical Toxicology 42 (Suppl.), 53–83.

Best, F.R., 1987. Radiotracer studies with carbon-14 labeled glycerol. Fate in smoke. In: Proceedings of the International Conference on the Physical and Chemical Processes Occurring in a Burning Cigarette. Meeting held at Wake Forest University, Winston Salem, NC April 26–29, 244–255.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicology Letters 24 (2–3), 119–124.

Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part: 1 cigarette design, testing approach, and review of results. Food and Chemical Toxicology 40 (1), 77–91.

Coggins, C.R., Ayres, P.H., Mosberg, A.T., Sagartz, J.W., Burger, G.T., Hayes, A.W., 1989. Ninety-day inhalation study in rats, comparing smoke from cigarettes that heat tobacco with those that burn tobacco. Fundamental and Applied Toxicology 13, 460–483.

CPSC, 1993. Toxicity testing plan for low ignition-potential cigarettes. Consumer Product Safety Commission.

Deichmann, W., 1940. Behavior in the animal organism (a review of the literature). Industrial Medicine 9, 60–67.

Deichmann, W., 1941. Glycerol-effects upon rabbits and rats. Indian Medicine and Surgery 10, 5–6.

Diana, J.N., Vaught, A., 1997. Research Cigarettes. The University of Kentucky Printing Services, Lexington, KY.

Doolittle, D.J., Lee, D.A., Lee, C.K., 1988. The genotoxicity of glycerol in an in vitro test battery. Food and Chemical Toxicology 26, 631–635.

Federal Trade Commission, 1967. August 1, 1967 Press Release.

Food and Drug Administration. 2004. Redbook 2000 Toxicological Principles for the Safety Assessment of Food Ingredients. Available from: <www.cfsan.fda.gov/~redbook/red-toca.html>.

Gager, P.L., Brunot, C.E., Carpenter, R.D., Resnik, F.E., Varsel, C.J. 1960. Cigarette smoke precursors. I. The contribution of the humectant glycerol to the acrolein content of cigarette smoke. Philip Morris Internal Report. Available from: <www.pmdocs.com>, Document ID number 2024925061/5067.

Gaworski, C.L., Dozier, M.M., Gerhart, J.M., Rajendran, N., Brennecke, L.H., Aranyi, C., Heck, J.D., 1997. 13-week inhalation toxicity study of menthol cigarette smoke. Food and Chemical Toxicology 35 (7), 683–692.

Gaworski, C.L., Heck, J.D., Bennett, M.B., Wenk, M.L., 1998. Toxicologic evaluation of flavor ingredients added to cigarette tobacco: skin painting bioassay of cigarette smoke condensate in SENCAR mice. Toxicology 139 (1–2), 1–17.

Gaworski, C.L., Dozier, M.M., Heck, J.D., Gerhart, J.M., Rajendran, N., David, R.M., Brennecke, L.H., Morrissey, R, 1999. Toxicologic evaluation of flavor ingredients added to cigarette tobacco: 13 week inhalation exposures in rats. Inhalation Toxicology 10, 357–381.

Gerarde, H.W., 1959. The pathogenesis of pulmonary injury in kerosene intoxication. Delaware State Medical Journal 31, 276–280.

Gori, G.B. 1977. Report No. 3. Toward less hazardous cigarettes. The third set of experimental cigarettes. DHEW, Publication No. 77-1280.

Greenspan, B.J., 1988. Inhalation Studies of Humectant Aerosols in Rats. Toxicologist 8, 255.

Guerrant, N.B., Whitlock, G.P., Wolff, M.L., Dutcher, R.A., 1947. Response of rats to diets containing varying amounts of glycerol and of propylene glycol. Bulletin of Natural Formulary Communication 15, 205–229.

Haag, H.B., Ambrose, A.M., 1937. Studies on the physiological effect of diethylene glycol. Journal of Pharmacology and Experimental Therapy 59, 93–100.

Heck, J.D., Gaworski, C.L., Rajendran, N., Morrissey, R.L., 2002. Toxicologic evaluation of humectants added to cigarette tobacco:13-week smoke inhalation study of glycerin and propylene glycol in Fischer 344 rats. Inhalation Toxicology 14 (11), 1135–1152.

Hine, C.H., Anderson, H.H., Moon, H.D., Dunlap, M.K., Morse, M.S., 1953. Comparative toxicity of synthetic and natural glycerin. Archieves of Industrial Hygiene and Occupational Medicine 7, 282–291.

Hoffmann, D., Hoffmann, I., 1997. The changing cigarette, 1950–1995. Journal of Toxicology and Environmental Health 50 (4), 307–364.

IARC., 1986. Chemistry and analysis of tobacco smoke. IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. 83–126. Lyon, IARC.

International Organization for Standardization. 1989. Cigarettes-determination of nicotine content in smoke condensates—gas chromatographic method. ISO 10315.

International Organization for Standardization. 1991a. Cigarettes—determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine. ISO 4387, second ed.

International Organization for Standardization. 1991b. Tobacco and tobacco products—atmosphere for conditioning and testing. ISO 3402. third ed.

International Organization for Standardization. 1991c. Routine analytical cigarette-smoking machine—definitions and standard conditions. ISO 3308. third ed.

International Organization for Standardization. 1995a. Cigarettes-determination of water in smoke condensates, Part: 2 Karl Fischer method. ISO 10362-2.

International Organization for Standardization. 1995b. Cigarettes—determination of carbon monoxide in the vapour phase of cigarette smoke-NDIR method. ISO 8454, second ed.

Inayama, Y., Kitamura, H., Ito, T., Kanisawa, M., 1986. Effects of glycerol on 4-nitroquinoline 1-oxide induced pulmonary tumorigenesis in ddy mice. Japan Journal of Cancer Research 77, 103–105.

John, J., 1981. Quantitative determination of product—precursor relationships for the dehydration reactions of two humectants glycerol and triethyleneglycol. 1981. Philip Morris Internal Report. Available from: <www.pmdocs.com>, Document ID number 2057953382/3386.

Johnson, V., Carlson, A.J., Johnson, A., 1933. Studies on the physiological action of glycerol on the animal organism. American Journal of Physiology 103, 517–534.

Kitamura, H., Inayama, Y., Ito, T., Yabana, M., Piegorsch, W.W., Kanisawa, M., 1987. Morphologic alteration of mouse Clara cells induced by glycerol: ultrastructural and morphometric studies. Experimental Lung Research 12, 281–302.

Kobashi, U.T., Doihara, S., Sugawara, S., Kaburaki, Y., 1965. Changes in the chemical composition of smoke from cigarettes imparted with polyols. Research Institute of Japan Monopoly Corporation 107, 319–323.

Kudo, K., Ito, R., 1972. Comparison of acute toxicity of polyglycerin, natural glycerin, and synthetic glycerin. Toho Igakkai Zasshi 19, 415–417.

Laurene, A.H., Cundiff, R.H., Greene, G.H., 1965. Determination of glycerol and propylene glycol in cigarette smoke. Tobacco Science 9, 1–4.

Lewis, D.J., 1981. Mitotic indices of rat laryngeal epithelia. Journal of Anatomy 132, 419–428.

Litton Bionetics Mutagenic evaluation of compound FDA 71-89, glycerin, 1975. NTIS PB-245 479. US Food and Drug Administration, Washington, DC.

Liu, C., 2004. Glycerol transfer in cigarette mainstream smoke. Beitrage Zur Tabakforschung International 21 (2), 111–116.

Maron, D.M., Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. Mutation Research 113 (3–4), 173–215.

OECD. Guideline 413., 1981. Subchronic inhalation toxicity: 90-day study. Organization for Economic Co-operation and Development. OECD Guidelines for Testing Chemicals. Paris.

OECD. Guideline 471., 1997a. Bacterial reverse mutation test. Organization for Economic Co-operation and Development. OECD Guidelines for Testing Chemicals. Paris.

OECD. Guideline 474., 1997b. Mammalian erythrocyte micronucleus test. Organization for Economic Co-operation and Development. OECD Guidelines for Testing Chemicals. Paris.

Oey, J. 1990. Cytotoxicity of the diluted mainstream and sidestream whole smoke of the research cigarettes Tear-1, -2, -3, -4, -5, -6, -7, -8, and -9. Acute in vitro exposure study using chinese hamster embryo lung V79 cells., Philip Morris Internal Report. Available from: <www.pmdocs.com>, Document ID number 2026047137/7307.

Renne, R.A., 1992. 2-Week and 13-week inhalation studies of aerosolized glycerol in rats. Inhalation and Toxicology 4, 95–111.

Rodgman, A., 2002. Some studies of the effects of additives on cigarette mainstream smoke properties. II. Casing materials and humectants. Beitrage Zur Tabakforschung International 20, 279–299.

Roemer, E., Tewes, F.J., Meisgen, T.J., Veltel, D.J., Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part 3: in vitro genotoxicity and cytotoxicity. Food and Chemical Toxicology 40 (1), 105–111.

Rustemeier, K., Stabbert, R., Haussmann, H.J., Roemer, E., Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part 2: chemical composition of mainstream smoke. Food and Chemical Toxicology 40 (1), 93–104.

Secretary of State for Health., March, 1997. Voluntary agreement on the approval and use of tobacco additives in tobacco products in the UK.

Select Committee on GRAS Substances (SCOGS), 1975. Life Sciences Research Office, Federation of American Societies of Experimental Biology, Evaluation of the Health Aspects of Glycerin and Glycerides as Food Ingredients, PF-254, 536.

Smyth, H.F., Seaton, J., Fischer, L., 1941. The single dose toxicity of some glycols and derivatives. Journal of Industrial Hygiene Toxicology 23, 259–265.

Staples, R., Misher, A., Wardell, J., 1967. Gastrointestinal irritant effect of glycerin as compared with sorbitol and propylene glycol in rats and dogs. Journal of Pharmaceutical Science 56, 398–400.

Stavanja, M.S., Ayres, P.H., Meckley, D.R., Bombick, B.R., Pence, D.H., Borgerding, M.F., Morton, M.J., Mosberg, A.T., Swauger, J.E., 2003. Toxicological evaluation of honey as an ingredient added to cigarette tobacco. Journal of Toxicology and Environmental Health Part A 66, 1453–1473.

Stein, Y.S., Antal, M.J., Jones, M., 1983. A study of gas phase pyrolysis of glycerol. Journal of Analytical and Applied Pyrolysis 4, 283–296.

Tewes, F. 1987. Mutagenicity of mainstream and sidestream whole smoke condensate of research cigarettes Tear-1, -2, -3, -4, -5, -6, -7, -8, and -9 on Salmonella typhimurium strains TA98, TA100, and TA102., Philip Morris Internal Report. Available from: <www.pmdocs.com>, Document ID number 2029145700/5717.

Vanscheeuwijck, P.M., Teredesai, A., Terpstra, P.M., Verbeeck, J., Kuhl, P., Gerstenberg, B., Gebel, S., Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part 4: subchronic inhalation toxicity. Food and Chemical Toxicology 40 (1), 113–131.

Vleeming, W., Schenk, E., Opperhuizen, A., 2004. Letter to the editor. Food and Chemical Toxicology 42 (5), 865–866.

Whitlock, G., Guerrant, N.B., Dutcher, R.A., 1944. Response of rats to diets containing propylene glycol and glycerol. Proceedings of the Society for Experimental and Biological Medicine 57, 124–125.

Young, J.T., 1981. Histopathologic examination of the rat nasal cavity. Fundamental and Applied Toxicology 1, 309–312.