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Food and Chemical Toxicology 43 (2005) 1303–1322
www.elsevier.com/locate/foodchemtox

Toxicologic evaluation of licorice extract as a cigarette ingredient ☆

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Received 20 September 2004; accepted 25 January 2005

Abstract

Licorice extract (block, powder or liquid) may be applied to cigarette tobacco at levels of about 1–4% to enhance and harmonize the flavor characteristics of smoke, improve moisture holding characteristics of tobacco, and act as a surface active agent for ingredient application. Neat material pyrolysis studies, and smoke chemistry and biological activity studies (bacterial mutagenicity, cytotoxicity, micronucleus, and sub-chronic inhalation) with mainstream smoke, or mainstream smoke preparations from cigarettes containing various target levels (1.5–12%) of the licorice extracts were performed to provide data for an assessment of the use of licorice extract as a cigarette tobacco ingredient. At simulated tobacco burning temperatures up to 900 °C all forms of neat licorice extract pyrolyzed extensively, yielding small amounts of benzene, toluene, phenol and acetaldehyde with no indication that licorice extracts would transfer intact to mainstream smoke. As a single ingredient added to cigarette tobacco, block licorice extract at a target level of 12.5% increased smoke constituents including selected PAH, arsenic, lead, phenol and formaldehyde (on a TPM basis), while licorice extract powder (target level of 8% tobacco) increased select PAH, phenol and formaldehyde (on a TPM basis). Lower target application levels (including typical application levels) of block, powder or liquid licorice extract did not significantly alter the smoke chemistry profile. Biological tests indicated no relevant difference in the genotoxic or cytotoxic potential of either mainstream smoke (or smoke preparations) from cigarettes with added licorice extracts compared to control cigarettes. In sub-chronic 90-day rat inhalation studies, the mainstream smoke from cigarettes with 12.5% added block and 8% added powder licorice extract contained higher formaldehyde concentrations compared to control cigarette smoke. Female rats in the 12.5% block licorice extract exposure group displayed an increased incidence and severity of epithelial hyperplasia in the nose (level 2), with no relevant respiratory tract changes in the 8% powder licorice extract exposed rats. At the lower licorice extract application levels (1.25–5%), there was no indication of increased formaldehyde concentration in the smoke atmosphere and no relevant changes in respiratory tract tissues. Mineralcorticoid-like effects which have been associated with excess licorice ingestion were not found in any of the smoke inhalation studies. The results of these studies with various forms of licorice extract applied to cigarette tobacco suggest that

Abbreviations: 11βHSD, 11β-hydroxysteroid dehydrogenase; °C, degrees centigrade; CAS, chemical abstract service; CFR, Code of Federal Regulations; CPSC, US Consumer Product Safety Commission; CO, carbon monoxide; EC_{50}, concentration that reduces the number of viable cell by 50% compared to the vehicle control; DMSO, dimethyl sulfoxide; FTC, Federal Trade Commission; GRAS, generally recognized as safe; GSD, geometric standard deviation; HCN, hydrogen cyanide; HPLC, high performance liquid chromatography; IARC, International Agency for Research on Cancer; ISO, International Organization for Standardization; °K, degrees Kelvin; MMAD, mass median aerodynamic diameter; MS, mass spectrometer; MW, molecular weight; 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; NMEA, *N*-nitrosomethylethylamine; NNN, N′-nitrosonornicotine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NO*x*, Nitrogen Oxides; NPI, N-nitrosopiperidine; NPRA, *N*-nitrosodi-*n*-propylamine; NPY, *N*-nitrosopyrrolidine; NRU, neutral red uptake; OECD, Organization for Economic Cooperation and Development; PAH, polycyclic aromatic hydrocarbons; PCE, polychromatic erythrocytes; SCOGS, Select Committee on GRAS Substances; SD, standard deviation; SE, standard error; TPM, total particulate matter.

☆ Presented in part as a poster at the 42nd annual meeting of the Society of Toxicology, March 9–13, 2003, Salt Lake City, UT, USA.

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0278-6915/$ - see front matter
doi:10.1016/j.fct.2005.01.012

adding licorice extract to cigarette tobacco at levels of ⩽5% does not discernibly alter the smoke chemistry or biological effects normally associated with mainstream cigarette smoke.

1. Introduction

Records of licorice cultivation date back to the third century (Olukoga and Donaldson, 1998). It is used in two primary forms: root and extract. Licorice root contains about 20% of water-soluble extractives much of which (typically 3–5% of the root, but up to 12% in some varieties) is composed of glycyrrhizin, a mixture of potassium and calcium salts of glycyrrhizic acid. Sugars (glucose and sucrose) are also present (Dewick, 1997). Glycyrrhizin constitutes 10–25% of licorice extract and is considered the primary flavor constituent (Chandler, 1985; Samuelsson, 1992; Stormer et al., 1993). Licorice extract is produced by shredding and extracting the root. The extracted liquor is filtered and then either spray dried to produce a powder or concentrated to produce a solid block which generally has a stronger flavor than the powder (Vora, 1984). Licorice extract is also sold as a liquid solid extract where the extracted material is dissolved/suspended in a solvent to produce a syrup-like material.

Licorice and its derivatives are generally recognized as safe (GRAS), and are used in a variety of foods, some over-the-counter drugs and in both traditional and herbal medicines (21 CFR 184.1408, 310.528, 310.544, 582.10, and 582.20). The acute oral toxicity potential of glycyrrhizic acid and licorice extract is low. In mice and rats the oral LD_{50} is in the g/kg range (Komiyama et al., 1977; SCOGS, 1974). Short-term studies in both animals and humans have clearly defined the hypermineralocortocoidism effects of glycyrrhizin consumption (Card et al., 1953; Girerd et al., 1958; Kobuke et al., 1985; Komiyama et al., 1977; Molhuysen et al., 1950). Hypertension, hypokalemia, edema, and loss of plasma renin activity appear to be the most common clinical signs of glycyrrhizin toxicity. Consumption of glycyrrhizic acid by mice for 96 weeks did not elicit carcinogenic or chronic toxic effects (Kobuke et al., 1985).

Glycyrrhizic acid is not a teratogen (Food and Drug Research Laboratories, 1972; Itami et al., 1985), does not induce heritable chromosomal defects in rats or mice (Sheu et al., 1986), and is not likely to be toxic to the developing rodent fetus. Immunological studies have indicated that glycyrrhizic acid can induce the production of γ-interferon (Abe et al., 1987), with some speculation that licorice extract may have immunostimulatory properties (Utsunomiya et al., 1997). In vivo and in vitro tests have shown that glycyrrhizic acid is non-genotoxic (Litton Bionetics, 1972; Oak Ridge National Laboratory, 1982; Stanford Research Institute, 1977; Yamaguchi and Watanabe, 1984; Zani et al., 1993) and may have anti-genotoxic properties (Tanaka et al., 1987).

Licorice extract has been used since the 1880's as an additive in cigarette and pipe tobaccos and snuff (Tilley, 1948). Licorice extract is used in cigarettes both as a flavor and casing material (a mixture of hygroscopic agents and flavors used to facilitate tobacco processing). All three forms (block, powder and liquid) may be used in the production of cigarette tobacco, but they are not necessarily interchangeable because of their different flavor characteristics. Specifically licorice extract provides the following attributes (Vora, 1984):

- Enhances and harmonizes the smoke flavor.
- Reduces dryness in the mouth and throat.
- Improves moisture holding characteristics of tobacco, thus increasing stability and shelf life.
- Acts as a surface active agent during the spraying process of casing ingredients, thus improving the rate of absorption of flavors uniformly and evenly into tobacco.
- Minimizes rough smoke character by balancing out the overall flavor profile of the tobacco smoke.

As stated above, licorice extract is GRAS. Because licorice extract is added to tobacco and potentially burned during the smoking process, it is not possible to justify cigarette use based solely upon its approved use in foods. While there are no regulatory requirements for testing cigarette ingredients, in 1997, the tobacco industry and the United Kingdom 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. Toxicology data on ingredients in the burnt and unburnt form known to the manufacturer are required to be submitted to member states of the European Union (2001), however, there are no specific study requirements or any guidelines for evaluation of the submitted data.

Previous studies have addressed various ingredients and mixtures of ingredients added to cigarettes (Baker et al., 2004a,b,c; 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 smoke chemistry of cigarettes containing

ingredients, they have not suggested any relevant increases in the measured 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 cigarette and potentially detect effects of the ingredient itself. The test cigarettes were designed to encompass representative use levels, as well as higher levels. While the use of multiple test levels seemingly provides the opportunity to potentially generate a dose-response in the effects, the maximum inclusion level was limited by the physical capability to make a cigarette which burned in a manner similar to the control cigarette (that is, the number of puffs and the amount of tar[1] being approximately equivalent). This limitation restricted the highest level that could be tested. Routinely, one would like to test the potential exposure level and some multiples of the exposure level to generate a dose–response and provide information for margins of exposure calculations. Since the licorice extract was being tested as part of a toxic matrix (smoke), it is not possible to test at extreme inclusion levels of the licorice extract without diluting the smoke and thus potentially 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 various forms of licorice extract on the chemistry of smoke, to examine the potential genotoxic and cytotoxic effects of smoke, and to evaluate the inhalation toxicity of smoke in a rat model.

2. Materials and methods

2.1. Licorice extract

Three different preparations of licorice extract were purchased and evaluated. The nature of the preparations was determined by the extraction and drying process. In the first case, the licorice extract solution was dried to a solid block, in the second the extract was fed to a spray drier to produce a powder and in the third case the extract was dissolved in a water-based solution to produce a syrup-like liquid material. For clarity, the three forms were referred to as ''Block Licorice Extract'', ''Powder Licorice Extract'' and ''Liquid Licorice Extract'', respectively. The mean glycyrrhizic acid content of the three forms of licorice extract was 10%, 7.5%, and 5.5%, respectively. The licorice extracts were purchased from commercial suppliers and were of food grade or greater purity.

2.2. Cigarette construction

Studies were conducted with 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%). Cigarettes were 84 mm in length (57 mm tobacco rod, 27 mm filter) and 25 mm in circumference. The average tobacco rod weights ($n = 3$) were 0.775 g, 0.710 g, and 0.713 g for the block, powder and liquid licorice extract cigarettes, respectively. 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 ranging from 1.25% to 12.5% licorice extract added to the tobacco during processing. The low level of each form of licorice extract represented typical application levels in our commercial products. The exaggerated higher levels were selected to maximize the potential to reveal a dose-response. A water, high fructose corn syrup and glycerin mixture was used as a carrier for application of the block licorice extract, with water only used as a carrier for the powder and liquid licorice extract. The tobacco used for the control cigarettes in each respective study was treated with an equal amount of the carrier. Following suspension of the licorice extract in the carrier, the suspension 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. Either of two University of Kentucky reference cigarettes, 1R4F or 2R4F, were utilized in the studies as internal references to monitor study consistency (Diana and Vaught, 1997).

2.3. Cigarette licorice extract analysis

Test cigarettes were analyzed using glycyrrhizic acid as a target marker (Zimmerman and Yang, 1991). Following tobacco and cigarette production, the tobacco was extracted with methanol and a sample of the extract injected on a Hewlett Packard model 1100 liquid chromatograph (ODS Hypersil Reverse Phase C18 column, Hewlett Packard) with a diode array detector (Agilent Technologies, Palo Alto, CA). Glycyrrhizic acid (Acros Organics, Morris Plains, NJ) was used as the standard.

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

2.4. Neat ingredient pyrolysis studies

Pyrolysis studies followed the approach described by Stotesbury et al. (1999). Neat samples of licorice extract 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 (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 chemistry

Smoke chemistry analyses were conducted on preparations of mainstream smoke, gas–vapor phase or particulate fractions collected during machine smoking to determine the potential effects of licorice extract on the toxicologically important constituents of smoke. While machine smoking may, or may not, represent actual human exposure situations, it provides a uniform situation for smoke constituent analysis. 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. TPM was measured gravimetrically. Tar was determined by subtracting the nicotine and water content from the TPM. A total of up to 50 analytes (including the elements and FTC analytes: tar, 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 U.S. Consumer Product Safety Commission (CPSC, 1993) and a monograph from the International Agency for Research on Cancer (IARC, 1986). FTC analytes (tar, nicotine, water, and CO) were determined according to ISO standards with only slight modifications (ISO 10362-2, ISO 8454, ISO 4387, ISO 10315). For elemental analysis, the mainstream cigarette smoke condensate was collected by electrostatic precipitation in quartz tubes. Arsenic, cadmium, nickel, and lead in the condensate were determined by inductively coupled plasma mass spectrometry (Chang et al., 2003). Chromium was determined by graphite furnace atomic absorption spectrometry (Torrence et al., 2002). A reference cigarette, 1R4F or 2R4F, was included as an internal control (results not presented).

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. A reference cigarette, 1R4F or 2R4F, was included as an internal control (results not presented). 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 different *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 licorice extract 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 BALB/c 3T3 mouse embryo 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 or 2R4F, was included as an internal control (results not presented). 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-h 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. Two or 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

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). 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.

2.9. 90-Day inhalation

The biological response of inhaled mainstream smoke was evaluated in three separate 90-day subchronic inhalation studies 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 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 block, powder or liquid licorice extract treated tobacco. For comparison, two control groups of rats were maintained; one exposed to smoke from a control cigarette prepared without licorice extracts, with another group sham exposed to air. A 1R4F or 2R4F cigarette smoke exposure group was included in each study as an internal control (results not presented). 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 licorice extract 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. 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 (1981), and for the larynx according to Lewis (1981).

2.10. Statistical evaluation

Mean values and standard deviations were calculated for all assays. The results were normalized on a TPM basis to allow for comparison across assays. 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. In the block licorice extract studies ordinal data were analyzed using the generalized Cochran–Mantel–Haenszel test for overall comparison followed by a pair wise comparison where appropriate. Results were evaluated for statistical significance at $p \leqslant 0.05$ without adjustment for multiple testing. A statistical analysis was performed on the histopathology data from the block licorice extract study. Significant results were considered as explorative indicators rather than confirmatory evidence of an effect except in cases where the parameters evaluated revealed a consistent, treatment-related response that was biologically meaningful. A difference from control of one severity unit was used as a guide in the evaluation of the powder and liquid licorice extract histopathological data. No other statistical evaluation was performed on these data.

3. Results

TPM is the general unit of dosing for the biological assays reported here with mainstream cigarette smoke. Smoke chemistry is generally measured on a per cigarette basis. However, to facilitate comparison, the smoke chemistry results are also presented in graphs on a normalized TPM basis. It is important to note that addition of licorice extract at typical use levels (1–2.5%) did not change the yield of the cigarettes, irrespective of the basis (TPM, tar, or cigarette basis). The results presented here were obtained from studies conducted over a period of 2 years and performed in two different laboratories. The two laboratories used essentially the same methods. Purkis et al. (2003) have

reviewed interlaboratory variability of smoke chemistry results and concluded that without standardized methods, it is not possible to make meaningful comparisons between laboratories. Some inter-study variability between the three licorice extract studies is therefore not unexpected.

3.1. Cigarette licorice extract analysis

Table 1 shows the target and measured amounts of the licorice extracts in the test cigarettes. Each group of cigarettes was made and tested separately. Although there were small differences between target and measured results, the differences were considered to be due to normal manufacturing variations and losses.

3.2. Pyrolysis studies

Combustion of tobacco has been reported to yield approximately 4000 chemicals (Hoffmann and Hoffmann, 1997). Neat licorice extract was pyrolyzed to determine any additional potential combustion products. Table 2 summarizes the chemicals identified in the pyrolyzed licorice extract samples. Although quantitation of the pyrolysis results was not performed, from the total ion chromatograms, 1-hydroxy-2-propanone, phenol, and 1,3,6-trimethylnapththalene appeared to be the most prevalent materials formed. Production of other minor pyrolysis products resulting from licorice extract pyrolysis (including benzene and acetaldehyde which have been identified as possible human or animal carcinogens) were not unexpected, since pyrolysis of organic materials may lead to formation of these chemicals (Baker and Bishop, 2004). The appearance of sulfur dioxide and methyl chloride in the liquid licorice extract sample was believed to be due to column bleed from a previous sample unrelated to this sample. Head space analysis of the sample at 100 °C did not reveal either of these chemicals (data not shown). The major component of licorice extract, glycyrrhizic acid, was not observed in the pyrolysis studies, suggesting that glycyrrhizic acid would not be present in mainstream cigarette smoke.

3.3. Smoke chemistry

The individual results of smoke chemistry studies for block, powder, and liquid licorice extract cigarettes are shown in Table 3 (cigarette basis) and in the radar charts in Figs. 1–3 (percentage in TPM relative to the control cigarette). The data are presented on a TPM basis to allow for comparison with the unit of dose in the other studies. The TPM, nicotine, water, and thus tar yields were nearly the same for the control and the test licorice extract test cigarettes. Inclusion of 12.5% block licorice extract appeared to reduce the amount of nicotine and CO in the smoke. This is thought to be due to a dilution of the tobacco in the cigarette which is added on a total weight basis. There also appeared to be an increase the amount of lead and arsenic in the smoke on a TPM basis. The medium level (3.75%) appeared to decrease the amount of cadmium and lead. All differences (with the exception of arsenic) between control and test cigarettes were minimal ($\leqslant$20%).

The smoke chemistry analytes were qualitatively examined to establish any pattern of consistent change (i.e. statistically significant changes which appeared to be licorice extract level-dependent). Table 4 presents those selected smoke chemistry analytes which appeared to show some relationship with licorice extract level. The data for the table have been ranked by increasing glycyrrhizic acid concentration rather than the type of licorice extract. This ranking shows a clear effect of adding licorice extract to tobacco in that increasing glycyrrhizic acid content produced more frequent patterns of statistically significant changes in some smoke constituent concentrations.

Block and powder licorice extract increased the amount of formaldehyde in the smoke at the highest levels tested. There was also an apparent increase in phenol and catechol at the medium and high levels for both block and powder. Selected PAH may have also been affected but the increases appeared to be minimal (less than ~20%). Liquid licorice extract did not produce any level-related increases in any smoke constituents. There was a pattern of reduction in a number of nitrogen containing constituents. The low level tested (typical use level) for each form of licorice extract did not

Table 1
Licorice extract content in prepared test cigarettes

Group	% Block licorice extract		% Powder licorice extract		% Liquid licorice extract	
	Target	Measured[a]	Target	Measured	Target	Measured
Control	0	<0.045 (<0.0045)	0	<0.060 (<0.0045)	0	<0.082 (<0.0045)
Low	1.25	0.94 (0.094)	2.0	1.45 (0.108)	2.5	1.89 (0.104)
Medium	3.75	2.69 (0.269)	4.0	4.81 (0.360)	3.75	2.81 (0.154)
High	12.5	9.00 (0.900)	8.0	7.26 (0.544)	5.0	4.04 (0.221)

[a] Measured values represent the mean of two analyses and were based on the measured glycyrrhizic acid content shown in the parentheses. The block licorice extract contained 10%, the powder 7.5% and the liquid 5.5% glycyrrhizic acid.

Table 2
Summary of compounds identified following pyrolysis of neat block, liquid and powder licorice extracts

Molecular weight	Identification	CAS #	Block licorice extract	Liquid licorice extract	Powder licorice extract
44	CARBON DIOXIDE	124-38-9	x[a]	x	x
42	1-PROPENE	115-07-1	x		
30	ETHANE	74-84-0			x
44	ACETALDEHYDE	75-07-0	x		x
50	METHYL CHLORIDE	74-87-3		x	
56	2-METHYL-1-PROPENE	115-11-7		x	
64	SULFUR DIOXIDE	7446-09-5		x	
90	1,3-DIHYDROXY-2-PROPANONE	96-26-4	x		
70	2-PENTENE	109-68-2	x		
58	ACETONE	67-694-1	x	x	x
82	2-METHYLFURAN	534-22-5	x	x	
86	DIACETYL	431-03-8	x	x	x
72	METHYL ETHYL KETONE	78-93-3	x	x	
78	BENZENE	71-43-2	x		
60	GLYCOLALDEHYDE	141-46-8	x	x	x
86	3-METHYLBUTANONE	563-80-4	x		
96	2,5-DIMETHYLFURAN	625-86-5	x	x	
60	ACETIC ACID	64-19-7	x	x	x
74	1-HYDROXY-2-PROPANONE	116-09-6	x	x	x
92	TOLUENE	108-88-3	x	x	x
67	PYRROLE	109-97-7	x		x
106	XYLENE	1330-20-7	x		
106	1,3-DIMETHYLBENZENE	108-38-3			x
76	PROPYLENE GLYCOL	57-55-6		x	
102	METHYL PYRUVATE	600-22-6	x	x	x
82	CYCLOPENTENONE	930-30-3	x	x	x
96	FURFURAL	98-01-1	x	x	x
98	FURFURYL ALCOHOL	98-00-0	x	x	x
100	3-HEXANONE	589-38-8		x	
110	5-METHYLFURFURAL	620-02-0		x	
116	ACETOL ACETATE	592-20-1	x		x
96	2-METHYL-2-CYCLOPENTEN-1-ONE	1120-73-6	x		
120	1,2,3-TRIMETHYLBENZENE	526-73-8	x		
120	1,2,5-TRIMETHYLBENZENE	108-67-8			x
98	1,2-CYCLOPENTANEDIONE	3008-40-0			x
96	3-METHYL-2-CYCLOPENTEN-1-ONE	2758-18-1	x		
112	2-HYDROXY-3-METHYL-2-CYCLOPENTEN-1-ONE	80-71-7	x		x
110	2,3-DIMETHYL-2-CYCLOPENTEN-1-ONE	1121-05-7	x		x
94	PHENOL	108-95-2	x	x	x
124	2-METHOXYPHENOL	90-05-1	x		x
92	1,2,3-PROPANETRIOL	56-81-5	x		
108	2-METHYLPHENOL	95-48-7	x	x	x
126	MALTOL	118-71-8	x	x	x
108	4-METHYLPHENOL	106-44-5	x	x	x
144	2,3-DIHYDRO-3,5-DIHYDROXY-6-METHYL-4H-PYRAN-4-ONE	28564-83-2		x	x
122	2,4-DIMETHYLXYLENOL	105-67-9	x	x	
122	4-ETHYLPHENOL	123-07-9	x	x	x
136	4-METHYL-BICYCLO[3.2.1]OCT-3-EN-2-ONE	62702-89-0	x		
136	2-ETHYL-5-METHYLPHENOL	1687-61-2			x
164	ORTHO HYDROXY-(E)-CINNAMIC ACID	614-60-8			x
126	5-HYDROXYMETHYL-2-FURFURAL	67-47-0		x	
150	2-METHOXY-4-VINYLPHENOL	7786-61-0			x
156	1,6-DIMETHYLNAPHTHALENE	575-43-9	x		
154	2,6-DIMETHYLPHENOL	91-10-1	x		
156	1,7-DIMETHYLNAPHTHALENE	575-37-1			x
110	1,3-BENZENEDIOL	108-46-3	x	x	x
170	2,3,6-TRIMETHYLNAPHTHALENE	829-26-5			x
162	LEVOGLUCOSAN	498-07-7	x		x

(*continued on next page*)

Table 2 (*continued*)

Molecular weight	Identification	CAS #	Block licorice extract	Liquid licorice extract	Powder licorice extract
136	p-HYDROXYACETOPHENONE	99-93-4		x	
184	7-ETHYL-1,4-DIMETHYLAZULENE	529-05-5		x	
162	BETA-D-GLUCOPYRANOSE	498-07-7		x	
166	4-HYDROXYBENZENEPROPANOIC ACID	501-97-3		x	

[a] 'x' indicates that the chemical was identified in the pyrolysate atmosphere.

produce any statistically significant level–related increases in any smoke constituent measured.

3.4. Salmonella typhimurium reverse mutation assay

In general, cigarette smoke condensate is mutagenic in certain *Salmonella* strains. A clear cut mutagenic response was obtained with S9 in strains TA98, TA100, and TA1537 for all cigarettes types (control and test). No, or only borderline, responses were seen in strains TA102 and TA1535. In all of the bacterial strains tested, the specific mutagenicity of the smoke condensate obtained from cigarettes prepared with block, powder or liquid licorice extract added to tobacco was not significantly increased from that of the respective control cigarettes (Table 5). There was a statistically significant reduction in the mutagenicity of the 12.5% block licorice extract cigarettes in strain TA 1537 in the presence of S9.

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). No statistically significant differences were observed between the test and control cigarettes regardless of the form of licorice extract or mainstream smoke fraction tested (Table 6).

3.6. Micronucleus assay

Mainstream smoke from cigarettes made with either powder or liquid licorice extract did not produce micronuclei in immature erythrocytes of rats after 90 days of nose-only inhalation exposure (Table 7). Block licorice extract was not tested for its potential effect on micronuclei formation.

3.7. Subchronic smoke inhalation

Table 8 summarizes the exposure atmosphere conditions for the inhalation studies. The overall mean TPM concentrations for the licorice extract test cigarettes were within ~3% of the target concentration of 150 mg/m^3. The overall mean nicotine concentrations for the test cigarette atmospheres were 9.3–10.6 mg/m^3, while the overall mean CO concentrations were 135–162 ppm. The particle size distribution was within the rat respirable range with mean MMADs of 0.28–0.41 μm and mean GSDs of 1.64–2.02. Consistent with the increased formaldehyde concentrations observed in the smoke chemistry studies with high level block or powder licorice extract, the mean formaldehyde concentration in the smoke atmosphere generated with these cigarettes was increased.

Exposure to the control or test cigarettes did not result in any smoke-related mortality during the three inhalation studies, nor were there any clinical observations related to licorice extract smoke exposure. Consistent with previous cigarette smoke inhalation studies (Vanscheeuwijck et al., 2002), male rats in all smoke exposed groups exhibited decreases in mean weekly body weights during the exposure (Fig. 4; representative data shown for block licorice extract only). However, no licorice extract-related body weight differences were seen between any of the test cigarette groups and the control cigarette group for either sex. Steady state carboxyhemoglobin concentration in the blood was slightly lower in the high powder and block licorice extract groups when compared to the control groups. This was consistent with the slightly lower CO concentrations in the exposure chambers. No other licorice extract-related, biologically relevant changes were observed for any hematology or clinical chemistry parameters measured at the end of the exposure period. Serum nicotine and cotinine levels in both males and females in all smoke exposed groups were increased from those of the sham control group throughout the exposure period; however, no biologically relevant differences were evident between any of the test cigarette groups and the control cigarette group for any of the parameters.

Tidal breathing parameters measured during the exposure period were generally decreased from pre-exposure baseline values in all cigarette smoke exposure groups; however, no differences were seen between the test cigarette and control cigarette groups (data not shown). Exposure to the licorice extract test cigarette smoke did not result in any smoke-related changes in organ weights or gross necropsy observations (data not shown).

Table 3
Mainstream smoke constituent concentrations (per cigarette) in control and test cigarettes containing licorice extract

Constituent	Units	Block licorice extract				Powder licorice extract				Liquid licorice extract			
		Control	Low	Medium	High	Control	Low	Medium	High	Control	Low	Medium	High
TPM	(mg/cig)	10.9 ± 0.3[a]	11.2 ± 0.2	11.0 ± 0.4	10.3 ± 0.2	8.74 ± 0.15	8.97 ± 0.091	9.25 ± 0.28*	9.29 ± 0.19*	8.21 ± 0.41	8.65 ± 0.39	8.46 ± 0.17	8.70 ± 0.31
Tar	(mg/cig)	8.99 ± 0.19	9.22 ± 0.17	9.11 ± 0.26	8.59 ± 0.15*	7.45 ± 0.099	7.47 ± 0.10	7.81 ± 0.23*	8.03 ± 0.24*	6.96 ± 0.30	7.23 ± 0.23	7.16 ± 0.17	7.40 ± 0.19
Nicotine	(mg/cig)	0.803 ± 0.026	0.824 ± 0.012	0.838 ± 0.023	0.718 ± 0.019*	0.783 ± 0.017	0.805 ± 0.016	0.796 ± 0.033	0.815 ± 0.004	0.744 ± 0.025	0.781 ± 0.22	0.748 ± 0.024	0.749 ± 0.024
Water	(mg/cig)	1.11 ± 0.08	1.12 ± 0.04	1.07 ± 0.11	1.03 ± 0.04	0.502 ± 0.096	0.700 ± 0.03*	0.641 ± 0.05*	0.444 ± 0.093	0.511 ± 0.11	0.483 ± 0.006	0.548 ± 0.022	0.559 ± 0.099
Carbon monoxide	(mg/cig)	12.3 ± 0.7	11.8 ± 0.3	11.8 ± 0.2	10.0 ± 0.7*	9.41 ± 0.47	8.73 ± 0.69	9.28 ± 0.10	8.70 ± 0.23	10.9 ± 0.38	10.6 ± 0.17	10.8 ± 0.44	10.9 ± 0.50
1,3-butadiene	(μg/cig)	35.1 ± 2.0	34.7 ± 4.4	33.5 ± 2.4	31.6 ± 1.4	36.7 ± 1.6	35.7 ± 3.1	33.2 ± 4.6	33.6 ± 1.8	27.5 ± 3.7	26.5 ± 1.6	28.6 ± 1.2	27.4 ± 1.7
Isoprene	(μg/cig)	325 ± 21	306 ± 11	351 ± 50	278 ± 11	368 ± 9.2	373 ± 26	343 ± 35	341 ± 18	342 ± 36	330 ± 13	352 ± 9.2	333 ± 18
Formaldehyde	(μg/cig)	24.4 ± 2.0	24.9 ± 2.5	26.8 ± 2.4	33.2 ± 2.6*	21.8 ± 2.1	21.9 ± 1.0	26.4 ± 1.9*	28.9 ± 2.6*	11.0 ± 0.8	12.3 ± 2.1	13.4 ± 0.4	12.7 ± 1.7
Acetaldehyde	(μg/cig)	607 ± 26	596 ± 22	598 ± 23	538 ± 30*	565 ± 20	546 ± 19	560 ± 29	548 ± 33	485 ± 36	522 ± 28	499 ± 8.8	475 ± 63
Acrolein	(μg/cig)	78.2 ± 4.8	77.1 ± 4.5	77.7 ± 4.1	75.3 ± 2.3	53.9 ± 2.5	51.2 ± 2.1	55.0 ± 3.1	54.9 ± 3.9	43.4 ± 3.7	46.6 ± 3.0	44.1 ± 1.8	41.6 ± 5.4
Propionaldehyde	(μg/cig)	46.4 ± 2.7	45.7 ± 1.9	46.5 ± 2.2	42.7 ± 3.2	50.0 ± 2.0	48.4 ± 1.8	50.2 ± 2.5	49.4 ± 3.0	40.0 ± 2.4	43.1 ± 2.2	41.0 ± 0.9	40.1 ± 4.6
Acrylonitrile	(μg/cig)	10.26 ± 0.26	9.97 ± 1.54	9.62 ± 0.93	8.91 ± 0.27	11.1 ± 0.68	10.9 ± 0.76	9.75 ± 0.95*	9.97 ± 0.35	10.9 ± 1.7	10.5 ± 0.67	11.3 ± 0.49	10.9 ± 1.3
Hydrogen cyanide	(μg/cig)	105.6 ± 6.4	102.9 ± 8.0	101.9 ± 1.8	82.4 ± 2.8*	91.0 ± 15	100 ± 3.6	95.3 ± 0.70	93.5 ± 5.2	107 ± 7.2	114 ± 8.4	118 ± 7.4	117 ± 18
2-nitropropane	(ng/cig)	10.44 ± 0.92	9.92 ± 0.92	9.91 ± 1.27	8.37 ± 1.46	12.6 ± 2.0	12.3 ± 1.7	13.7 ± 2.3	13.3 ± 1.3	19.9 ± 2.3	20.8 ± 1.7	24.8 ± 4.0	20.8 ± 1.4
o-toluidine	(ng/cig)	40.6 ± 5.0	45.8 ± 5.2	46.3 ± 4.1	47.6 ± 5.7	44.0 ± 2.8	48.2 ± 2.8	44.3 ± 1.7	46.6 ± 3.0	43.2 ± 2.2	45.1 ± 2.9	46.7 ± 1.1	45.6 ± 5.8
2-naphthylamine	(ng/cig)	4.85 ± 0.97	5.26 ± 0.77	5.27 ± 0.65	5.33 ± 1.02	6.19 ± 0.51	6.71 ± 0.41	5.50 ± 0.30	6.00 ± 0.48	6.37 ± 0.76	6.43 ± 0.70	6.57 ± 0.1	7.08 ± 0.84
4-aminobiphenyl	(ng/cig)	0.860 ± 0.130	0.942 ± 0.153	0.862 ± 0.152	0.964 ± 0.127	1.29 ± 0.12	1.49 ± 0.12*	1.34 ± 0.024	1.18 ± 0.078	1.40 ± 0.10	1.53 ± 0.11	1.57 ± 0.69	1.60 ± 0.10
o-anisidine	(ng/cig)	ND	ND	ND	ND	2.31 ± 0.27	2.82 ± 0.24*	2.18 ± 0.22	2.27 ± 0.30	2.39 ± 0.26	2.53 ± 0.25	2.58 ± 0.13	2.36 ± 0.16
Vinyl chloride	(μg/cig)	27.9 ± 1.5	26.6 ± 3.6	25.1 ± 2.3	24.2 ± 1.4	<19.8	<19.8	<19.8	<19.8	<19.8	<19.8	<19.8	<19.8
Nitrogen oxides	(ng/cig)	235 ± 11	230 ± 10	226 ± 7	187 ± 10*	249 ± 9.4	238 ± 9.0	237 ± 4.8	209 ± 5.0*	280 ± 7.4	285 ± 8.5	291 ± 7.3	289 ± 0.011
Benzene	(μg/cig)	45.4 ± 1.4	45.4 ± 1.3	43.9 ± 3.4	41.6 ± 0.4	37.8 ± 2.8	40.1 ± 1.9	34.8 ± 3.9	35.0 ± 1.4	36.8 ± 4.2	36.0 ± 2.5	39.0 ± 1.5	38.1 ± 3.5
Toluene	(μg/cig)	82.1 ± 6.3	84.2 ± 5.4	82.5 ± 6.5	77.9 ± 1.8	59.6 ± 6.0	61.4 ± 4.1	55.9 ± 5.4	57.2 ± 2.2	61.4 ± 7.7	59.2 ± 4.5	63.5 ± 3.8	63.6 ± 6.7
NDMA	(ng/cig)	<5.00[b]	<5.00[b]	<5.00[b]	<5.00[b]	<1.4	<1.4	<1.4	<1.4	2.76 ± 0.17	2.06 ± 0.078*	2.03 ± 0.31*	2.03 ± 0.19*
NMEA	(ng/cig)	<10.00[b]	<10.00[b]	<10.00[b]	<10.00[b]	<5	<5	<5	<5	<5	<5	<5	<5
NDEA	(ng/cig)	<7.00[b]	<7.00[b]	<7.00[b]	<7.00[b]	<3.6	<3.6	<3.6	<3.6	<3.6	<3.6	<3.6	<3.6
NPRA	(ng/cig)	<11.00[b]	<1.00[b]	<11.00[b]	<11.00[b]	<15	<15	<15	<15	<15	<15	<15	<15
NBUA	(ng/cig)	<9.00[b]	<9.00[b]	<9.00[b]	<9.00[b]	<5.7	<5.7	<5.7	<5.7	<5.7	<5.7	<5.7	<5.7
NPY	(ng/cig)	<7.00[b]	<7.00[b]	<7.00[b]	<7.00[b]	2.81 ± 0.34	4.29 ± 0.12*	2.71 ± 0.13	2.21 ± 0.83	8.59 ± 0.60	6.29 ± 0.33*	6.33 ± 0.50*	6.20 ± 0.90*
NPI	(ng/cig)	<8.00[b]	<8.00[b]	<8.00[b]	<8.00[b]	<4	<4	<4	<4	<4	<4	<4	<4
NNN	(ng/cig)	90.2 ± 7.8	88.0 ± 7.1	89.7 ± 4.8	67.5 ± 6.0*	133 ± 3.8	130 ± 4.3	117 ± 6.5*	97.3 ± 4.9*	146 ± 15	139 ± 4.3	132 ± 7.9	126 ± 7
NNK	(ng/cig)	97.4 ± 6.6	92.9 ± 6.8	106.9 ± 8.7	84.6 ± 5.0	109 ± 2.5	106 ± 4.9	102 ± 3.8*	95.7 ± 3.1*	134 ± 28	117 ± 4.3	108 ± 5.4	105 ± 3.1
Phenol	(μg/cig)	9.18 ± 0.34	9.04 ± 0.32	10.22 ± 0.39	11.09 ± 0.28*	13.6 ± 0.52	15.0 ± 0.69*	16.1 ± 0.66*	17.2 ± 0.61*	12.2 ± 0.85	12.8 ± 0.98	12.7 ± 0.51	12.8 ± 1.2
Catechol	(μg/cig)	40.7 ± 0.8	42.1 ± 0.7*	43.1 ± 0.7*	41.9 ± 0.1*	42.3 ± 0.67	46.0 ± 0.87*	45.5 ± 1.3*	47.4 ± 0.83*	41.3 ± 1.8	41.5 ± 1.0	42.4 ± 0.9	42.0 ± 2.4
Dibenz(*a*,*j*)acridine	(ng/cig)	<2.72[b]	<2.72[b]	<2.72[b]	<2.72[b]	<5	<5	<5	<5	ND	ND	ND	ND
Benzo(*a*)anthracene	(ng/cig)	11.5 ± 0.4	10.6 ± 0.5	11.4 ± 0.3	11.4 ± 0.5	7.89 ± 0.36	8.59 ± 0.45*	9.94 ± 0.31*	9.57 ± 0.33*	9.53 ± 0.27	10.2 ± 0.36*	9.83 ± 0.25	10.7 ± 0.25*
Benzo(*b*)fluoranthene	(ng/cig)	ND	ND	ND	ND	4.29 ± 0.34	4.46 ± 0.27	4.88 ± 0.26	4.46 ± 0.18	4.24 ± 0.17	4.45 ± 0.12	4.36 ± 0.15	4.64 ± 0.15*
Benzo(*k*)fluoranthene	(ng/cig)	11.5 ± 0.4	10.6 ± 0.5	11.4 ± 0.3	11.4 ± 0.5	ND	ND	ND	ND	1.35 ± 0.029	1.44 ± 0.043*	1.39 ± 0.023	1.52 ± 0.039*
Benzo(-)fluoranthenes	(ng/cig)	7.00 ± 0.38	7.14 ± 0.21	7.06 ± 0.37	7.00 ± 0.36	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(*j*)fluoranthene	(ng/cig)	ND	ND	ND	ND	ND	ND	ND	ND	2.80 ± 0.19	3.04 ± 0.15	2.92 ± 0.13	3.21 ± 0.16*
Benzo(*a*)pyrene	(ng/cig)	5.74 ± 0.43	5.92 ± 0.23	6.22 ± 0.25	6.4 ± 0.11*	5.28 ± 0.16	5.77 ± 0.43	6.92 ± 0.17*	6.45 ± 0.27*	5.80 ± 0.24	6.29 ± 0.36	6.08 ± 0.33	6.70 ± 0.23*
Indeno(1,2,3-*cd*)pyrene	(ng/cig)	2.72 ± 0.17	2.87 ± 0.09	2.89 ± 0.09	2.9 ± 0.09	2.32 ± 0.12	2.43 ± 0.14	2.91 ± 0.16*	2.68 ± 0.066*	2.38 ± 0.064	2.53 ± 0.079*	2.49 ± 0.074	2.67 ± 0.047*
Dibenz(*a*,*h*)anthracene	(ng/cig)	0.64[b]	0.61[b]	<0.60[b]	<0.60[b]	<5	<5	<5	<5	0.59 ± 0.022	0.59 ± 0.059	0.61 ± 0.054	0.67 ± 0.022
5-methylchrysene	(ng/cig)	<7.60[b]	<7.60[b]	<7.60[b]	<7.60[b]	<10	<10	<10	<10	<4.8	<4.8	<4.8	<4.8
Dibenzo(*a*,*l*)pyrene	(ng/cig)	ND	ND	ND	ND	ND	ND	ND	ND	<0.29	<0.29	<0.29	<0.29
Dibenzo(it a,*e*)pyrene	(ng/cig)	ND	ND	ND	ND	ND	ND	ND	ND	<0.61	<0.61	<0.61	<0.61
Dibenzo(*a*,*i*)pyrene	(ng/cig)	ND	ND	ND	ND	ND	ND	ND	ND	<0.92	<0.92	<0.92	<0.92
Dibenzo(a,h)pyrene	(ng/cig)	ND	ND	ND	ND	ND	ND	ND	ND	<1.9	<1.9	<1.9	<1.9
Arsenic	(ng/cig)	3.10 ± 0.11	3.30 ± 0.10	2.94 ± 0.08	5.02 ± 0.24*	NE	NE	NE	NE	NE	NE	NE	NE
Cadmium	(ng/cig)	47.14 ± 0.68	43.22 ± 1.3*	44.44 ± 1.79	46.43 ± 2.03	NE	NE	NE	NE	NE	NE	NE	NE
Chromium	(ng/cig)	1.4[b]	<1.2	1.46[b]	<1.2	NE	NE	NE	NE	NE	NE	NE	NE
Nickel	(ng/cig)	<3	<3	<3	<3	NE	NE	NE	NE	NE	NE	NE	NE
Lead	(ng/cig)	15.54 ± 0.53	14.08 ± 0.20*	13.96 ± 1.04*	16.74 ± 0.84	NE	NE	NE	NE	NE	NE	NE	NE

ND = not determined (validated method not available at time of assay); NE = not evaluated.

* Significantly different from the respective control cigarette $p \leqslant 0.05$.

[a] Mean ± standard deviation, $n = 4$–10 determinations per analyte with 4–20 cigarettes being smoked per determination.

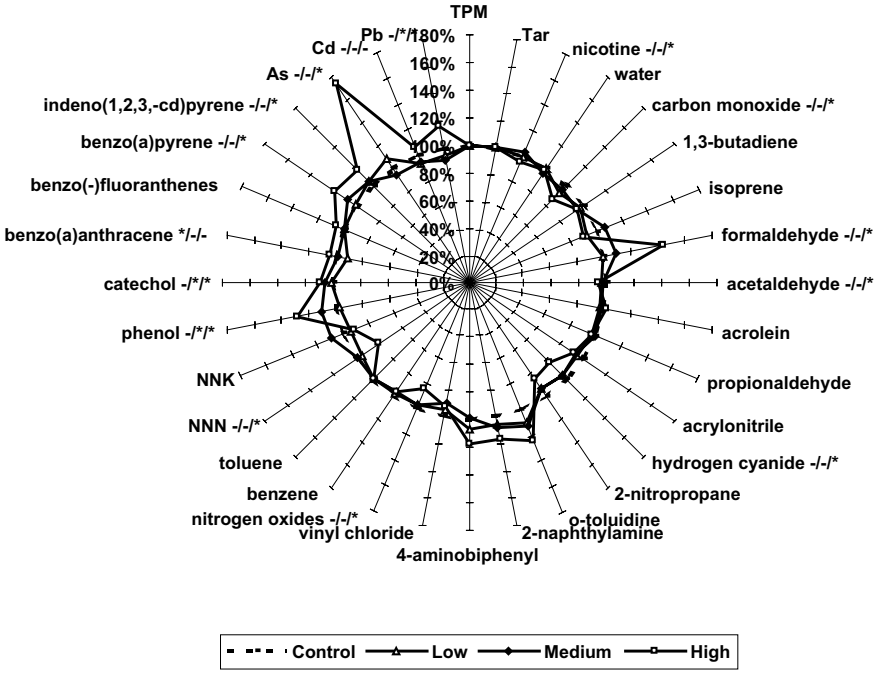

Fig. 1. Relative changes in smoke chemical constituents of block licorice extract treated cigarettes. This radar plot is constructed by setting the control cigarette analyte to 100% and then normalizing each licorice extract analyte to respective control cigarette value. Data were calculated and compared on an equal TPM basis relative to the control cigarette. Target block licorice extract levels were: low = 1.25%, medium = 3.75% and high = 12.50%. Symbols for the test cigarettes represent mean increases or decreases compared to control samples. * = Significantly different from control ($p \leqslant 0.5$) are indicated for low/medium/high levels.

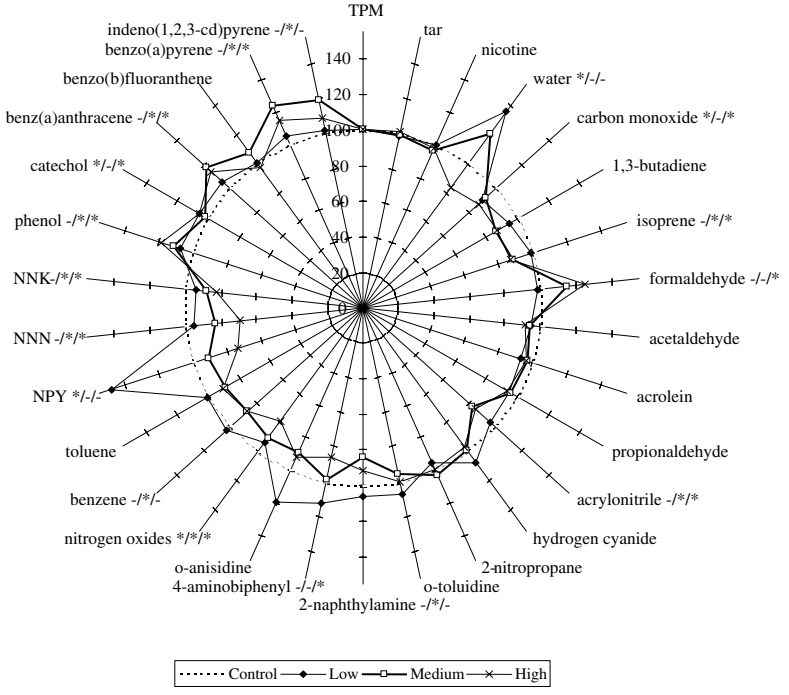

Fig. 2. Relative changes in smoke chemical constituents of powder licorice extract treated cigarettes. Data were calculated and compared on an equal TPM basis relative to the control cigarette. Target powder licorice extract levels were: low = 2%, medium = 4% and high = 8%. Symbols for the test cigarettes represent mean increases or decreases compared to control samples. * = Significantly different from control ($p \leqslant 0.5$) are indicated for low/medium/high levels.

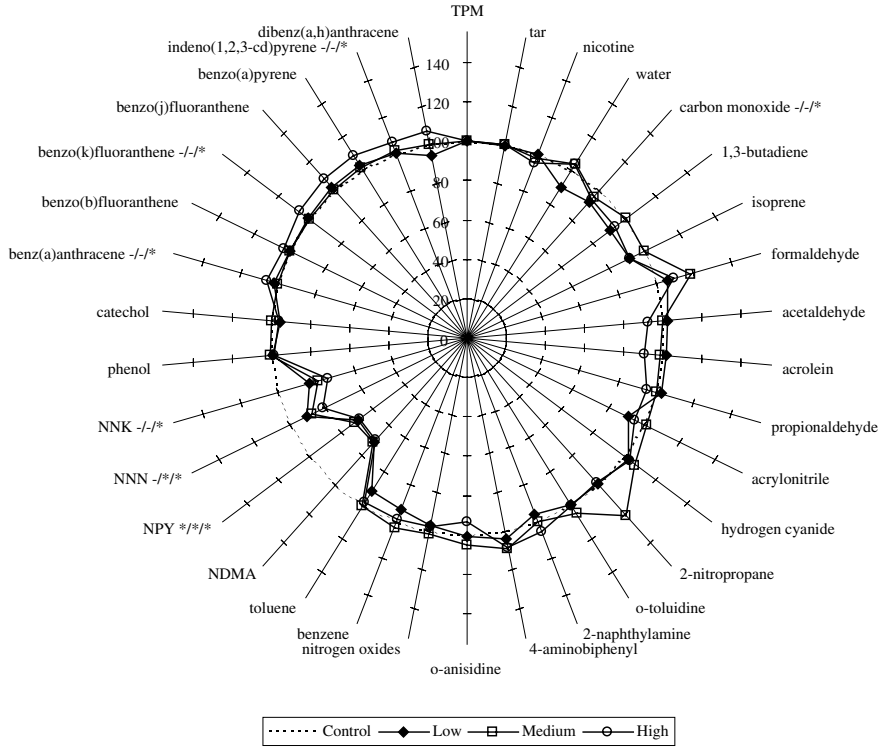

Fig. 3. Relative changes in smoke chemical constituents of liquid licorice extract treated cigarettes. Data were calculated and compared on an equal TPM basis relative to the control cigarette. Target liquid licorice extract levels were: low = 2.5%, medium = 3.75% and high = 5.0%. Symbols for the test cigarettes represent mean increases or decreases compared to control samples. * = Significantly different from control ($p \leqslant 0.5$) are indicated for low/medium/high levels.

Table 4
Summary of statistically significant changes in smoke constituents (TPM basis) ranked by glycyrrhizic acid content of the licorice extract cigarettes

Constituent	Glycyrrhizic acid content (%)								
	0.094	0.104	0.108	0.154	0.221	0.269	0.360	0.544	0.900
Formaldehyde								+23%	+42%
Phenol						+10%	+12%	+17%	+26%
Catechol						+4%		+4%	+8%
Indeno(1,2,3,-*cd*)pyrene							+20%		+14%
Benzo(*a*)pyrene							+25%	+13%	+18%
Benz(*a*)anthracene							+20%	+12%	
NNN				−13%	−19%		−16%	−32%	−22%
NNK				−11%	−16%		−10%	−19%	
NO*x*			−4%				−9%	−22%	−17%
HCN									−18%
Acrylonitrile							−16%	−17%	
Isoprene							−11%	−14%	
CO								−14%	−15%
Nicotine									−6%
Cigarette description	Low block	Low liquid	Low powder	Medium liquid	High liquid	Medium block	Medium powder	High powder	High block

Note: Percent changes represent those statistically significant increases (+) or decreases (−) that were considered to be related to the licorice extract level within an individual study.

Microscopic examination of the tissues of rats exposed to smoke indicated exposure-related changes limited to the respiratory tract. All findings in the respiratory tract were comparable in spectrum and severity to those seen in previous inhalation studies conducted by the testing laboratories 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 smoke from

Table 5
Mutagenic activity of mainstream smoke condensate from control and test cigarettes containing licorice extract

S9	Licorice extract	Group (target % licorice extract)	Bacterial strain specific mutagenicity (per mg TPM)[a]				
			TA98	TA100	TA102	TA1535	TA1537
Yes	Block	Control	4230 ± 190.2	2152 ± 130.1	0 ± 39.6	−3 ± 2.8	448 ± 7.1
		Low (1.25%)	4128 ± 282.8	1963 ± 89.1	25 ± 12.0	1 ± 0.0	386 ± 67.2
		Medium (3.75%)	4110 ± 304.1	2148 ± 157.0	−8 ± 13.4	−4 ± 2.1	357 ± 15.3
		High (12.5%)	3932 ± 111.0	2131 ± 91.2	30 ± 11.3	0 ± 1.4	224 ± 66.5*
	Powder	Control	2897 ± 516.9	803 ± 216.4	−99 ± 154.1	−1 ± 4.2	262 ± 84.1
		Low (2%)	1952 ± 707.8	789 ± 101.1	−38 ± 73.5	2 ± 0.0	356 ± 89.1
		Medium (4%)	2406 ± 301.2	776 ± 62.9	13 ± 31.8	2 ± 3.5	213 ± 28.3
		High (8%)	2125 ± 770.7	663 ± 409.4	−25 ± 23.3	1 ± 2.1	309 ± 101.8
	Liquid	Control	2039 ± 613.1	500 ± 334.5	43 ± 9.9	−6 ± 2.1	262 ± 215.0
		Low (2.5%)	1908 ± 195.9	685 ± 26.9	63 ± 4.2	−4 ± 3.5	317 ± 70.0
		Medium (3.75%)	1756 ± 574.2	528 ± 77.8	−3 ± 7.1	−4 ± 4.2	293 ± 98.3
		High (5%)	1992 ± 357.8	461 ± 96.9	18 ± 103.2	−7 ± 4.9	269 ± 114.6
No	Block	Control	12 ± 4.2	121 ± 0.0	11 ± 19.0	4 ± 2.1	8 ± 0.0
		Low (1.25%)	4 ± 0.0	140 ± 7.8	7 ± 2.8	2 ± 1.4	9 ± 2.8
		Medium (3.75%)	14 ± 0.0	63 ± 14.1	−3 ± 9.9	1 ± 0.7	9 ± 4.2
		High (12.5%)	12 ± 0.7	94 ± 8.5	9 ± 21.9	3 ± 0.7	6 ± 3.5
	Powder	Control	6 ± 9.2	106 ± 32.5	−91 ± 67.2	2 ± 2.8	3 ± 0.0
		Low (2%)	−13 ± 2.8	−10 ± 20.5	−47 ± 8.5	1 ± 4.2	−4 ± 6.4
		Medium (4%)	1 ± 4.9	51 ± 19.8	−133 ± 109.6	1 ± 0.0	−1 ± 4.2
		High (8%)	−3 ± 9.2	81 ± 44.5	−100 ± 169.7	4 ± 0.7	−1 ± 0.7
	Liquid	Control	10 ± 7.8	62 ± 0.0	−17 ± 17.7	3 ± 1.4	3 ± 2.8
		Low (2.5%)	2 ± 0.7	88 ± 35.5	−14 ± 60.1	2 ± 2.1	2 ± 6.4
		Medium (3.75%)	5 ± 5.7	−7 ± 77.1	3 ± 6.4	5 ± 0.7	4 ± 1.4
		High (5%)	3 ± 4.9	53 ± 127.3	−24 ± 72.1	3 ± 1.4	−1 ± 4.9

* Batch 1 and batch 2 statistical comparisons were both significantly different from control ($p \leqslant 0.05$).
[a] Specific mutagenicity (regression coefficient) calculated from approximately linear part of the dose–response curve using Poisson weights, mean ± SD, $n = 2$.

Table 6
Cytotoxicity of mainstream smoke from control cigarettes or cigarettes containing licorice extract

Group	$1/EC_{50}$ (ml/mg TPM)					
	Block licorice extract		Powder licorice extract		Liquid licorice extract	
	Particulate phase	Gas vapor phase	Particulate phase	Gas vapor phase	Particulate phase	Gas vapor phase
Control	8.10 ± 0.45[a]	7.43 ± 0.56	10.43 ± 1.56[b]	6.22 ± 0.52	8.64 ± 0.73[b]	5.10 ± 0.71
Low	8.57 ± 0.28	7.29 ± 0.88	11.64 ± 3.92	5.71 ± 0.85	12.04 ± 7.39	5.40 ± 3.18
Medium	8.54 ± 0.24	7.52 ± 1.26	11.16 ± 3.77	5.86 ± 1.08	7.42 ± 0.60	5.61 ± 2.77
High	8.86 ± 0.17	7.76 ± 1.06	10.15 ± 0.94	6.11 ± 0.57	9.39 ± 0.50	5.26 ± 0.97

[a] Mean ± SD, $n = 2$–3 independent batch collections.
[b] Mean ± SE, $n = 2$–3 independent batch collections.

the licorice extract studies are shown in Table 9. The anterior sections of the nose displayed the highest degree of histopathologic change related to smoke exposure, with the incidence of epithelial hyperplasia and metaplasia approaching 100% in the smoke exposed animals. The severities of these changes were judged to be mild to moderate. Mild to moderate squamous and epithelial hyperplasia was also prevalent in the floor, vocal folds, and vocal cords of the larynx of smoke exposed animals. Histopathologic observations in the lung were limited to mild hyperplasia of the goblet cells in the bronchi and bronchiole, and accumulations of macrophages many of which were pigmented. With the exception of slighter higher incidence and severity of respiratory cell hyperplasia in the nasal sections of rats exposed to smoke from the cigarettes containing target levels of 12.5% block licorice extract, inclusion of licorice extract did not appreciably change the type or extent of histopathological changes normally seen in rat smoke inhalation studies.

Overall, the results of the studies with three forms of licorice extract indicate that addition of licorice extract $\leqslant 5\%$ would not be expected to significantly alter the toxicity of the smoke.

Table 7
Micronuclei frequency in bone marrow of rats exposed to 150 mg TPM/m^3 mainstream smoke from control cigarettes or cigarettes containing licorice extract

Licorice extract	Group (target % licorice extract)	Sex	% Micronucleated PCE[a]
Powder	Control	M	0.12 ± 0.103
	Low (2%)	M	0.13 ± 0.052
	Medium (4%)	M	0.18 ± 0.140
	High (8%)	M	0.15 ± 0.099
	Control	F	0.18 ± 0.088
	Low (2%)	F	0.28 ± 0.189
	Medium (4%)	F	0.21 ± 0.107
	High (8%)	F	0.28 ± 0.197
	Positive control[b]	M	1.97 ± 0.541
Liquid	Control	M	0.12 ± 0.108
	Low (2.5%)	M	0.11 ± 0.058
	Medium (3.75%)	M	0.07 ± 0.038
	High (5%)	M	0.12 ± 0.133
	Control	F	0.10 ± 0.055
	Low (2.5%)	F	0.11 ± 0.074
	Medium (3.75%)	F	0.14 ± 0.080
	High (5%)	F	0.12 ± 0.075
	Positive control[b]	M	1.16 ± 0.541

[a] Mean ± SD, n = 5–6 rats/sample.
[b] Animals were injected intravenously with cyclophosphamide (30 mg/kg i.p.) 24 h prior to necropsy.

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 and propylene glycol are typically added to increase the moisture holding capacity of the tobacco and aid to in processing, while flavor ingredients may be used to complement the subjective characteristics of the smoke. These ingredients include non-volatile materials like sugars and licorice extract, and highly volatile aromatic materials like menthol. Other kinds of ingredients used to enhance the flavor of tobacco smoke include foods such as chocolate and cocoa, and spices such as vanilla, nutmeg, and ginger. Most of the volatile ingredients applied to cigarette tobacco would not be expected to pyrolyze extensively during smoking and would be expected to transfer intact to the smoke; however, less volatile like licorice extract would be expected to pyrolyze in the burning area of the cigarette (Baker and Bishop, 2004; Green et al., 1989).

Smoke inhalation studies in rats and smoke condensate mouse skin painting studies conducted with cigarettes having licorice extract added to the tobacco as a component of an ingredient mixture have previously been reported (Baker et al., 2004a; Carmines, 2002; Gaworski et al., 1998, 1999). Here, various forms of licorice extract were evaluated as single tobacco ingredients in a series of studies design to detect if the

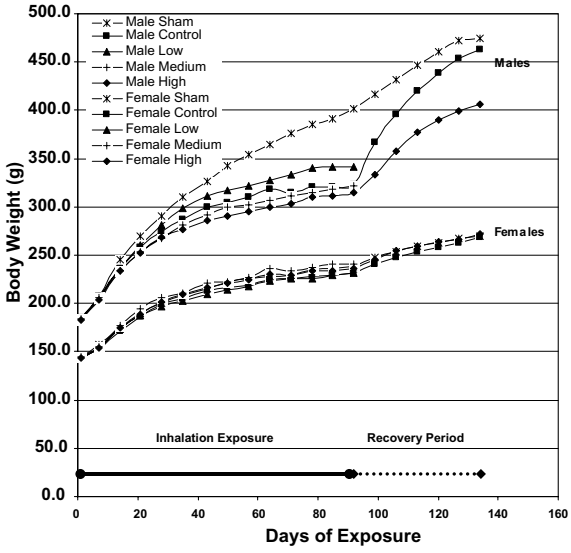

Fig. 4. Mean weekly body weights for block licorice extract smoke exposed rats.

Table 8
Smoke atmosphere conditions measured in the 90-day nose-only inhalation studies

Licorice extract	Group (target % licorice extract)	TPM (mg/m^3)	CO (ppm)	Nicotine (mg/m^3)	Formaldehyde (mg/m^3)	MMAD (μm)	GSD
Block	Control	150 ± 5[a]	153 ± 6	10.6 ± 0.6	0.33 ± 0.02	0.40	1.68
	Low (1.25%)	150 ± 5	151 ± 7	10.1 ± 1.2	0.37 ± 0.03	0.40	1.64
	Medium (3.75%)	148 ± 6	143 ± 6	10.3 ± 1.2	0.33 ± 0.02	0.41	1.66
	High (12.5%)	149 ± 4	135 ± 5	10.2 ± 0.8	0.49 ± 0.03	0.39	1.65
Powder	Control	145 ± 7	156 ± 9	9.6 ± 1.2	0.29 ± 0.03	0.28	1.65
	Low (2%)	146 ± 7	162 ± 11	9.6 ± 1.1	0.31 ± 0.03	0.30	2.02
	Medium (4%)	154 ± 6	153 ± 9	10.0 ± 2.0	0.34 ± 0.07	0.28	1.73
	High (8%)	151 ± 6	143 ± 8	10.6 ± 1.2	0.38 ± 0.04	0.30	1.80
Liquid	Control	146 ± 6	147 ± 6	9.5 ± 0.7	0.20 ± 0.06	0.30	1.85
	Low (2.5%)	152 ± 8	143 ± 7	9.4 ± 1.1	0.21 ± 0.06	0.32	1.83
	Medium (3.75%)	153 ± 6	144 ± 7	9.8 ± 0.9	0.26 ± 0.06	0.32	1.76
	High (5%)	149 ± 7	144 ± 7	9.3 ± 1.0	0.23 ± 0.07	0.29	1.70

[a] Mean ± SD.

Table 9
Summary of major histopathological changes noted in rats exposed to 150 mg TPM/m^3 mainstream smoke from control cigarettes or cigarettes containing licorice extract

Licorice extract	Sex	Site	Histopathological change	Sham	Control	Low	Medium	High
Block	Male	Nose level 1	Respiratory cell hyperplasia	1/10 (0.1)[a]	10/10 (3.0)	9/9 (3.2)	10/10 (3.3)	10/10 (3.2)
			Goblet cell hyperplasia	0/10	8/10 (1.9)	9/9 (2.3)	9/10 (2.1)	9/10 (2.2)
			Squamous metaplasia	0/10	10/10 (2.3)	9/9 (2.4)	10/10 (2.1)	10/10 (1.7)
			Loss of goblet cells	0/10	3/10 (1.0)	2/9 (0.7)	1/10 (0.4)	2/10 (0.7)
		Nose level 2	Respiratory cell hyperplasia	0/10	4/10 (0.4)	4/9 (0.4)	3/10 (0.3)	6/10 (0.6)
			Squamous metaplasia	0/10	0/10	0/9	0/10	0/10
			Olfactory—atrophy	0/10	7/10 (1.9)	3/9 (0.3)	5/10 (1.7)	3/10 (1.1)
			Olfactory—squamous metaplasia	0/10	9/10 (2.3)	8/9 (2.1)	9/10 (2.1)	10/10 (2.7)
		Nose level 3	Olfactory—atrophy	0/10	5/10 (2.1)	3/9 (0.8)	5/10 (1.3)	4/10 (0.8)
			Squamous metaplasia	0/10	9/10 (1.6)	8/9 (1.4)	7/10 (1.2)	7/10 (1.4)
		Larynx	Arythenoid projections—hyperplasia	0/10	3/10 (0.3)	1/9 (0.1)	3/10 (0.3)	2/10 (0.2)
			Arythenoid projections squamous metaplasia	0/10	7/10 (1.2)	6/9 (1.2)	5/10 (0.5)	6/10 (0.9)
			Floor—hyperplasia	0/10	10/10 (3.1)	9/9 (3.2)	10/10 (3.0)	10/10 (3.1)
			Vocal Cords—squamous hyperplasia	0/10	10/10 (2.5)	9/9 (3.2)	10/10 (3.0)	10/10 (3.1)
			Vocal Cords—pseudo hyperplasia	0/10	0/10	1/9 (0.1)	4/10 (0.4)	3/10 (0.3)
			Vocal Cords—squamous metaplasia	0/10	10/10 (1.5)	8/9 (1.3)	6/10 (0.8)	7/10 (1.0)
			Vocal Folds—squamous hyperplasia	0/10	9/10 (1.9)	9/9 (2.3)	10/10 (2.2)	10/10 (2.3)
		Trach. bifurcation	Reserve cell hyperplasia	0/10	2/10 (0.2)	4/9 (0.4)	2/10 (0.2)	1/10 (0.1)
			Goblet cell hyperplasia	1/10 (0.1)	10/10 (3.3)	9/9 (3.8)	10/10 (2.3)	10/10 (3.4)
			Squamous hyperplasia	0/10	0/10	1/9 (0.1)	0/10	0/10
		Lung	Bronchi—reserve cell hyperplasia	0/10	1/10 (0.1)	0/9	0/10	0/10
			Bronchi—goblet cell hyperplasia	1/10 (0.1)	10/10 (4.2)	9/9 (4.0)	9/10 (3.2)	10/10 (4.3)
			Alveolar macrophages	0/10	6/10 (0.6)	7/9 (0.8)	4/10 (0.4)	4/10 (0.4)
	Female	Nose level 1	Respiratory cell hyperplasia	0/10	9/10 (2.2)	9/9 (3.3)*	10/10 (2.4)	9/9 (3.4)*
			Goblet cell hyperplasia	0/10	9/10 (1.4)	9/9 (1.7)	7/10 (1.4)	8/9 (1.8)
			Squamous metaplasia	0/10	10/10 (2.2)	9/9 (1.9)	10/10 (1.8)	9/9 (2.4)
			Loss of goblet cells	0/10	6/10 (2.0)	4/9 (1.4)	2/10 (0.8)	5/9 (1.8)
		Nose level 2	Respiratory cell hyperplasia	0/10	3/10 (0.3)	2/9 (0.2)	1/10 (0.1)	7/9 (0.8)*
			Squamous metaplasia	0/10	0/10	0/9	1/10 (0.1)	0/9
			Olfactory—atrophy	0/10	4/10 (1.6)	3/9 (1.2)	2/10 (0.6)	4/9 (1.8)
			Olfactory—squamous metaplasia	0/10	10/10 (2.2)	9/9 (2.1)	10/10 (2.5)	9/9 (2.3)
		Nose level 3	Olfactory—atrophy	0/10	10/10 (2.0)	9/9 (2.1)	8/10 (1.5)	8/9 (1.6)
			Squamous metaplasia	0/10	10/10 (2.0)	9/9 (2.1)	8/10 (1.5)	8/9 (1.6)
		Larynx	Arythenoid projections—hyperplasia	0/10	3/10 (0.3)	0/9	0/10	0/9
			Arythenoid projections—squamous metaplasia	0/10	7/10 (1.6)	5/9 (1.0)	7/10 (1.1)	7/9 (1.1)
			Floor—hyperplasia	0/10	10/10 (3.2)	9/9 (2.8)	10/10 (2.9)	9/9 (2.9)
			Vocal cords—squamous hyperplasia	0/10	10/10 (2.3)	9/9 (2.2)	10/10 (2.3)	9/9 (2.3)
			Vocal cords—pseudo hyperplasia	0/10	4/10 (0.4)	4/9 (0.4)	4/10 (0.4)	2/9 (0.2)
			Vocal cords—squamous metaplasia	0/10	6/10 (0.6)	4/9 (0.9)	5/10 (0.7)	6/9 (1.0)
			Vocal folds—squamous hyperplasia	0/10	10/10 (2.1)	8/9 (2.0)	10/10 (1.7)	9/9 (2.0)

		Trach. bifurcation	Reserve cell hyperplasia	0/10	1/10 (0.1)	1/9 (0.1)	4/10 (0.4)	3/9 (0.3)
			Goblet cell hyperplasia	3/10 (0.6)	9/10 (2.1)	9/9 (2.7)	10/10 (3.5)	9/9 (3.6)
			Squamous hyperplasia	0/10	0/10	1/9 (0.1)	1/10 (0.1)	0/10
		Lung	Bronchi—reserve cell hyperplasia	0/10	2/10 (0.2)	0/9	1/10 (0.1)	3/9 (0.3)
			Bronchi—goblet cell hyperplasia	1/10 (0.1)	10/10 (4.3)	9/9 (4.1)	10/10 (4.0)	9/9 (4.6)
			Alveolar macrophages	0/10	9/10 (1.0)	8/9 (0.1)	9/10 (0.9)	5/9 (0.6)
Powder	Male	Nose level 1	Squamous metaplasia	0/10[b]	9/10 (1.80)	10/10 (1.70)	9/10 (1.80)	10/10 (1.90)
			Inflammation, subacute,	0/10	7/10 (1.20)	9/10 (1.20)	6/10 (0.80)	8/10 (1.00)
			Hyperplasia/hypertrophy, goblet cell	7/10 (1.00)	8/10 (2.00)	8/10 (1.80)	8/10 (1.10)	9/10 (2.10)
		Nose level 2	Hyperplasia, respiratory epithelium	0/10	10/10 (1.80)	10/10 (2.50)	10/10 (2.00)	10/10 (2.20)
			Atrophy, olfactory epithelium	0/10	1/10 (0.20)	1/10 (0.20)	1/10 (0.20)	4/10 (0.90)
		Larynx	Vocal folds—hyperplasia, epithelium	1/10 (0.10)	10/10 (1.40)	9/10 (1.50)	7/8 (1.38)	10/10 (1.20)
		Trachea	Goblet cell activity	4/10 (0.80)	5/10 (0.60)	9/10 (1.60)[+]	9/10 (1.60)[+]	9/10 (1.20)
		Lung	Accumulation, macrophage, focal	0/10	7/10 (1.80)	3/10 (0.60)[+]	5/10 (1.20)	5/10 (1.40)
			Goblet cell activity	8/10 (0.80)	6/10 (0.80)	10/10 (1.30)	10/10 (2.00)[+]	8/10 (1.10)
	Female	Nose level 1	Squamous metaplasia	0/10	10/10 (1.50)	8/10 (1.30)	10/10 (1.90)	10/10 (1.90)
			Inflammation, subacute,	0/10	9/10 (1.10)	8/10 (0.90)	6/10 (0.80)	9/10 (1.00)
			Hyperplasia/hypertrophy, goblet cell	0/10	7/10 (1.50)	5/10 (0.60)	5/10 (0.60)	6/10 (0.90)
		Nose level 2	Hyperplasia, respiratory epithelium	0/10	10/10 (2.00)	10/10 (1.70)	10/10 (1.40)	10/10 (1.50)
			Atrophy, olfactory epithelium	0/10	7/10 (1.60)	1/10 (0.30)[+]	3/10 (0.60)[+]	4/10 (1.00)
		Larynx	Vocal folds—hyperplasia, epithelium	0/9	8/10 (1.50)	8/10 (1.20)	10/10 (1.60)	7/9 (1.11)
		Trachea	Goblet cell activity	4/10 (0.40)	9/9 (2.00)	8/10 (1.70)	10/10 (2.00)	8/10 (1.70)
		Lung	Accumulation, macrophage, focal	0/10	7/10 (1.80)	6/10 (2.10)	6/10 (2.10)	5/10 (1.70)
			Goblet cell activity	6/10 (0.60)	8/10 (1.00)	8/10 (1.00)	7/10 (0.70)	9/10 (1.30)
Liquid	Male	Nose level 1	Squamous metaplasia	0/10	10/10 (2.70)[b]	10/10 (1.60)[+]	10/10 (1.90)	10/10 (2.20)
			Inflammation, subacute, submucosa	1/10 (0.30)	8/10 (1.60)	4/10 (0.70)	0/10[+]	5/10 (0.90)
			Hyperplasia/Hypertrophy, goblet cell	1/10 (0.10)	9/10 (2.10)	8/10 (1.20)	6/10 (0.70)[+]	8/10 (2.10)
		Larynx	Vocal folds—hyperplasia, epithelium	1/10 (0.10)	7/9 (1.00)	10/10 (1.60)	9/10 (2.00)[+]	8/10 (1.10)
		Trachea	Goblet cell activity	1/10 (0.10)	7/8 (1.25)	9/10 (1.10)	7/8 (1.25)	6/9 (1.00)
		Lung	Accumulation, macrophage, focal	0/10	8/10 (1.50)	6/10 (0.80)	4/10 (1.00)	5/10 (0.50)[+]
	Female	Nose level 1	Squamous metaplasia	0/10	10/10 (2.40)	10/10 (1.40)[+]	10/10 (1.70)	10/10 (2.40)
			Inflammation, subacute, submucosa	1/10 (0.30)	5/10 (0.70)	7/10 (0.90)	5/10 (0.60)	7/10 (0.70)
			Hyperplasia/hypertrophy, goblet cell	0/10	7/10 (1.00)	6/10 (0.70)	4/10 (0.50)	8/10 (1.30)
		Larynx	Hyperplasia, cuboidal epithelium	0/10	10/10 (1.30)	6/9 (1.00)	9/10 (1.60)	9/9 (1.22)
		Trachea	Goblet cell activity	2/10 (0.20)	10/10 (2.20)	8/9 (1.56)	6/9 (1.00)[+]	7/10 (1.10)[+]
		Lung	Accumulation, macrophage, focal	0/10	8/10 (1.50)	9/10 (1.40)	6/10 (1.50)	8/10 (1.30)

[*] Statistically different from control at $p \leqslant 0.05$.

[+] Group severity different by at least 1.00 from Control group severity; Interpreted as a finding of probable biological significance.

[a] Incidence (mean group severity score graded on a 5 point grading scale of 1 = minimal and 5 = severe).

[b] Incidence (mean group severity score graded on a 4 point grading scale of 1 = minimal and 4 = severe).

ingredient alone altered the smoke chemistry or biological effects associated with cigarette smoke.

Neat licorice extract pyrolyzed under simulated smoking temperatures suggested that the licorice extract would yield chemicals consistent with normal pyrolysis of organic material. All of the chemicals identified in the pyrolysis studies have been previously identified in tobacco smoke. No glycyrrhizic acid was observed in the pyrolysate. The fate of glycyrrhizic acid and glycyrrhetinic acid during the smoking of cigarettes has previously been studied by adding these acids directly to cigarettes (Sakagami, 1973). It was found that glycyrrhizic acid decomposed to glycyrrhetinic acid and was transferred into the mainstream smoke as such. When glycyrrhetinic acid itself was added to cigarettes, it was transferred intact to the mainstream smoke. In both cases, the glycyrrhetinic acid found in the smoke condensate was small (2% of the applied material), and it was concluded that the glycyrrhizic acid in licorice root used for tobacco flavoring was mostly decomposed on smoking. These studies used high levels of the pure chemicals applied directly to cigarettes. No other direct data exists for the transfer of glycyrrhetinic acid or glycyrrhizic acid into smoke from licorice extract applied to cigarettes.

Chemical analysis of the smoke from cigarettes containing various forms of licorice extract at typical application levels did not indicate any substantial increases in the amounts of the measured smoke constituents considered to be toxic. However, as the amount of licorice extract was increased (up to 10 fold the typical use level), a pattern of increased formaldehyde, phenol and 4-aminobiphenyl appeared. There was also an increase in some of the PAH and decrease in nitrogen containing chemicals at exaggerated use levels. Licorice extract is reported to contain up to 4% sugar (Dewick, 1997), and the combustion of sugar has been postulated to contribute to the increase of formaldehyde (Baker and Smith, 2003).

Smoke chemistry analysis also suggested an increase in arsenic and lead in the cigarettes containing 12.5% block licorice extract. A potential explanation for this is that licorice extract is derived from plant material, and that it may contain elements due to normal plant nutrient uptake. This uptake is expected to be dependent on soil chemistry and plant physiology.

Wynder and Hoffmann (1967) have previously speculated that, because of the structure of glycyrrhizic acid, pyrolysis of licorice extract would lead to the formation of PAH. Rodgman (2002) reviewed the effect of licorice on mainstream smoke properties suggesting that pyrolysis studies on licorice could lead to PAH production. Under the conditions of our pyrolysis experiment, no multi-ring aromatic molecules larger that substituted naphthalenes were produced. Smoke chemistry studies on cigarettes with block and powder licorice extract resulted in an increase in indeno(1,2,3,-*cd*)pyrene, benzo(*a*)pyrene, and benz(*a*)anthracene on a TPM basis at licorice extract addition levels in the range of 4–12.5%. No increases in PAH production were observed at the lower typical tobacco use levels. Since it is hypothesized that the PAH are being produced from glycyrrhizic acid, it is interesting to look at the relationship of the increase in PAH production relative to the actual glycyrrhizic acid content. This information is shown in Table 4. It appears that below 0.36% glycyrrhizic acid in the tobacco, no statistical significant increase in PAH was observed. Typical use levels of licorice extract, up to a glycyrrhizic acid content of 0.269%, did not increase the PAH levels. Application of exaggerated amounts of licorice extract above 4% may contribute to the formation of select PAH.

It has been suggested (Vleeming et al., 2004) that it is more appropriate to evaluate the smoke chemistry results on a tobacco weight basis rather than relative to the cigarette or TPM. The test cigarettes used in this series of studies were manufactured to a constant weight with the licorice extract displacing tobacco. If the licorice extract were inert and did not combust, the smoke constituents and toxicity should be reduced. As indicated above, the licorice extract appears to combust to materials that are consistent with the combustion of organic matter. That is, licorice extract would be expected to burn in a manner similar to tobacco. Fig. 5 presents the results of the block licorice extract smoke chemistry when calculated on a tobacco basis. The yield of each constituent was first divided by the amount of tobacco in the cigarette after removal of the weight of the licorice extract. Each value was then divided by the value for the control cigarette. As can be seen, the relative amounts of each smoke constituent are not significantly affected by calculation on a tobacco weight basis (compare Figs. 1 and 5). Based on these results calculated in this manner, there does not appear to be any effect of adding licorice extract that is not observed when the data are calculated on a cigarette or TPM basis. Calculation in this manner does not appear to underestimate the amount of the smoke constituents as suggested by Vleeming et al. (2004).

Mutagenicity, clastogenicity, and in vitro cytotoxicity studies did not reveal any increases in the biological activity of various fractions of smoke from the licorice extract cigarettes. This is in agreement with previous work with groups of cigarette ingredients (Roemer et al., 2002; Baker et al., 2004a) where no effects were observed.

In the 90-day rat inhalation studies, the smoke from cigarettes with 12.5% block and 8% powder licorice extract contained higher formaldehyde concentrations when compared to the control cigarette smoke (see Table 8). Female rats in the 12.5% block licorice extract exposure group displayed an increased incidence and

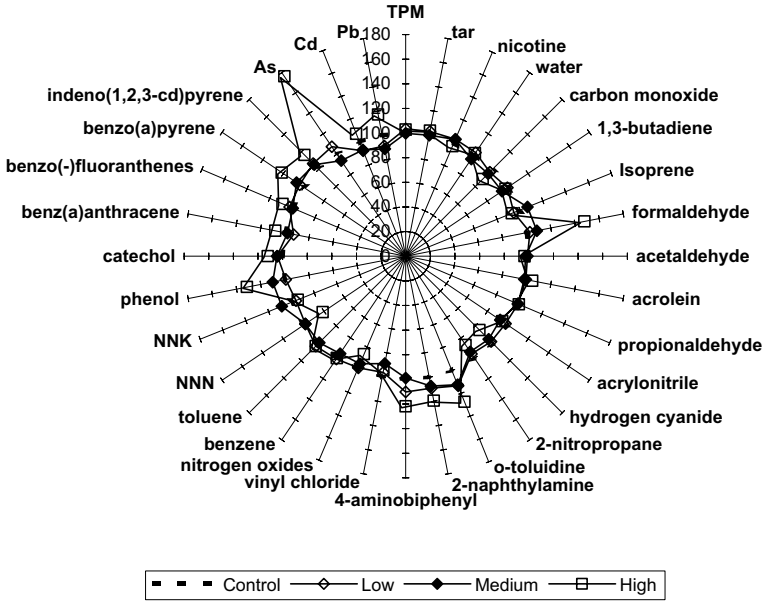

Fig. 5. Relative changes in smoke chemical constituents of block licorice extract treated cigarettes calculated on a tobacco basis. This radar plot was constructed by first dividing the measured cigarette yield by the average weight of tobacco in the cigarette excluding the weight of the licorice extract. The values for the control cigarette were set to 100% and each licorice extract analyte was divided by the control cigarette value. Target block licorice extract levels were: low = 1.25%, medium = 3.75% and high = 12.50%.

severity of epithelial hyperplasia in the nose (level 2), while male rats in this exposure group did not. The average smoke level of formaldehyde in the control, low and mid groups was about 0.33 mg/m^3 (~0.27 ppm). With high block licorice extract smoke, the average formaldehyde level was 0.49 mg/m^3 (~0.41 ppm). Extensive studies have been performed on the toxicity of formaldehyde. These studies have demonstrated that the effect of formaldehyde on the respiratory epithelium is concentration dependent and a not a function of concentration × time (Appelman et al., 1988; Swenberg et al., 1983; Wilmer et al., 1989). The principal effects of formaldehyde after 13 weeks of exposure are metaplasia and hyperplasia in the respiratory epithelium. These effects are seen after exposure to 10 ppm (6 h/day, 5;days/week) but not 1 or 0.1 ppm (Appelman et al., 1988). In a 13 week study designed to look at the effects of intermittent vs. continuous exposure to formaldehyde, Wilmer et al. (1989) found the non-toxic effect level to be 2 ppm. Rusch et al. (1983) exposed rats, hamsters and monkeys to about 0.2, 1.0, or 3.0 ppm formaldehyde for 22 h/day, 7 days/week for 26 weeks. The 3 ppm level produced effects in the rats and monkeys but not in the hamsters. Based on these findings, it is unlikely that an increase of about 0.1 ppm of formaldehyde alone could be responsible for the changes observed in the high block licorice extract smoke exposed animals. Studies where the nasal mucosa has been damaged by electrocoagulation followed by exposure to formaldehyde have been conducted (Appelman et al., 1988; Woutersen et al., 1989). The major conclusion is that damaged mucosa is more susceptible to the cytotoxic effects of formaldehyde than undamaged mucosa. Under the conditions of the studies reported here, it is possible that other cytotoxic constituents of smoke are making the nasal epithelium more sensitive to the toxic effects of formaldehyde. It is unlikely that the small increase in formaldehyde concentration (<1 ppm) of the smoke alone is responsible for the histopathologic changes in the female rats of the 12.5% block licorice extract group. There were no significant findings in the 8% powder licorice extract exposed rats. At the lower licorice extract application levels (1.25–5%), there was no indication of increased formaldehyde concentration in the smoke atmosphere and there were no significant effects noted in respiratory tract tissues.

Licorice extract and glycyrrhizic acid are known to produce mineralocorticoid-like effects in animals and humans at high doses by inhibition of 11β-hydrosteroid dehydrogenase (11β-HSD) (Stormer et al., 1993; Whorwood et al., 1993). No mineralocorticoid effects were observed in the 90-day nose only inhalation studies reported here even at cigarette inclusion levels up to 10× the typical level. Maser (2004) has suggested that glycyrrhizic acid inhibition of 11β-HSD could result in an increase in the levels of the lung carcinogen NNK by inhibiting its detoxification to the NNK alcohol (NNAL). He postulates that inclusion of licorice extract in cigarettes potentiates the carcinogenic response in humans. This supposition requires two assumptions: that glycyrrhizic acid transfers intact to cigarette smoke, and that the dose in humans would be sufficient to

inhibit 11β-HSD resulting in more NNK. Typical use levels of licorice extract resulted in approximately 0.1 mg of glycyrrhizic acid per gram of tobacco (Table 1). The test cigarettes used in these studies contained 0.7–0.8 g of tobacco. Thus, typical cigarettes would be expected to contain at most 0.08 mg of glycyrrhizic acid. Our pyrolysis studies with licorice extract did not indicate that glycyrrhizic acid would be expected to survive cigarette smoking temperatures and transfer intact to smoke. Sakagami (1973) studied the transfer of high levels of pure glycyrrhizic acid to cigarettes and concluded that at most 2% could transfer. Using this information it is possible to calculate the maximum potential level of glycyrrhizic acid in smoke: 0.0016 mg/cigarette (2% × 0.08 mg). Assuming that a person smokes 2 packs of cigarettes per day and that all of the glycyrrhizic acid is absorbed, the daily human dose might be 0.064 mg/day (40 cigarettes × 0.0016 mg/cigarette). Bernardi et al. (1994) evaluated the effects of graded doses of pure licorice root extract in order to identify the dosages leading to mineralocorticoid-like side effects in humans. Four groups of six volunteers were fed licorice root extract containing 108, 217, 380 or 814 mg glycyrrhizic acid daily for 4 weeks. No significant effects occurred in the groups receiving the two lowest doses. One subject from the 380 mg/day group and two subjects from the 814 mg/day group were forced to withdraw from the study due to headaches, hypertension, hypokalemia and/or edema. A significant depression of plasma renin activity was found only in those subjects of the two highest dose groups. The authors concluded that the untoward effects of pure licorice root extract were dose-related and were less common than after the intake of comparable amounts of pure glycyrrhizic acid. A minimum dose of 380 mg glycyrrhizic acid/day was needed to produce the effects. Based on this data it seems highly unlikely that the use of licorice extract in cigarettes will result in smoke delivery levels of glycyrrhizic acid that could inhibit 11β-HSD.

Bates et al. (1999) have speculated (not peer reviewed) that licorice extract may cause bronchodilation thereby making it easier to inhale cigarette smoke. In the present series of inhalation studies, the respiratory physiology of rats exposed to smoke from cigarettes with up to ten fold higher levels of licorice extract was not different from the control animals. There was no indication that inclusion of licorice extract in the tobacco had any effect on tidal volume, respiratory frequency or minute volume. The statement that glycyrrhizic acid acts as a bronchodilatator could not be substantiated in the literature. There are no biological data to support the contention that licorice extract acts as a bronchodilator.

These studies were designed to evaluate the potential effects of licorice extract added to cigarette tobacco at typical use levels. Higher application levels were included to demonstrate a margin of exposure and also to maximize the potential of detecting an effect within the cigarette smoke atmosphere. The exaggerated levels of licorice extract did not produce any new or unique effects beyond those which are typically seen with any of the assays used. The increases in some smoke constituents are consitent with normal combustion processes. There was no change in the in vitro biologic activity as measured by cytotoxicity or bacterial reverse mutation assays. There also were no effects on micronuclei formation in vivo. The exaggeratted level of block licorice at a target level of 12.5% appeared to increase the incidence and severity of respiratory cell hyperplasia in nose level 2 in the female rats inhaling smoke for 90 days, which may be related to the increased formaldehyde levels in the smoke. The histopathologic lesion itself is commonly seen in smoke exposed animals and does not, in our opinion, represent a biologically significant effect unique to licorice extract. The results of these studies therefore suggest that addition of licorice extract to cigarettes at typical use levels of $\leqslant$5% (about 0.269% glycyrrhizic acid) does not discernibly alter the smoke chemistry profile of the selected major toxic constituents of smoke, nor lead to an increase in the biological activity of smoke as measured by the series of in vitro and in vivo assays utilized in this evaluation.

Acknowledgements

The authors thank the The Burdock Group for reviewing the published literature on licorice extract. We also thank Karen Edwards and Linda Wettle for their help in preparing the licorice extract test cigarettes, the Philip Morris USA Analytical Methods and Applications group for analysis of the test cigarettes for glycyrrhizic acid and for the pyrolysis studies. We acknowledge the study directors and their technical staff at Philip Morris Research Laboratories, Philip Morris USA and Illinois Institute of Technology and Research Institute for their excellent technical assistance in conducting the studies: D. Veltel (block licorice extract cytotoxicity); B. Gingras (powder and liquid licorice extract cytotoxicity); K. Rustemeier (block licorice extract smoke chemistry); M. Muzzio (powder and liquid licorice extract smoke chemistry); M. Chang (block licorice metals in smoke); T. Meisgen (block licorice extract mutagenicity); N. Lyang (powder and liquid licorice extract mutagenicity); E. Van Miert (block licorice extract inhalation); W. Johnson (powder licorice extract inhalation); and N. Al-Humadi (liquid licorice extract inhalation).

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