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Research Section

Comparison of the Cytotoxic and Mutagenic Potential of Liquid Smoke Food Flavourings, Cigarette Smoke Condensate and Wood Smoke Condensate

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Abstract—Although products of pyrolysis are often cytotoxic and mutagenic, the relationship between the type of material pyrolysed and the toxicity of the resulting pyrolysis products is poorly understood. The objective of this study was to evaluate and compare the cytotoxicity and mutagenicity of several types of common pyrolysis products. The cytotoxicity and mutagenicity of these products were assessed by using neutral red uptake and Ames mutagenicity assays, respectively. The biological activities of four liquid smoke food flavourings (LSF) were compared with two other pyrolysis-derived materials; cigarette smoke condensate (CSC) and a wood smoke condensate (WSC). Results indicated all of the mixtures exhibited a concentration-dependent cytotoxic response. The CSC and WSC were less cytotoxic than three of the LSFs, but more cytotoxic than one of the brands. The CSC was mutagenic in two *Salmonella* strains; however, none of the LSFs or WSC was mutagenic using TA98, and only three of the LSFs were positive with TA100. The six pyrolysis-derived materials evaluated in this study showed differing patterns and magnitudes of cytotoxicity and mutagenicity. These results indicate that the cytotoxicity and mutagenicity of complex mixtures derived from pyrolysis products are affected by the type of material pyrolysed and/or the method used to prepare the mixture. The cytotoxic potential of some commercial smoke flavourings is greater than cigarette smoke condensate and several of the food flavourings are mutagenic in one *Salmonella* strain. © 1999 Elsevier Science Ltd. All rights reserved

Keywords: cigarette smoke; liquid smoke flavourings; cytotoxicity; neutral red; mutagenicity.

Abbreviations: CHO = Chinese hamster ovary; CSC = cigarette smoke condensate; K1R4F = Kentucky Reference cigarette; LSF = liquid smoke flavourings; WSC = wood smoke condensate; TPM = total particulate matter.

INTRODUCTION

The pyrolysis of plant and/or wood products yields a complex mixture of chemicals. Two examples of complex plant pyrolysis mixtures include liquid smoke flavourings and cigarette smoke condensate. These complex pyrolysis mixtures contain a variety of chemical classes and some fractionation has been done to separate compounds by their polarity, acidity or vapour pressure (Guerin, 1980; LaVoie *et al.*, 1980).

The mutagenic potential of several of these complex pyrolysis mixtures has been reported. Braun

et al. (1987) examined wood smoke extracts and reported that these mixtures were mutagenic in cultured human lymphoblasts but not mutagenic in a bacterial assay. Alfheim *et al.* (1984) examined organic extracts of wood combustion emissions and determined the polar fractions exhibited direct mutagenicity in an Ames/*Salmonella* bacterial assay while fractions containing polycyclic aromatic azarenes, and aromatic amines required metabolic activation. Lewis *et al.* (1988) conducted a study on the mutagenicity of wood smoke and motor vehicle emissions. Their results indicated that ambient aerosol samples of wood smoke and motor vehicle emissions were mutagenic. Cigarette smoke condensate

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has been reported to be mutagenic in the Ames *Salmonella* mutagenicity assay but negative in the mammalian HGPRT (hypoxanthine-guanine phosphoribosyl-transferase) mutation assay (DeMarini, 1982; Doolittle *et al.*, 1990).

Although the mutagenicity of cigarette and wood smoke mixtures has been reported, only a few studies examined the cytotoxicity of these complex mixtures, and we know of no studies which have directly compared the mutagenicity and cytotoxicity of wood smoke condensate (WSC), liquid smoke flavourings (LSF) and cigarette smoke condensate (CSC). The assessment of cytotoxicity is important to determine toxic mechanisms not directly affecting the genetic material. The specific objective of this study was to assess the cytotoxicity and mutagenicity of commercially available liquid smoke flavourings and to compare the magnitude and pattern of the response with cigarette smoke condensate.

MATERIALS AND METHODS

Cigarette smoke condensate

Kentucky Reference 1R4F (K1R4F) cigarettes which represent the average tar (9.5 mg/cig), nicotine (0.82 mg/cig) and CO (9.8 mg/cig) values for US market cigarettes (Steele *et al.*, 1995), were obtained from the University of Kentucky Tobacco and Health Research Institute (Lexington, KY, USA). Cigarette smoke condensate was prepared by smoking the cigarettes using the Federal Trade Commission conditions of a 35-ml puff taken over a duration of 2 sec, once every 60 sec. The total particulate matter (TPM) from 10 K1R4F cigarettes was collected onto a Cambridge filter pad. TPM was extracted from the pad with dimethyl sulfoxide for a theoretical concentration of 10 mg TPM/ml by shaking for 25 min. An actual concentration of 8.4 mg/ml was achieved, which was determined gravimetrically. Dilutions used in the neutral red cytotoxicity assay and the Ames *Salmonella*/microsome assay were prepared fresh from this stock solution and were stored at -70°C .

Wood smoke condensate

The wood smoke condensate used in this study was purchased from Sigma Chemicals (St Louis, MO, USA). The product was prepared from hardwoods by distillation and filtration to remove "insoluble tars" and subsequent removal of low boiling substances (Sigma Biochemicals and Reagents for Life Science Research, 1998, cat. #W 2000).

Liquid smoke flavourings

Four liquid smoke flavourings were purchased from a national grocery store chain. The Food and Drug Administration (1981) described the general procedure for processing liquid smoke flavourings as follows. Smoke produced by the controlled com-

bustion of hickory or maple sawdust is passed through a settling chamber to remove heavy tars and wood ash, then upward through a column of inert material counter-current to a stream of water. The aqueous smoke extract is filtered to remove tars and resins, then is stored to allow it to age. It is filtered again prior to packaging to remove resinous materials that may have formed. This method is the one used most often by US manufacturers of liquid smoke products.

Concentration of particulate matter

The concentration of the particulate matter in each sample was determined gravimetrically as follows: aluminium weigh pans were dried in a 40°C oven. Once a constant weight was achieved, four 5-ml aliquots each of LSF, WSC and CSC sample were put into the dried weigh pans. These samples were dried until they reached a constant weight (120 hr). The concentrations for these samples are presented in Table 1.

Neutral red assay

The neutral red studies used Chinese hamster ovary cells (WBL strain), kindly provided by Covance Laboratories (Vienna, VA, USA). The cells were cultured in Ham's F12 medium (JRH Biosciences, Lenexa, KS, USA) supplemented with 10% foetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA). The cells were grown at 37°C , 95% air and 5% CO_2 in a humidified incubator.

The neutral red cytotoxicity assay (Borenfreund and Puerner, 1984) was modified by Bombick and Doolittle (1995) and is briefly described as follows. CHO cells were plated into 96-well microtitre plates (Falcon, Fisher Scientific) at a density of 10,000 cells in $200\ \mu\text{l}$ medium per well. The plates were incubated for 24 hr to allow cell attachment and the medium was aspirated with a stainless-steel manifold (Fisher Scientific) attached to a vacuum system. $200\ \mu\text{l}$ of the test chemical in fresh medium were added. Each sample was tested in duplicate at concentrations of 0 (control), 10, 25, 50, 75, 100 and $150\ \mu\text{g/ml}$. After a 24-hr exposure period to each of the LSF, WSC and CSC samples, the test sample was removed and replaced with $200\ \mu\text{l}$ of the neutral red working solution. The working solution is 1.5% neutral red (w/v) (Sigma Chemical Company, St Louis, MO, USA; cat. #N 2884) added

Table 1. Concentration (mg/ml) of particulate matter from wood smoke condensate (WSC), liquid smoke flavouring (LSF) and K1R4F cigarette smoke condensate (CSC)

Sample	Concentration (mg/ml)
Wood smoke condensate	186 mg/ml
Brand A liquid smoke flavouring	74 mg/ml
Brand B liquid smoke flavouring	50 mg/ml
Brand C liquid smoke flavouring	60 mg/ml
Brand D liquid smoke flavouring	44 mg/ml
K1R4F cigarette smoke condensate	8.4 mg/ml

to Ham's F12 medium without FCS. The plates were incubated for 3 hr at 37°C in a tissue culture incubator. The neutral red solution was removed and 200 μ l of the 1% formaldehyde (Sigma) fixative were added to each well for 1 min. The fixative was removed and 100 μ l of the 1% acetic acid solvent were added to the cells. The solvent for the assay was made by adding 10 ml glacial acetic acid (Fisher Scientific) to 990 ml of a 50% ethanol/50% water solution (Quantum Chemical Co., Tuscola, IL, USA). The microtitre plates were placed on a shaker for 10 min, and then the absorbance of each well was measured at 540 nm on a microplate reader (Titertek Multiskan MCC, Flow Laboratories, McLean, VA, USA). ANOVA followed by the Scheffé test was used to determine the dose at which the compound became cytotoxic ($P < 0.05$). An EC_{50} value was also determined from the data using probit analysis (Finney, 1971; Gad and Weil, 1986). The EC_{50} is the concentration of the test sample necessary to cause a 50% reduction in growth of the cell population.

Ames assay

The Ames *Salmonella*/microsome plate assay (Maron and Ames, 1983) was used to evaluate mutagenic activity. All samples were tested in *S. typhimurium* strains TA98 and TA100, both with and without mammalian S9 activation. The test sample and the S9 mix, a liver homogenate (Moltox, Annapolis, MD, USA), were premixed with the test bacteria. The S9 concentration was

5% (v/v) in the S9 mix and 0.05 ml of the mix was added to each tube. The mixture was shaken and allowed to incubate for 20 min at 37°C.

2 ml molten top agar was added to the mixture and then poured onto minimal glucose agar plates. The LSF and WSC were tested in triplicate at concentrations of 0, 125, 250, 500, 750 and 1000 μ g/plate. The K1R4F CSC was tested at 0, 25, 50, 75, 100, 125 and 250 μ g/plate in triplicate. The plates were incubated for 48 hr at 37°C.

After the incubation period the plates were read using an Artek colony counter. A sample was considered to be mutagenic if it induced a concentration-dependent increase in revertant numbers with at least one concentration being two times the solvent control. Positive and negative controls were carried out in addition to the test samples. Estimates of mutagenicity on a revertant/mg basis were obtained by linear regression using Bernstein's method for point rejection (Bernstein *et al.*, 1982). This model bases the statistical analysis on the linear portion of the dose-response curve.

RESULTS

The neutral red cytotoxicity assay results of the LSF, WSC and the K1R4F CSC are shown in Fig. 1. The WSC, LSF Brand A, LSF Brand D and CSC exhibited statistically significant ($P < 0.05$) cytotoxicity beginning at 25 μ g/ml, while LSF Brands B and C demonstrated cytotoxicity ($P < 0.05$) at concentrations as low as 10 μ g/ml.

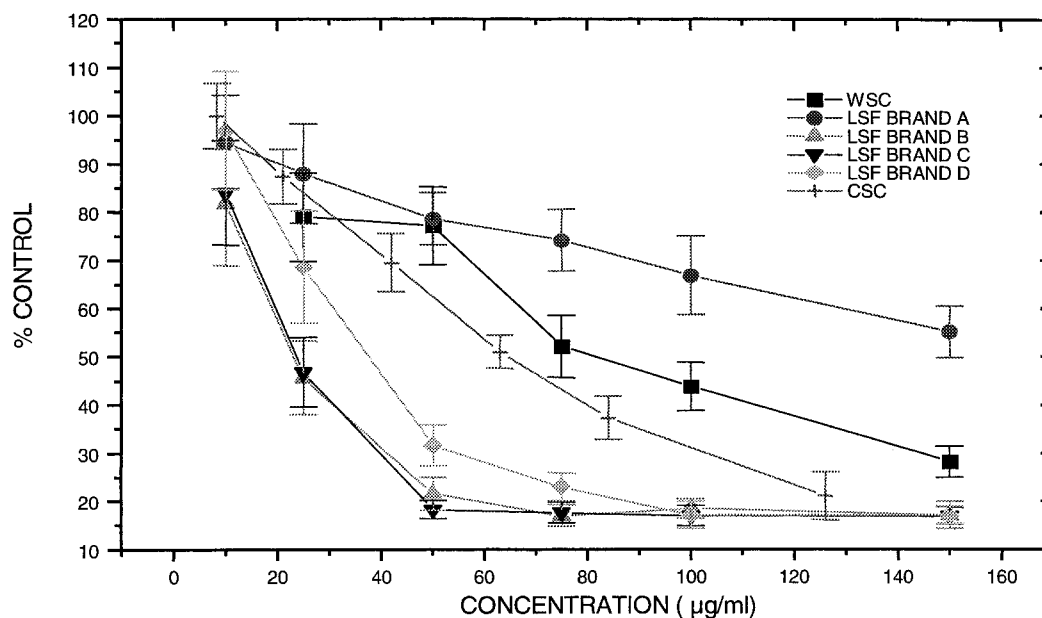


Fig. 1. Dose-response relationship of the wood smoke condensate (WSC), liquid smoke flavouring (LSF) and K1R4F cigarette smoke condensate (CSC) from the neutral red assay (mean \pm SD). CHO cells were plated in 96-well microtitre plates at a density of 10,000 cells/200 μ l media. The cells were exposed to the test samples for 24 hr. The neutral red cytotoxicity assay was performed and the results read on a microplate reader at 540 nm.

Table 2. Cytotoxicity expressed as EC₅₀ values ($\mu\text{g/ml}$) for WSC, LSF and K1R4F CSC

Sample	EC ₅₀ ($\mu\text{g/ml}$)*
Brand A liquid smoke flavouring	230.8 (extrapolated value)**
Brand B liquid smoke flavouring	25.6
Brand C liquid smoke flavouring	26.2
Brand D liquid smoke flavouring	44.3
K1R4F cigarette smoke condensate	62.8
Wood smoke condensate	85.8

*Concentration of the sample ($\mu\text{g/ml}$) to cause a 50% reduction in the growth of the cell population. The EC₅₀ values are determined by performing linear regression on the log of the dose concentration versus the probit number for each of the percent of control averages. **Extrapolated value is above the highest concentration of 150 $\mu\text{g/ml}$ tested.

EC₅₀ values were calculated using the dose-response probit regression lines and are shown in Table 2. The EC₅₀ values show the CSC (62.8 $\mu\text{g/ml}$) and WSC (85.8 $\mu\text{g/ml}$) to be intermediate in cytotoxicity when compared with the four liquid smoke flavouring samples.

The Ames assay with S9 activation was conducted on the four commercial liquid smoke flavourings, WSC and the CSC using *S. typhimurium* strains TA98 and TA100. The four LSF samples and the WSC sample were not mutagenic with TA98 (Table 3). Liquid smoke flavourings Brands B, C and D were mutagenic with TA100 yielding 458, 559 and 447 revertants/mg, respectively (Table 3). The WSC and Brand A LSF were not mutagenic with TA100 according to the criteria used (i.e. doubling of the background). Reduced background lawn (an indication of toxicity) were observed with LSF Brand B at the 1000 $\mu\text{g/plate}$ dose level and Brand C at the 750 and 1000 $\mu\text{g/plate}$ level. The other samples did not exhibit any toxicity up to the 1000 $\mu\text{g/plate}$ dose level. The CSC was mutagenic in both TA98 and TA100.

The liquid smoke samples, WSC and CSC were also analysed by the Ames assay without S9 activation on the same strains. The four liquid smoke flavourings and the wood smoke condensate were observed to be non-mutagenic at the concentration tested in strains TA98 and TA100. The CSC was mutagenic to strain TA98 (124 revertants/mg) but non-mutagenic to TA100. Toxicity was observed

with CSC at the highest dose level (250 $\mu\text{g/plate}$) with TA100.

DISCUSSION

Complex mixtures produced during pyrolysis are often toxicologically active. Commonly encountered pyrolysis products that are mutagenic and toxic include smoke from burned wood, automobile exhaust and cigarette smoke (Ames, 1987). Pyrolysis products arising during cooking of food can be a source of mutagens and toxins (Doolittle, *et al.*, 1989; Sugimura, 1990) and an individual may consume several grams of burned and/or browned material in their diet each day (Ames, 1983).

Alfheim *et al.* (1984) tested the mutagenicity of emissions from wood combustion that had been fractionated by HPLC. The fractions were tested in a modified Ames *Salmonella*/microsome assay using strain TA98 with and without S9 activation. The results revealed that direct mutagenic activity was noted in the most polar fractions, whereas indirect mutagenic activity was associated with the fractions containing polycyclic aromatic hydrocarbons and with polar fractions consisting of aza-arenes and aromatic amines. Alfheim *et al.* point out that although tester strain TA98 (frameshift type) appeared to account for the majority of the mutagenic activity in their samples base-pair substitution mutations were not determined in this study. Two tester strains were used to examine both frameshift and base-pair substitutions in our work.

Lewis *et al.* (1988) reported that ambient aerosol samples of wood smoke were mutagenic. These conclusions were made by using *S. typhimurium* TA98 both with and without S9 activation. Samples tested using S9 were found to be significantly more mutagenic than those tested without S9. This conclusion agrees with our results that *S. typhimurium* strains TA98 and TA100 exhibit a more sensitive response in the presence of S9. The study that Lewis *et al.* conducted used ambient aerosols and our study used condensates.

Braun *et al.* (1987) conducted a study on the mutagenicity of commercial wood smoke flavouring using a forward mutation assay with *S. typhimurium* strain TM677, which detects a variety of inactivat-

Table 3. Results of the Ames assay expressed as revertants/mg (rev/mg*)

Sample	TA98		TA100	
	+S9	-S9	+S9	-S9
Wood smoke condensate	negative**	negative	negative	negative
Brand A liquid smoke flavouring	negative	negative	negative	negative
Brand B liquid smoke flavouring	negative	negative	458	negative
Brand C liquid smoke flavouring	negative	negative	559	negative
Brand D liquid smoke flavouring	negative	negative	447	negative
K1R4F cigarette smoke condensate	1732	124	1660	negative

*Calculated from the linear regression using Bernstein's method for point rejection (Bernstein *et al.*, 1982). **According to the criteria used, none of the dose levels tested (0-1000 $\mu\text{g/ml}$) elicited a response which was double the background.

ing mutations. There was no detectable bacterial mutagenic behaviour in the hickory smoke flavouring using this assay and strain. However, Braun *et al.* (1987) did report wood smoke flavouring to be mutagenic in a lymphoblast assay. The results from this study and the others mentioned above suggest that different forms of wood smoke produce different mutagenic responses.

Our study indicates TA100 detected mutagens in three of the liquid smoke samples and no activity with TA98 with any of the LSFs. This demonstrates that a more specific bacterial mutagenicity assay finds the smoke flavourings to have mutagenic potential, specifically a mutation mechanism indicating base-pair substitutions.

There have been no reports on the cytotoxicity of various wood smoke flavours. However, there have been reports on the cytotoxicity of cigarette smoke condensates (Bombick, B.R. *et al.*, 1998; Bombick, D.W. *et al.*, 1997, 1998a,b). Results from this study indicated a range of cytotoxicity of the wood smoke flavourings with the 1R4F CSC cytotoxicity intermediate within this range. The wide range of cytotoxic responses observed with the wood smoke flavourings and 1R4F CSC may be due to the varied chemical composition of these mixtures. For example, acetaldehyde concentrations range from 0.6 to 658 mg/litre in the various mixtures (1R4F CSC has a concentration of 627 mg/litre) (Guillen and Ibargoitia, 1996a,b, 1998; Guillen *et al.*, 1995; R.J. Reynolds, 1988). Cresols and phenol concentrations were eight and three times higher, respectively, in the wood smoke flavourings than the 1R4F CSC (Guillen and Ibargoitia, 1996a,b, 1998; Guillen *et al.*, 1995; R.J. Reynolds, 1988). Catechol concentrations were often higher in the 1R4F CSC compared with the WSF. Because of the various types of processes and temperatures used to make these wood smoke flavourings, differences in chemical composition of the mixtures may result (Guillen and Ibargoitia, 1996b).

The results of these studies clearly indicate that complex mixtures derived from different sources of pyrolysis differ in their cytotoxic and mutagenic potential. Further toxicological studies on various pyrolysis products, combined with examination of the chemical composition of the mixtures, will lead to a better understanding of the toxic mechanisms.

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