Toxicity Testing Plan

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U.S. CONSUMER PRODUCT

SAFETY COMMISSION

IN CONSULTATION WITH THE

U.S. DEPARTMENT OF HEALTH

AND HUMAN SERVICES

AUGUST 1993

Fire Safe Cigarette Act of 1990

Under the Cigarette Safety Act of 1984 (P.L. 98-567), the Technical Study Group on Cigarette and Little Cigar Fire Safety (TSG) found that it is technically feasible and may be commercially feasible to develop a cigarette that will have a significantly reduced propensity to ignite furniture and mattresses. Furthermore, they found that the overall impact of such a cigarette on other aspects of the United States society and economy may be minimal.

Recognizing that cigarette-ignited fires continue to be the leading cause of fire deaths in the United States, the Fire Safe Cigarette Act of 1990 (P.L. 101-352) was passed by the 101st Congress and signed into law on August 10, 1990. The Act deemed it appropriate for the U.S. Consumer Product Safety Commission to complete the research recommended by the TSG and provide. by August 10, 1993, an assessment of the practicality of a cigarette fire safety performance standard.

Three particular tasks were assigned to the National Institute of standards and Technology's Building and Fire Research Laboratory:

- develop a standard test method to determine cigarette ignition propensity,
- compile performance data for cigarettes using the standard test method, and
- Conduct laboratory studies on and computer modeling of ignition physics to develop valid, user-friendly predictive capability.

Three tasks were assigned to the Consumer Product Safety Commission:

- design and implement a study to collect baseline and follow-up data about the characteristics of cigarettes, products ignited, and smokers involved in fires,
- develop information on societal costs of cigarette-ignited fires, and
- in consultation with the Secretary of Health and Human Services, develop information on changes in the toxicity of smoke and resultant health effects from cigarette prototypes.

The Act also established a Technical Advisory Group to advise and work with the two agencies.

This report is one of six describing the research performed and the results obtained. Copies of these reports may be obtained from the U.S. Consumer Product Safety Commission. Washington, DC 20207.

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Part I

VOLUME 5
PART I

TOXICITY TESTING PLAN FOR LOW IGNITION-POTENTIAL CIGARETTES

U.S. Consumer Product Safety Commission and its
Expert Panel, in consultation with the
U.S. Department of Health and Human Services

May 5, 1993

HEALTH EFFECTS ASSESSMENT PLAN

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May *5*, 1993

EXECUTIVE SUMMARY

I. Introduction

The Fire-Safe Cigarette Act of 1990 requires the U.S. Consumer Product Safety Commission (CPSC), in consultation with the Secretary of the U.S. Department of Health and Human Services (DHHS), to develop information on changes in the toxicity of smoke and resultant health effects of cigarettes with a reduced ability to start fires. The Act states that CPSC "shall not obligate more than $50,000 to develop such information." The Technical Advisory Group (TAG) established by the Act agreed that this amount precluded any significant testing of prototypes. The Act succeeds the Cigarette Safety Act of 1984 which established a Technical Study Group to examine the feasibility of developing cigarettes with lowered ignition potential. The Technical Study Group concluded it is technically feasible and may be commercially feasible to develop cigarettes that will have a significantly reduced propensity to ignite upholstered furniture or mattresses.

The Act expresses a consideration for the possible nationwide health implications of changes resulting from the market substitution/entrance of low-ignition cigarette types. There were about 50 million smokers in the U.S. in 1991, according to the National Cancer Institute. The primary concern is that a small increase in the risk of a serious health effect, due to new cigarette types, could result in a great increase in human mortality and morbidity and thus overbalance the benefits that would be achieved from the reduction of fires.

CPSC staff, in consultation with DHHS and with the concurrence of the TAG, decided that in view of the statutory $50,000 limitation, a plan must be developed for the toxicological work needed. CPSC convened an expert panel to assist in the development of the plan. The panel was composed of knowledgeable scientists in the field of cigarette toxicity testing. These members were nominated by TAG members and selected by the CPSC staff.

This report discusses significant issues and recommends testing necessary for the comprehensive assessment of health effects of low-ignition potential cigarette smoke. It is not intended to be a detailed manual of cigarette toxicity testing, although some necessary technical information is presented.

11. General Discussion

Several adverse health effects of serious concern are the basis for considering the various existing toxicity tests. These effects include: lung and throat cancer, chronic obstructive lung disease, heart and vessel disease, male and female reproductive effects, fetal growth retardation, and psychophysiological addiction, as indicated in Chapter A. Not all of these health effects can be addressed at this time due to the impracticality or non-existence of adequate tests, expenses, or time needed for testing. Therefore, only the tests believed to be practical are recommended. Estimates of costs and times needed for testing are included in Chapters B and D-F.

Major issues surrounding the testing include sidestream smoke, bases of comparisons, analytical vs. in vitro vs. in vivo testing, machine reflection of human smoking behavior, design or performance-based testing, screening paradigms, and disclosure of new additives or increased levels of existing additives, as discussed in Chapter A. Since low ignition-potential cigarettes might cause changes in smoking behaviors and therefore modify the toxicity, altered human behavior may become a significant factor in exposure, as discussed in Chapter C. Since the smoke is collected by mechanically smoking the cigarettes, the apparatus should be set to reflect smoking behavior as closely as technically feasible.

Two methods presently exist for the mechanical smoking of cigarettes, as noted in Chapter B. The Federal Trade Commission (FTC) method, established in **1969**, is used in the United States, and the CORESTA method (ISO **3308-1991)** is mainly used in Europe. The FTC method is described in Chapter B and is very similar to the CORESTA method. Both methods analyze for tar, nicotine, carbon monoxide, and moisture content.

In light of present knowledge on the adverse health effects and toxic constituents of cigarette smoke, further testing beyond the Federally mandated requirements for tar, nicotine, and carbon monoxide levels is needed to evaluate the toxicity. Levels of key chemical constituents known to be associated with adverse health effects need to be measured, as described in Chapter D. Cigarette smoke is a complex mixture of more than **3,500** chemicals containing at least 35 known carcinogens, and analysis of a limited number of individual chemicals may not predict the net toxic effects of the smoke. In order to address certain conglomerative toxicities of the non-gaseous constituents, in ***vitro*** and animal testing are needed, as described in Chapters E and F. Limited whole-animal testing is necessary because of the complexity of the biological systems and a variety of toxic reactions caused by cigarette smoke. As an example, pulmonary inflammation testing requires intact immune, respiratory, and cirulatory systems to be simultaneously present.

The CPSC staff recommends the following guidance plan after reviewing the considerations of its expert panel and DHHS.

111. Assessment Plan

This plan provides guidance for the development of data needed to evaluate the changes in toxicity associated with low ignition-potential cigarettes. Performance-based, rather than design-based, testing will be used to provide data specific to cigarette prototypes. A screening paradigm that requires acceptable performance levels by a candidate cigarette type at one tier of tests before proceeding with the next tier is recommended. This would allow early rejection of candidates evaluated as unacceptable. However, definition of acceptable levels of performance is beyond the scope of this plan and the direction given by the Act. Therefore, the tests are presented in a sequence of tiers for screening without ascribing acceptable levels of performance at each tier.

Results of the recommended testing will be used to assess the relative toxicity of low-ignition potential cigarettes. The toxicity of a candidate low ignition cigarette should be compared to:

1) the specific marketed brand/type intended for replacement, or comparable marketed brands/types for a non-replacement candidate, and
2) standard reference cigarettes, such as the University of Kentucky standard cigarettes mentioned in Chapter E, for quality control.

There are insufficient test methods and data on exposure to cigarette smoke and resultant effects for the direct translation of the results into absolute risks to humans. Since the overall health goal is to avoid the production of greater or perhaps new toxicities than that caused by existing cigarettes, a comparative approach of assessing toxicity is appropriate.

Selection of the guidance plan tests assumes that no new additives would be present in the candidate cigarettes and that presently used additives would not exceed the levels in the current cigarettes. Since toxic effects not considered by this guidance plan could also occur, it is recommended that additives exceeding the current maximum levels of use on a per unit weight of tobacco basis must be disclosed to the U.S. Department of Health and Human Services. Confidential business information status may be requested for the data disclosed.

A. Smokina machine

The FTC method described in Chapter B is the basis for the mechanical generation of smoke constituents. Puff volume,

frequency, and draw velocity may be modified as dictated by behavioral data developed from human testing (Tier 111), as described in Chapter C. Unless consistent correlation of testing results of mainstream and sidestream smokes can be shown, both must be separately collected and tested.

B. Description of Tiers

An outline of four tiers is presented in Table 1. A description of the tiers follows.

Tier I - Analvses of chemicals

All constituents will be reported as per unit weight of tobacco burned and per cigarette. Moisture, nicotine, tar (total particulate matter- dry), and carbon monoxide will be measured according to the FTC method, as described in Chapter B. Nitric oxide will also be measured using the detector attachment to the smoking machine. The gaseous phase will be analyzed for acidity, reduction/oxidation potential, hydrogen cyanide, volatile hydrocarbons, aldehydes, and volatile nitrosamines, as described in Chapter D. The tar will be analyzed for phenols, catechols, polyaromatic hydrocarbons, and tobacco-specific nitrosamines (Chapter D).

Tier II - In vitro tests

The tar will be assayed for mutagenic activity with Ames' Salmonella test with strains TA98, 100, and 1535. The tar will also be assayed for malignant cell transforming activity, using C3H/10T1/2 mouse embryo fibroblast cells. Both mutagenicity and cell transformation assays are described in Chapter E.

Tier III - Human smoking behavior

Humans are typically the last experimental tier in testing products with potential human health effects. An example is the premarket testing of new drugs. Human testing to collect topographical data is limited to a couple of weeks of exposure.

Smoking behavior, including puff volume, frequency, and draw velocity of a selected group of human volunteers would be monitored, as outlined in Chapter C. Carbon monoxide (breath or blood) and cotinine (urinary, salivary, or blood) will serve as biological markers of exposure to the smoke. If the smoking behavior data is significantly different from the FTC smoking machine settings such that an increase in exposure to the analyzed chemicals might result, then the machine must be set to reflect these data before generating smoke constituents for further Tier I and II testing and then animal testing.

Tier IV - Animal tests

Inflammatory lung response to cigarette smoke in C57Bl mice will be assayed as described in Chapter E. Tumor formation in the upper respiratory tract of random-bred golden Syrian hamsters from inhalation exposure and the skin, lungs, and other tissues of Swiss albino Ha/ICR/Mil strain mice from skin painting exposure will be examined. These two carcinogenicity tests are described in Chapter F.

All testing must conform to good laboratory practices, humane laboratory animal methods, and informed human consent procedures accepted within the scientific community. Evaluations of toxicity must be conducted by scientists possessing appropriate toxicological qualifications.

IV. First implementation step

Table 2 is a collection of direct cost estimates for Tiers I, 11, and IV. No estimates are available for Tier 111. Completion of all four testing tiers by successful low ignition potential cigarette candidates might be considered expensive relative to the present level of testing required by FTC ($330K for Tiers I, 11, and IV **vs.** $3.5K for FTC; Table *2)*. Therefore, a stepwise implementation of the plan is suggested.

Table 1
Health Effects Assessment Plan
Outline of Tiers

- Tier I - Analyses of chemicals
  - Whole smoke
    - acidity (pH)
    - reduction/oxidation potential
  - Gas phase
    - gases
      - carbon monoxide
      - hydrogen cyanide
      - nitric oxide
    - aldehydes
      - acetaldehyde
      - acrolein
      - proprionaldehyde
    - volatile hydrocarbons
      - benzene
      - toluene
      - 1,3-butadiene
      - isoprene
    - volatile nitrosamines
      - N-nitrosodiethylamine
      - N-nitrosodimethylamine
      - N-nitrosopyrrolidine
  - Particulate phase
    - catechol
    - nicotine
    - phenols, as phenol
    - polyaromatic hydrocarbon
      - benzo(a)pyrene
    - tar-FTC
    - tobacco specific nitrosamines
      - N'-nitrosonornicotine
      - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

- Tier 11- In Vitro Tests
  - Salmonella mutagenicity (Ames' assay)
  - mouse embryo fibroblast cell transformation assay

- Tier III - Human Smoking Behavior
  - cotinine
  - carbon monoxide
  - topography

- Tier IV - Animal Tests
  - mouse inflammatory lung response
  - hamster upper respiratory tract carcinogenicity
  - mouse skin painting carcinogenicity

Table 2
Estimated Direct Costs in 1993 U.S. Dollars
per brand or prototype

$	
Tier I - Analyses of chemicals = $9,500	
3500	FTC-required tar, nicotine, and carbon monoxide
	Whole smoke
250	acidity (pH)
500	reduction/oxidation potential
	Gas phase
	gases
350	hydrogen cyanide
400	nitric oxide
700	aldehydes
600	volatile hydrocarbons
800	volatile nitrosamines
	N-nitrosodiethylamine
	N-nitrosodimethylamine
	N-nitrosopyrrolidine
	Particulate phase
350	catechol
250	nicotine
500	phenols, as phenol
500	benzo(a)pyrene
800	tobacco specific nitrosamines
Tier 11- In Vitro Tests = $9,350	
1850	Salmonella mutagenicity (Ames' assay)
7500	mouse embryo fibroblast cell transformation assay
Tier IV - Animal Tests = $309,000	
50K	mouse inflammatory lung response
220K	hamster upper respiratory tract carcinogenicity
39K	mouse skin painting carcinogenicity
$327,850	total for Tiers I, 11, and IV

A practical selection of recommended tests should comprise a first step in the implementation of this health effects assessment plan. Subsequent steps should consider the testing recommended by this plan. The first step should include:

Smoke and condensate generated by machine according to the FTC protocol

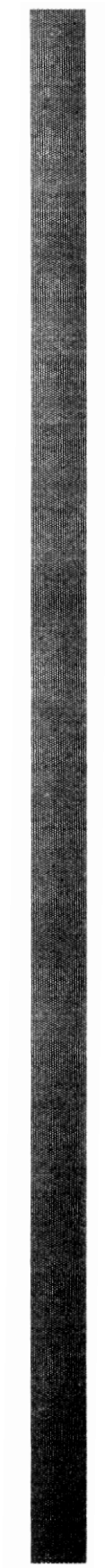
Tier I **$5,050**
- tar-FTC
- nicotine
- carbon monoxide
- whole smoke pH
- benzo(a)pyrene
- tobacco specific nitrosamines
  - N'-nitrosonornicotine
  - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

Tier **II $1,850**
- Salmonella mutagenicity ("Ames") assay

=======
$6,900 Estimated total per brand or prototype

The rationale for selecting these tests extends beyond cost and time duration considerations. Levels of specific chemicals (Tier I) as well as an indication of the genotoxicity of the mixture (Tier 11) are needed. Tar, nicotine, and carbon monoxide are presently required by FTC. The pH of the whole smoke is relevant to nicotine uptake. Benzo(a)pyrene is a known animal and human carcinogen; however, cigarettes are not the only source of exposure. The tobacco-specific nitrosamines are potent animal carcinogens and tobacco is the only known source of human exposure. No data are available on the human carcinogenicity of these nitrosamines.

Chapter A



OVERVIEW AND MAJOR CONSIDERATIONS IN THE TOXICITY TESTING OF LOW IGNITION-POTENTIAL CIGARETTES

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ABSTRACT

Both mainstream and sidestream cigarette smoke are complex chemical mixtures. In view of this chemical complexity, it should be no surprise that cigarette smoke has multiple, diverse effects on human health. Nor should it be unexpected that multiple chemicals in cigarette smoke contribute to any single adverse health effect.

The diverse human health consequences of cigarette smoking are briefly reviewed. Many experimental laboratory models have been developed to study the mechanisms of cigarette smoke-induced disease. These laboratory models are not always convertible into practical, standardized test systems that quantitatively compare one cigarette prototype with another. In view of the multiplicity of health effects and mechanisms of smoke-induced health damage, no single test or battery of tests can capture all possible health endpoints.

While analyses of smoke constituents and studies in laboratory animals are feasible, human epidemiological studies are not practical for short-term assessment of small differences in the toxic effects of various cigarette prototypes. Cigarette smoke samples for chemical analysis and biological testing need to be collected in a manner that approximates human cigarette puffing as closely as technically feasible.

In formulating a testing plan, the CPSC essentially has two options: a design-based testing plan, in which individual, pre-selected cigarette design parameters, such as paper porosity or percent expanded tobacco, are systematically varied and tested; and a performance-based testing plan, in which complete cigarette prototypes, and not individual design parameters, are evaluated.

Some testing protocols entail a "screening paradigm." Multiple tests are performed in sequence. If a prototype fails any particular test in the sequence, the prototype is rejected and no further tests are performed. Other multi-test protocols allow for tradeoffs among costs and benefits. An unfavorable result at any point along the testing sequence does not necessarily result in rejection.

The only governmentally-mandated, health-oriented testing of commercial cigarette brands is the measurement and reporting of "tar," nicotine and carbon monoxide in mainstream smoke by the Federal Trade Commission. With this exception, none of the toxicity tests described by the Expert Panel are routinely performed on existing cigarette brands by any governmental agency. The contents of currently marketed cigarettes are proprietary information. Specific additives, tobacco

composition, and other design features are not publicly disclosed.

SCOPE OF THE EXPERT PANEL REPORT

This report addresses: scientific aspects of the design of cigarette toxicity testing systems; the selection and sequencing of particular tests; the reliability, feasibility, and costs of particular tests; and the interpretation, limitations, uses and misuses of test results. In addition to the present Overview chapter, the report contains specific chapters on:

(i) collection of smoke samples from prototype cigarettes for toxicity testing, by Dr. Harold Pillsbury (Chapter B);

(ii) measuring the dosage of smoke constituents actually absorbed by human smokers of different cigarette prototypes, by Dr. David Burns (Chapter C);

(iii) measuring the amounts of specific chemicals contained in the collected smoke, by Dr. Dietrich Hoffmann (Chapter D);

(iv) toxicity testing in single-cell ("in vitro") systems, by Dr. Gary Gairola (Chapter E);

(v) toxicity testing in whole animal ("in vivo") systems, by Dr. Dietrich Hoffmann (Chapter F); and

(vi) research needs for developing methods to collect additional data (Chapter G, input needed).

The Expert Panel has not made policy recommendations. The Panel members did not perform any testing of prototypes in connection with this Report.

SOURCES OF INFORMATION

In preparing this Report, the Expert Panel relied upon: the Final Report of the Technical Study Group on Cigarette and Little Cigar Fire Safety under the Cigarette Safety Act of 1984 [28]; background papers issued in connection with the Technical Study Group Report [17;27]; reports issued by the National Institute for Standards and Technology (and its predecessor, the National Bureau of Standards) in connection with low-ignition potential cigarettes [11;18]; communications from members of the TAG, CPSC staff and DHHS Staff; the published scientific literature; as well as its own expertise and experience. No proprietary or confidential information was requested, offered, or considered.

MAINSTREAM VERSUS SIDESTREAM CIGARETTE SMOKE

Both smokers and nonsmokers can incur adverse health effects from the smoke of burning cigarettes. Smokers inhale mostly

"mainstream (MS) smoke" that is drawn through the burning tobacco column and filter tip and exits through the mouthpiece of the cigarette. Nonsmokers inhale mostly "sidestream (ss) smoke" that is emitted into the surrounding air between puffs from the end of the smoldering cigarette. Sidestream smoke is the major source of "environmental tobacco smoke (ETS)."

While ss and MS smoke have qualitatively similar chemical compositions, the respective quantities of individual smoke constituents can be quite different [35, Chapt.3; 37, p.88]. For example, in studies of nonfilter cigarettes smoked by machines, the yield of carbon monoxide (CO) in sidestream smoke was 2.5 to 4.7-fold that of MS smoke, while the corresponding SS/MS ratio for N-Nitrosodimethylamine (NDMA), an animal carcinogen, was 20 to 100 [35, pp.130-131]. In one compilation of toxic and tumorigenic agents in cigarette smoke, the SS/MS ratio ranged from 0.03 to 130 [14].

Cigarette modifications that reduce the yields of "tar," nicotine and CO in mainstream smoke do not necessarily reduce the corresponding yields in sidestream smoke. In one study of U.S. commercial cigarettes, the SS/MS ratios for carbon monoxide were 2.1 and 2.7, respectively, in two nonfilter cigarettes; 3.5 in a conventional filter cigarette; and 26.8 in a perforated filter cigarette. The SS/MS ratios for NDMA were 23.6 and 139 in the nonfilter cigarettes; 50.4 in the filter cigarette; and 167 in the perforated filter cigarette [35, p.131]. The exposure to sidestream smoke constituents, though, may be greatly reduced depending on distance from the cigarette and ventilation characteristics.

Modifications of cigarette design intended to reduce ignition potential may likewise have different effects on the compositions of MS and *ss* smoke. In principle, ignition-reducing chemical agents added to the tobacco column or paper wrapper, such as metals and silicates, may transfer differently into MS and *ss* smoke.

A number of devices have been developed to collect samples of ss smoke for chemical analysis [7]. However, there are no regularly published data on the composition of *ss* smoke of U.S. cigarette brands. By contrast, the Federal Trade Commission regularly publishes machine-measured yields of "tar," nicotine and CO of the MS smoke of U.S. commercial cigarettes, as described later in this Report. Still, a testing plan for low-ignition potential cigarette prototypes needs to consider both MS and SS smoke.

RANGE OF HUMAN HEALTH CONSEQUENCES

Cigarette smoke (whether MS or SS) is not a homogeneous entity, but a complex mixture of substances. Some smoke components, such as CO, hydrogen cyanide and nitrogen oxides, are gases. Others, such as nicotine and polyaromatic hydrocarbons (PAH), are contained in the submicron-sized solid particles that are suspended in the smoke. Still others, such as formaldehyde and benzene, are volatile chemicals contained in the liquid-vapor portion of the smoke aerosol [37, p.79; 39, Chapt.14]. In view of this chemical complexity, it should be no surprise that cigarette smoke has multiple, diverse effects on human health. Nor should it be unexpected that multiple chemicals in cigarette smoke contribute to any one adverse health effect.

Among the major health effects of cigarette smoke that need to be considered in the development of a toxicity testing plan are the following: cancer; non-cancerous lung diseases; atherosclerotic diseases of the heart and blood vessels; and toxicity to the human reproductive system.

Cancer

Cigarette smoking causes cancers of the lung, esophagus, larynx, oral cavity, bladder, and pancreas in male and female smokers. Smoking has reported to increase the risks of cancers of the kidney, liver, anus, male penis, and female uterine cervix, as well as leukemia [13;31;37;38]. Cigarette smoking is far and away the major cause of lung cancer in the U.S., accounting for 90 percent of cases in men and 79 percent in women [37, p.156].

Numerous epidemiological studies covering the experience of millions of men and women over many years show that smokers' risks of developing cancer increase with the number of cigarettes smoked daily, with the lifetime duration of smoking, and with early age of starting smoking. Smoking cessation gradually reduces cancer risk [37;38]. Filter-tipped and low "tar" cigarettes reduce cancer risk somewhat. Cigarette smoking interacts with other causative agents, including alcohol, asbestos, certain viruses, and certain workplace exposures, in the development of human cancers [31;34;37].

Mainstream cigarette smoke contains over three dozen distinct chemical species considered to be tumorigenic in humans or animals [14; 31, pp.192-218; 37, p.86]. Some of these chemicals are alone capable of initiating tumors in laboratory animals; others can promote the development of previously initiated cancers. As described later in this Report, condensates collected from cigarette smoke cause mutations and damage to DNA in laboratory assays of mutagenesis [12], as well as malignant transformation in laboratory tests of a chemical's ability to induce malignant changes in mammalian cells [3;8].

Undiluted mainstream cigarette smoke is too toxic to be tolerated by laboratory animals such as rodents. In long term experiments with diluted smoke, these animals still do not inhale the smoke in the same way as humans. In natural human smoking, the smoke is puffed in volumes of about 30 to 70 ml; the puffed smoked is temporarily retained in the smoker's mouth, after which it may be inhaled deeply into the lungs. By contrast, some laboratory animals breath by panting, while others are obligate nose breathers. Even with installation of smoke through artificial airways, it can be quite difficult to get the animals to inhale deeply, as human smokers do. Accordingly, the distribution and retention of smoke components in the respiratory systems of laboratory animals may not mimic natural human smoking.

Nevertheless, as described later in this Report, significant progress has been made in the design of inhalation devices that can expose laboratory animals, especially rodents, to diluted smoke for long periods. Long-term smoke inhalation regularly induces tumors of the larynx in Syrian golden hamsters. Direct installation of cigarette tar into the airways of laboratory animals causes lung cancers [14;31]. As discussed later in this Report, the most widely used experimental system is the mouse skin bioassay, in which cancers are induced by the repeated application of condensates of cigarette smoke to the shaved skins of mice.

Independent scientific agencies have concluded that environmental tobacco smoke causes lung cancer in nonsmokers [22;35]. *ss* smoke, like MS smoke, contains numerous tumorigenic agents.

Non-Cancerous Lung Diseases

Cigarette smoking is the main cause of chronic obstructive lung disease (COLD), also called chronic obstructive pulmonary disease (COPD) [33]. Smoking accounts for 84 percent of COLD deaths in men and 79 percent in women [37, Chapt.3].

COLD is a slowly progressive illness that develops after repeated insults to the lung over many years. In the early years after starting to smoke, an individual may report no symptoms. Even at this early stage, however, breathing tests can often detect abnormalities in the small, terminal airways of the lung [2;26;33], and these abnormalities have been directly observed in autopsy studies of young smokers who died suddenly [23]. For smokers in their twenties, there is already a dose-response relation between the extent of abnormal lung tests and the number of cigarettes smoked daily. In random population surveys, from 17 to 60 percent of adult smokers under age 55 have detectable small airways dysfunction [33, pp.27-32].

Over the course of two decades or more of smoking, a constellation of chronic respiratory changes develops. This picture of chronic lung injury includes: (i) mucus hypersecretion, with chronic cough and phlegm; (ii) airway thickening and narrowing, resulting in obstruction to airflow during expiration; and (iii) emphysema, i.e., abnormal dilation of the air spaces at the end of the respiratory tree, with destruction of the walls lining the air sacs, resulting in further airflow obstruction. These changes can cause significant respiratory impairment, disability, and death. While individual patients vary in the relative contribution of these three changes, those with clinically severe COLD typically have all three.

While a minority of cigarette smokers will develop clinically severe COLD, some chronic deterioration in lungstructure or function is demonstrable in the majority of long-term smokers [33, Chapt.2]. Some smokers show more chronic cough and phlegm, others more airway obstruction. In general, breathing function declines as a person's cumulative exposure to smoke, measured in pack-years, increases [6].

Cigarette smoke produces pathological changes in the lungs of smokers by a number of different mechanisms [38, pp.282-285]. Cigarette smoke is toxic to the small hairlike cilia that line the central breathing passages. These cilia, in combination with mucus secretions, defend against deep inhalation of foreign material [33, p.279]. Smoking also induces many abnormalities in the inflammatory and immune systems within the lung [34, p.256]. In particular, cigarette smoke causes inflammatory cells to produce an enzyme called elastase. The enzyme elastase in turn breaks down elastin, an important protein that lines the elastic walls of the air sacs [9; 33, p.431]. Moreover, oxidants present in cigarette smoke can inactivate a separate protective enzyme called alpha-1-antitrypsin, which inhibits the destructive action of elastase [16; 33, p.434].

Researchers have produced various types of acute and chronic lung injury in laboratory animals exposed to cigarette smoke [33, pp.286,428,432,436]. But they have had difficulty inducing genuine emphysema from cigarette smoke alone. As in experimental models of cancer, the laboratory animals do not inhale the smoke deeply. Moreover, very long smoke exposures may be required, as is the case in humans. In one experimental study, hamsters exposed either to low doses of elastase or low doses of smoke alone did not develop emphysema, but the combination of low doses of cigarette smoke and elastase caused emphysema-like changes [15]. A later chapter in this Report describes a laboratory test for the acute inflammatory effects of cigarette smoke on the lung, in which mice are exposed to cigarette smoke through a nose-only system.

A large number of organic and inorganic chemicals in the gaseous, volatile and particulate phases of cigarette smoke appear to contribute to its toxicity to the respiratory system [33, pp.289,415], including hydrocarbons, aldehydes, ketones, organic acids, phenols, cyanides, acrolein, and nitrogen oxides. Some components contribute to the development of chronic mucus hypersecretion in the central airways, while others play a greater role in the production of small airway abnormalities and emphysematous injury to the peripheral air sacs [33, p.425]. As noted above, oxidizing agents in smoke inhibit the enzymes that defend against the destruction of lung elastin.

Passive exposure to environmental tobacco smoke produces respiratory irritation in nonsmokers, particularly in the children of smoking parents [33, Chapt.7; 35, p.37]. Infants and children of smoking parents are at increased risk of acute respiratory infections, chronic cough and wheezing, and measurable declines in lung function [35, pp.38-59]. These early-life infections can have long-term adverse effects. In adults passively exposed to ETS, some studies have reported measurable changes in lung function. Overall, the effect appears to be too small to implicate passive smoking alone as a cause of full-blown COLD [35, p.62].

Atherosclerotic Cardiovascular Diseases

Cigarette smoking is a major contributing cause to coronary heart disease, stroke, and other atherosclerotic diseases of the circulatory system [32;37].

Atherosclerosis is a chronic disease that can affect the arterial blood vessels in virtually every part of the human body, including the coronary arteries that supply blood to the heart muscle; the aorta that carries the blood directly from the heart; the carotid arteries that carry blood to the brain; and the iliac and femoral arteries that carry blood to the legs.

The common underlying lesion of atherosclerosis is the plaque, which occurs within the wall of the affected artery. As the plaque enlarges and matures, the artery becomes narrowed, and blood flow is reduced. If the narrowed artery carries blood to the heart, then chest pain on exertion (angina) is produced. If the affected artery carries blood to the leg, then calf pain on walking (claudication) is produced. If the affected artery carries blood to the brain, then transient neurological symptoms, such as fainting, loss of vision, movement, or speech (transient ischemic attacks) are produced. If the affected artery carries blood to a man's penis, impotence can result.

A sufficiently narrowed artery is susceptible to complete blockage by a superimposed blood clot. If the blocked artery carries blood to the heart, then a heart attack (myocardial

infarction) is produced. A blockage of an artery supplying a limb can produce gangrene. A blockage to the arteries supplying the brain can cause a stroke.

The most important form of atherosclerosis in the U.S. is coronary atherosclerosis. Its manifestations, which include angina, heart attack, heart failure, and sudden death, are described by the inclusive term coronary heart disease (CHD). Atherosclerosis involving the arteries supplying the brain is a form of cerebrovascular disease (CVD). Atherosclerosis involving the arteries to the limbs is called peripheral vascular disease (PVD).

Atherosclerotic plaques take years to develop. The earliest lesion is called a fatty streak, which consists of deposits of cholesterol within the arterial wall. These fatty streaks can be observed in young people with no symptoms, and even in children. There is a progressive inflammatory reaction to the fatty deposits, and a collection of fibrous debris, muscle cells, and more fatty deposits is incorporated into the developing plaque.

Cholesterol is a fatty substance that does not dissolve readily in water. It circulates in the blood mostly by attaching to specialized proteins. These cholesterol-protein complexes, which also contain other fatty substances, form particles of various sizes, which are called lipoproteins. The lipoprotein particles are classified by their density. There are very-low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particles.

The fundamental event in the initiation of a fatty cholesterol deposit appears to be the transfer of LDL particles from the blood across the inner lining (endothelium) of the arterial wall. This transfer may require prior injury to the inner lining of the artery, in order to expose the raw surface to LDL transfer. When a person's blood cholesterol is measured, the amount that is specifically attached to LDL is called the LDL-cholesterol, or popularly the "bad cholesterol."

On the other hand, HDL particles work in the opposite direction, removing cholesterol from LDL and transporting it back to the liver. Because of this reverse-transport function of HDL, the amount of cholesterol attached to HDL is popularly termed the "good cholesterol."

In epidemiological studies of humans, certain measurable personal characteristics have been consistently found to be predictors of the risks of atherosclerotic disease. These predictors are sometimes called risk factors. For example, male gender is a risk factor for coronary heart disease. This does not mean that maleness per se causes CHD. Still, the fact that

women have lower rates of CHD, and that their risk of CHD increases after menopause, indicates that sex hormones are important in the development of the disease. Likewise, elevated blood pressure is a risk factor for CHD (and for strokes). Again, this does not mean that hypertension per se causes CHD. However, higher pressures in the arterial system tend to damange the inner lining (endothelium) of arteries, thus contributing to the development of plaque formation, arterial narrowing and blockage. Because atherosclerosis entails a sequence of pathological events over an extended period of time, it is to be expected that multiple environmental agents and personal characteristics can affect the course of the disease.

In numerous epidemiologic studies of millions of people, cigarette smokers have been found to have higher rates of heart attack, sudden death, and other manifestations of CHD. They also have higher rates of stroke, peripheral vascular disease, and other atherosclerotic lesions [32;37;39]. In a study of over one million people followed during 1982-1986, currently smoking men had a 94 percent greater risk of CHD than lifelong nonsmokers; while currently smoking women had a 78 percent greater risk. In smokers under age 65, men had a 181 percent greater risk, and women a 200 percent greater risk [37, Chapt.3].

Cigarette smoking is sometimes called an "independent risk factor" for CHD because smokers' CHD rates are found to be higher even when other risk factors such as gender, blood pressure, and cholesterol level are taken into account. It is sometimes called a "modifiable risk factor" because one can reduce or stop smoking. While smoking obviously cannot be a cause of CHD in someone who never smoked, it can be an important contributor to CHD in a smoker. Among 540 thousand deaths from CHD in the U.S. in 1905, an estimated 115 thousand would not have occurred but for the presence of cigarette smoking [37].

Cigarette smoke appears to enhance the atherosclerotic process by several different mechanisms [38, p.192]. Cigarette smoking affects cholesterol metabolism. Smokers have repeatedly been observed to have lower HDL-cholesterol levels [41]; and smoking cessation raises HDL-cholesterol [25]. In animal models, cigarette smoke can damage the inner lining of blood vessels, thus enhancing the transfer of LDL and the development of underlying plaques [19;42]. Cigarette smoking can also affect the blood clotting system, including the adherence of blood platelets to the lining of arterial blood vessels [24;32] and the formation of blood clots that block a narrowed artery. Cigarette smoke can also cause spasm of the coronary arteries.

Many chemical components of cigarette smoke have been implicated in the development of atherosclerotic disease. Nicotine, the major psychoactive component of smoke, causes powerful changes in heart rate and blood circulation. Nicotine

appears to cause injury to the arterial lining [19;42]. Carbon monoxide in cigarette smoke binds to the hemoglobin in red blood cells, thereby reducing the oxygen- carrying capacity of the blood. Hydrogen cyanide, nitrogen oxides, and chemical components of cigarette "tar" have also been implicated [32]. Oxidants in cigarette smoke may also promote plaque formation.

Cisarette Smokins and Human Reproduction

Cigarette smoking adversely affects sexual and reproductive function in women in a number of different ways.

Cigarette smoking appears to impair female fertility [1; 5; 21; 30, p.235]. Among the possible mechanisms are direct toxicity to female eggs, interference with motility in the female reproductive tract, and alterations in immunity that predispose female smokers to infections that block the Fallopian tubes [4].

Maternal cigarette smoking has serious adverse effects on the outcome of pregancy. These include: retarded fetal growth; low birthweight; spontaneous abortion; certain complications of pregancy, labor and delivery, such as bleeding during pregnancy and prolonged premature rupture of membranes; and infant death [30, p.188; 37, p.71; 38, Chapt.8; 39, Chapt.8]. Direct nicotine toxicity has been suggested as a mechanism for spontaneous abortion [38, p.372]. While a smoking-induced reduction in maternal weight gain contributes to fetal growth retardation [30, p.202; 40], the evidence points to oxygen starvation of the fetus and placenta as important factors. Carbon monoxide in cigarette smoke can cross the placenta and bind to the hemoglobin in fetal blood. Smoking causes constriction of the umbilical arteries, impairing placental blood flow. Nicotine, which also crosses the placenta, can have a number of toxic effects on the fetus [30, p.229]. Cyanide, another component of cigarette smoke, has also been implicated.

Currently smoking women enter nonsurgical menopause about one to two years earlier than nonsmokers [38, p.397]. Heavy smokers experience an even earlier menopause than light smokers. This effect has important consequences for women's health, because the rates of osteoporosis and atherosclerotic cardiovascular diseases increase after menopause. One proposed mechanism for early menopause is that polyaromatic hydrocarbons (PAH) in smoke are directly toxic to ovarian follicles [20].

Cigarette smoking may also affect male reproductive performance. In a number of studies, men who report impotence (i.e., the inability to maintain an erection sufficient for intercourse) were more likely to be cigarette smokers. This association between smoking and impotence is particularly common among men who have high blood pressure or diabetes, and appears to be a consequence of increased atherosclerotic disease in the

blood vessels supplying the genitalia, rather than an effect on sexual drive.

Nicotine as a vsychoactive druq

The psychoactive drug in cigarette smoke is nicotine. Cigarette smoking is a highly controlled form of self-administration of this drug. Nicotine use is self- reinforcing. Attempts to stop smoking lead to craving, withdrawal symptoms, and high rates of relapse [36].

RESEARCH MODELS VERSUS STANDARDIZED TEST SYSTEMS

As the foregoing brief review indicates, there are many laboratory and animal models of the mechanisms of cigarette-induced human toxicity, and there are many methods of studying the health effects of smoking in humans. However, not all of these models and methods are easily converted into inexpensive, practical, standardized tests that quantitatively compare one cigarette prototype with another.

THE MULTIPLICITY OF TESTING PROTOCOLS

It is unlikely that any battery of standardized, practical tests will be able to gauge all important dimensions of human cigarette toxicity. Exhaustive testing of every conceivable dimension of toxicity is a "bottomless pit." From the scientific standpoint, there will necessarily be some stopping point to testing.

At present, there exists a wide range of testing protocols, reflecting different dimensions of human toxicity. These testing protocols will be considered in detail in later sections of this Report. In general, tests of cigarette toxicity include:

(1) Chemical and physical analyses of MS and SS smoke collected by smoking machines under standardized conditions. These tests include quantitative measurement of known smoke constituents, qualitative analyses for new chemicals, and studies of particle size distribution.

(2) Studies of the dosage of specific smoke constuents actually received by human smokers or by nonsmokers exposed passively to environmental tobacco smoke.

(3) Laboratory tests of the effects of whole smoke or fractions of smoke on individual cells and tissues. The individual cells can be single-cell organisms, such as bacteria. They can be cells extracted from a specific organ of an animal

and preserved in tissue culture. Tests that do not entail exposure to an entire living animal are called in vitro tests.

(4) Laboratory tests of the effects of smoke or smoke fractions in whole animals. These include short-term tests to study specific mechanisms of disease or to assess acute toxicity, and long-term tests to assess the effects of chronic exposure.

The multiplicity of human health endpoints, as well as the wide range of available tests, means that a particular cigarette prototype may appear more toxic in some tests, equally toxic in other tests, and less toxic in still others.

EPIDEMIOLOGY

Human epidemiological studies play a central role in generating and testing hypotheses about causation of disease; in identifying groups of people who at higher or lower risks of disease; in estimating quantitatively the risks of specific diseases in relation to different levels of toxic exposure; and in evaluating the effects of preventive measures.

Epidemiological studies are more limited in assessing the differences in the toxic effects of various types or brands of cigarettes. For example, to determine whether brand "A" causes less lung cancer than brand "B," a researcher would have to identify and compare long-term smokers exclusively of brand "A" with long-term smokers of brand "B" alone. If the expected differences in cancer rates are small, then large numbers of long-term smokers of each brand need to be identified.

Epidemiologic methods are impractical for testing the comparative effects of prototype cigarettes that have not already been marketed and smoked by consumers.

ABSOLUTE RISK VERSUS RELATIVE RISK

Human epidemiology can be used to estimate quantitatively the risk of specific diseases to human smokers. For example, in a study of smoking practices and mortality rates among 1.2 million U.S. adults followed during 1982-1986, about 0.8 percent of current male smokers aged 65 or more died of lung cancer each year [37, p.143]; while the comparable annual lung cancer death rate was about 0.04 percent among men aged 65 or more who never smoked. These quantitative risk estimates are often termed "absolute risks." The fact that the continuing smokers' risk of lung cancer was 20-fold that of nonsmokers is an expression of "relative risk."

Estimating absolute risks from nonhuman toxicity studies is much more complicated. For example, the smoke from prototype

cigarette "Z" might contain 0.05mg of benzo(a)pyrene (BaP), a known carcinogen, while the smoke from a control cigarette might contain 0.02mg of BaP. To estimate human lung cancer risks from these data alone would require a number of assumptions relating the dose of BaP to the incidence lung cancer in humans.

Toxicity studies can give estimates of relative risk, but applying these estimates directly to humans requires caution. While prototype "Z" had 2.5-fold as much BaP as the control cigarette, we cannot automatically conclude that their relative risks of lung cancer in humans is 2.5. The relative concentrations of benz(a)anthracene, another carcinogen in the "polyaromatic hydrocarbon" group, might be higher or lower. Estimating relative risks from toxicity studies entails combining estimates from different sources [8].

HUMAN BEHAVIORAL RESPONSES ARE IMPORTANT IN TESTING

Testing plans require samples of cigarette smoke, which can then be analyzed chemically or biologically. The results of such testing may hinge critically on the method of collecting the sample. Smoke samples from cigarettes are generally collected from smoking machines, not from living smokers. As discussed in more detail later in this Report, it is important that such samples be collected in a manner that mimics human smoking as closely as is technically feasible.

The study of the ways in which humans consume cigarette smoke is called smoking topography. Many variables are involved, even in the smoking of a single cigarette: the intensity of the draw on the column of smoke during a single puff; the duration of the puff; the volume of smoke in each puff; the intervals between puffs; and the number of puffs taken per cigarette. These variables, as well as other physiological factors, affect the actual dosages of smoke constituents that are inhaled, absorbed, and retained in the smoker's body. The study of the actual dosages of smoke constituents received by human smokers is called smoking dosimetry.

No two humans smoke cigarettes exactly the same way. Puffing intensity, duration and volume, as well as inter- puff intervals and puffs per cigarette, vary among human smokers. Accordingly, no protocol for machine-based collection of cigarette smoke can accurately mimic all human smoking. Toxicity testing of machine-collected smoke samples may not accurately gauge a particular smoker's risk, but rather an average or representative smoker's risk.

Toxicity testing ordinarily requires a uniform method of collecting smoke samples. To compare the "tar," nicotine and *co* yields of the smoke of prototype "X" with those of a control

cigarette, one uses the same smoking machine to smoke both cigarettes under the same conditions according to the same protocol. For example, under the current FTC protocol, a smoking machine takes one puff each minute. The puff volume is 35ml; and the puff duration is 2 seconds. As described lqter in this Report, the smoking machine continues to take puffs on the test cigarette until a pre-specified butt length is achieved.

Different cigarette prototypes or design modifications may affect the ways that people smoke cigarettes. This can complicate the choice of test conditions for collecting smoke samples from prototype cigarettes. For example, when cigarette prototype "X" is smoked by machines under standard FTC conditions, the amount of nicotine in the smoke may appear to be reduced. But human smoking topography may show that smokers actually take deeper puffs on prototype "X" than the 35 ml-puffs taken by the smoking machines. Human dosimetry may further show that the amount of nicotine actually absorbed from prototype "X" is not reduced. Alternatively, prototype "Y" may contain more "tar" per machine-smoked puff. But human topography may show that smokers take fewer puffs on that prototype, so that the total yield of "tar" per cigarette is not increased.

For these reasons, human smoking topography and dosimetry may need to be a part of cigarette testing for increased toxicity.

DESIGN-BASED TESTS VERSUS PERFORMANCE-BASED TESTS

Section 2(c) of the Fire Safe Cigarette Act of 1990 mandates the development of information on "changes" in toxicity of smoke and resultant health effects of cigarette "prototypes." Such information can be acquired by design- based testing, which assesses the effects of a specific, known modification (or a combination of modifications) in cigarette design.

The "tar," nicotine and CO analyses of "Series 1" and "Series 2" experimental cigarettes performed by the National Bureau of Standards (NBS) are examples of design-based testing [11, Tables 2-5, 2-6, 2-7, 3-10, 3-11, and 3-12]. In Series 1, for instance, NBS analyzed five different dimensions of cigarette design: tobacco leaf composition (burley vs. flue cured); tobacco density (decreased by tobacco expansion); paper porosity; the presence of citrate additive to the cigarette wrapping paper; and the circumference of the tobacco column. Experimental cigarettes were produced that contained modifications in one or more of these design dimensions. The modified cigarettes could then be compared to each other and to control cigarettes with no modifications. By such comparison, NBS estimated that lower tobacco density decreased the "tar" yield per smoke puff; while

low paper permeability increased "tar" yield per puff [11, Table 3-12].

The **NBS** Series-1 tests did not encompass all possibilities in design-based testing. A specific newchemical additive could be incorporated into a test cigarette, whose smoke would be compared with that of a control cigarette that is otherwise identical. In the **NBS** Series 1, the experimental cigarettes were all filter- tipped, with the individual tows, plug wraps, and plasticizer levels selected by the participating cigarette manufacturers [11, p.33]. The effects of lower-porosity wrapping paper could also have been assessed in nonfilter cigarettes, or perforated filter-tip cigarettes. This might be important if the presence or type of filter affected ignition propensity [11, p.65]

From the scientific standpoint, design-based testing is advantageous when there is a limited practical range of cigarette design modifications, and when such design modifications are publicly known. For example, if changes in tobacco packing density and paper permeability were the only feasible design modifications under consideration, and if the proposed methods of tobacco expansion and paper manufacture were specifically disclosed, then the effects of such design changes could be assessed. However, if a specific cigarette prototype entailed tobacco expansion combined with proprietary changes in tobacco leaf composition, cigarette paper, and filter design, then designed-based testing may be impractical.

The alternative is performance-based testing, in which individual cigarette prototypes-- not design technologies-- are assessed. Such testing may be more appropriate when there are many different cigarette prototypes, each with complex design changes, and when the specific changes are proprietary or not fully disclosed. **NBS's** analyses of patented cigarettes [11, Table 3-14] more closely resembles performance-based testing. In that case, inventors submitted their own prototypes, along with unmodified control cigarettes. While **NBS** appears to have tested these patented prototypes for ignition propensity only, analyses of "tar," nicotine and CO in such patented cigarettes would constitute performance-based testing.

Accordingly, in design-based testing, information might be acquired on the effects of changes in paper porosity on smoke carbon monoxide. By contrast, in performance-based testing, information is acquired on the CO delivery of prototype "X."

In performance-based testing, there is no unique or natural control cigarette. As in the **NBS** testing of patented cigarettes, the smoke of prototype **"X"** could be compared to a control cigarette that incorporates none of the proposed modifications. But this alternative is not necessarily so simple. Prototype **"X"**

could include modifications designed to: (i) reduce ignition potential; (ii) reduce smoke toxic constituents; and (iii) improve consumer acceptability. A comparable control cigarette may be unmodified in one or all of these dimensions. Such controls may not correspond to any currently marketed cigarette brand. Alternatively, the prototype "X" could be compared to other existing marketed cigarettes; to another prototype "Y;" or to pre-set standard cigarette. Thus, in performance-based testing, one could conclude that prototype "Z" delivered more or less nicotine than any other prototype; than the average marketed cigarette; or than some value set by a public or private standard-setting body.

TESTING PROTOCOLS: SCREENING VERSUS TRADEOFFS

Testing is expensive and time-consuming. Accordingly, most testing protocols entail a sequence of tests. The order of testing is usually influenced by the cost and time required. If human subjects are involved, then risk and ethical considerations are important.

For example, in the screening of environmental agents for their carcinogenic potential (e.g., under the Toxic Substances Control Act), bacterial mutagenesis and other short-term tests for genotoxicity are performed first. After that, whole animal exposure studies of acute toxicity may be considered. Thereafter, longer term whole-animal studies of carcinogenicity may be undertaken. In the screening of investigational new drugs, human studies are undertaken only after laboratory and whole animal studies are completed.

Some testing protocols entail a "screening paradigm." If a substance or product fails any particular test in the sequence, the product is rejected and no further tests are performed. For example, in the testing of cigarette prototypes, analytical studies of smoke components might be performed initially, followed by short-term mutagenicity studies, followed then by long-term bioassays of carcinogenicity in animals, followed by studies of smoke dosimetry in humans.

In the screening paradigm, a cigarette prototype "Z" that initially yielded an excess of carcinogenic polyaromatic hydrocarbons (PAH) might be rejected, and no further testing performed. Alternatively, if cigarette prototype "Q" yielded no excess of toxic compounds on chemical analysis, then testing of prototype "Q" would proceed to the next level.

In contrast to screening protocols, other testing protocols allow for tradeoffs among costs and benefits. A positive test at any point along the testing sequence does not necessarily result in rejection. For example, prototype "R" may have performed

exceptionally in tests of low- ignition potential, but it yielded an excess of tobacco-specific nitrosames in chemical analysis. Such a finding might not lead to automatic rejection of prototype "R." Instead, testing would continue, and the positive analytical test result would be weighed against other evidence.

Conservatively designed protocols may be appropriate when the potential adverse health effects of a new product or new design are more important than its potential benefits for fire safety. On the other hand, if a relatively small increase in "tar" or nicotine delivery is to be gauged against a major reduction in ignition potential, then some form of cost-benefit analysis will be required.

SPECIAL ASPECTS OF CIGARETTE PROTOTYPE TESTING

The only governmentally-mandated, health-oriented testing of the finished cigarette product is the measurement and reporting of tar, nicotine and carbon monoxide by the Federal Trade Commission. While USDA and DHHS may conduct research programs on the health effects of smoking, no other federal or state agency is currently required to perform tests for toxicity on various brands of marketed cigarettes. With the exception of standardized machine measurements of "tar," nicotine and CO, none of the toxicity tests described by the expert panel are routinely performed on existing cigarette brands by any governmental agency.

Some low-ignition protype cigarettes may contain additives that are not in currently marketed cigarettes. Such additives may have qualitatively different health effects than those discussed above. Neither performance-based nor design-based testing solves the problem of evaluating the health effects of new, undisclosed cigarette additives. For example, if a new inorganic compound, such as a metal salt, were added to the cigarette tobacco, then one might have supplement the test battery with additional studies of acute or chronic toxicity to kidney, liver and other organs. In cases where new additives are involved, and not merely a quantitative change in existing design parameters, disclosure of contents is required for adequate toxicity testing.

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GLOSSARY AND ABBREVIATIONS

benzo(a)pyrene (BaP): a carcinogenic chemical in cigarette smoke, a member of the class of polyaromatic hydrocarbons.

benzo(a)anthracene: a carcinogenic chemical in cigarette smoke, a member of the class of polyaromatic hydrocarbons.

carbon monoxide (CO): a gas found in cigarette smoke.

condensate: the portion of whole smoke that condenses upon passage of the smoke through a cold trap.

dosimetry of smoking: study of the actual dosages of smoke constituents inhaled, absorbed and retained by human smokers.

environmental tobacco smoke (ETS): mostly sidestream smoke, but also exhaled mainstream smoke, as well as some gaseous and

vapor-phase constituents of smoke that diffuse through the cigarette paper wrapper into the surrounding air.

genotoxicity tests: tests of the propensity of cigarette smoke, smoke particles, or smoke condensate to damage the genetic material (DNA) of the test cell; a more general term than mutagenicity tests.

mainstream (MS) smoke: smoke that is drawn through the burning tobacco column and filter tip and exits through the mouthpiece of the cigarette.

"in vitro" test: a test that is performed on single cells or organs derived from an animal (or human), as opposed to an "in vivo" test that is performed on an entire living animal (or human). Tests performed on primitive single-celled organisms, such as bacteria or yeast, are classified as "in vitro" tests.

"in vivo" test: a test that is performed in a whole, living animal (or human), as opposed to an "in vitro" test.

mutagenesis tests: tests for the propensity of cigarette smoke, smoke particulates, or smoke condensate to cause mutations in the genetic material (DNA) of the test cell. A widely used mutagenesis test is the Ames test, which is performed on special strains of the Salmonella bacterium.

N-Nitrosodimethylamine (NDMA): an animal carcinogen.

particulate phase: the portion of cigarette smoke that is trapped by a standard Cambridge filter at room temperature.

polyaromatic hydrocarbons: a class of carcinogenic chemicals found in cigarette smoke. An example is benzo(a)pyrene.

sidestream (ss) smoke: smoke that is emitted into the surrounding air between puffs from the end of the smoldering cigarette.

topography of smoking: study of the ways that humans consume cigarette smoke, including the intensity of the draw on the column of smoke during a single puff; the duration of the puff; the volume of smoke in each puff; the intervals between puffs; the number of puffs taken per cigarette; and the number of cigarettes smoked daily.

tumorigenic: causing tumors or cancers in laboratory animals or humans; used synonymously here with "carcinogenic."

vapor phase: the gaseous and vaporizable chemicals in cigarette smoke that pass through a standard Cambridge filter at room temperature.

Chapter B

SMOKING MACHINE PARAMETERS FOR COLLECTION OF TOTAL PARTICULATE MATTER AND GASES FROM LOW IGNITION-POTENTIAL CIGARETTES

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Under contract to the U.S. Consumer Product Safety Commission
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Introduction

The official Federal Trade Commission method (FTC, 1969, 1979, 1980) to determine tar, nicotine, and carbon monoxide in cigarette smoke is presented as a basis for the method of collecting total particulate matter and gases from low-ignition potential cigarettes. The FTC method is very similar to the Centre de *Coopération* pour les Recherches *Scientifiques* au Tabac (CORESTA) Standard Method (CORESTA, 1968) used in Europe. One of these two methods or a slight modification thereof is used in all countries that test cigarettes.

Differences between the FTC and the CORESTA methods are small. The environmental rooms under the CORESTA method are maintained at 22 f3 °C and 55-65% relative humidity. The FTC method requires conditions of 75 ±2 °F (23.9 ±1.1 °C) and relative humidity of 60 ±2%. Under the FTC method, cigarettes are smoked to a butt length of 23 mm or the overwrap plus 3 mm, whichever is longer. Using the CORESTA method, cigarettes with a filter length exceeding 15 mm are smoked to the length of filter plus 8 mm and cigarettes with extra long filter tips are smoked to the length of the tipping plus 3 mm.

The Filtrona-400 smoking machine used in the CORESTA method requires an increased draft over the burning cigarette, whereas the FTC method does not. The increased draft is not encountered under normal smoking conditions. It causes the cigarette to burn faster, reducing the number of puffs and lowering the yield of total particulate matter (TPM) and gases. This air flow over the cigarette is needed to match the results of the Filtrona smoking machine to that of the Borgwaldt smoking machine. The Filtrona and Borgwaldt are the only two existing commercial manufacturers of smoking machines.

The following is a summary of the FTC protocol.

Materials and Methods

1) Environmental Room: A room where cigarette conditioning and smoking is conducted. The room should be maintained at 75 f2 °F (23.9 ±1.1 °C) and 60% f2% relative humidity.

2) Smoking Machine: The cigarette smoking machine should be similar to the Filtrona machine used by FTC (Pillsbury, 1969). This machine can smoke 20 cigarettes at one time, one in each port. Each port can be fitted with a filter holder and filter pad for the collection of TPM. Gases pass through the pad and are collected in specially designed plastic bags (Filtrona).

3) Smoke Collection Trap: The polyacrylic plastic holders (Wartman, **1959)** can be obtained from the manufacturer of the smoking machine (Filtrona).

4) Filter Disks: Filter disks (pads) are made from a fiberglass sheet pre-cut to a diameter of approximately **44** mm. The filters collect at least **99.9%** of all particles larger than 0.3 um in diameter (Ogg, **1964).** The filter disks fit into plastic holders. The particles collected on the pad are referred to as total particulate matter (TPM).

5) Standard solutions:

A) Extraction solution: This solution contains extractant and internal standards- 2-propanol containing 1 mg anethole (p-propenylanisole, 1-methoxy-4-propenylbenzene) per mL as an internal standard for nicotine and **20** mg ethanol per mL as an internal standard for water.

B) Moisture content: Standards are prepared by adding measured amounts of water into measured volumes of extraction solution. A standard curve is constructed from the ratio of peak heights of the water to the peak height of ethanol against the amount of water added to the extraction solution, after correcting with a solvent blank.

C) Nicotine: A stock solution contains **2.500** g nicotine in 100 mL of extraction solution. Working solutions are made from 1, 2, **3, 4,** and **5** mL of the stock solution diluted and brought to 100 mL volume with extraction solution. A standard curve is constructed as with moisture content.

6) Carbon monoxide: Gases from the plastic bags in the smoking machine are passed into an infrared detector. The detector is calibrated using a carbon monoxide gas standard.

7) Gas chromatograph:

A) Moisture content analysis: The **6** ft X **1/8"** (1.8 m X 0.32 cm) diameter column is packed with 80-100 mesh porous polymer (Porapak Q). Operating temperature for the column is set at 200 °C, injection port at **240** °C, and thermal conductivity detector at 210 °C. The helium carrier gas flow is about 100 mL per minute.

B) Nicotine analysis: The **6** ft X **1/8"** (1.8 m X 0.32 cm) diameter column is packed with 2% KOH and 10% polyethylene glycol (Carbowax **20M)** on **45-60** mesh acid washed diatomaceous earth. The column temperature is set at **165** °C, and the injection port and the flame ionization detector are set at **200-250** °C. Helium carrier gas flow is about **40** mL per minute.

8) "Monitor" cigarettes: These are cigarettes with known tar, nicotine and carbon monoxide yields. Monitor cigarettes serve as "standards" to ensure that the smoking machine is operating properly. True standard reference cigarettes are mentioned in Dr. Gairola's chapter on Short-term Toxicity Tests. No fewer than four ports should be used for monitors per 20 port machine on each run.

9) Run: This is a complete smoking of 100 cigarettes- five of the same type in each of the 20 ports (4 monitor and 16 test cigarettes).

Samples

Cigarette quantities: A minimum of **150** cigarettes and preferably 200 cigarettes of each type are needed for the FTC specified tests. This would ensure that at least 100 cigarettes of each type were succesfully smoked for one run. Typically, some test pads are discarded due to cigarette lighting failures, port leaks, or other technical problems.

Sample preparation and selection: Store all cigarette samples and monitors in an environmental room or chamber for not less than 24 hours before marking or smoking. Cigarettes should remain in the environmental room until they are smoked. Select only cigarettes without physical damage. Cigarettes should be marked to either a butt length of **23** mm or the overwrap plus 3 mm, whichever is longer. The insertion depth of about 9 nun is also marked. Mark the perforations for easy identification by the technician during placement into the holder. The perforations must not be occluded or compressed by the holder since this would affect the smoke yield.

Machine Smoking of Cigarettes

1) Puff volume: **35 mL ± 0.5** mL

2) Puff duration: 2 sec ± **0.2** sec, measured under actual machine smoking conditions. Resulting draw velocity is about **17.5 mL** per sec.

3) Puff frequency: One puff per 60 sec ± 1 sec.

Weigh the filter assembly to the nearest **0.05** mg and connect it to the smoking machine so that the cigarette and filter assembly are held horizontally. Test the smoking apparatus and filter assembly for leaks. Insert a cigarette through the hole in the rubber membrane until the butt end is inserted approximately 9 mm, such that the butt end does not contact the filter disk. Light the cigarette at the beginning of the first

puff. Smoke five cigarettes per pad. If the cigarettes are very low in tar, more cigarettes may be smoked per pad providing the pad does not wet through. The cigarettes should be protected from drafts, other than normal convection, during smoking.

Results

After five or more cigarettes are smoked in each port, each pad is extracted with the extraction solution. The extracted material is analyzed for moisture content and nicotine levels. Other extracted materials, such as described in the Analyses chapter, may also be analyzed. Part of the gas phase, which has been accumulated in the bag, is passed through an infrared detector for the determination of carbon monoxide. Although not required by FTC, nitric oxide may be measured by a chemiluminescent detector designed by Filtrona, specifically for the smoking machine. The gas may also be analyzed for substances indicated in Dr. Hoffman's Analysis chapter. Tar, nicotine and carbon monoxide are reported as mg per cigarette.

TPM (total particulate matter): Immediately after smoking the cigarettes disconnect the filter assembly from the smoking machine. Record the weight gain of the filter assembly to the nearest 0.05 mg and divide this by the number of cigarettes smoked to determine TPM per cigarette.

Extraction: Immediately after weighing, place the filter pad in a dry, rubber-stoppered 25 mL flask. Wipe out the filter assembly with one-fourth of an unused pad and place this into the flask. Add **10.0** mL of extraction solution and shake for 30 minutes.

Water: A **1-10** uL aliquot of the extract is withdrawn through the stopper and injected into the chromatograph. Compare the resulting peak against the standard curve to determine the moisture content.

Nicotine: A **1-10** uL aliquot of the extract is withdrawn through the stopper and injected into the chromatograph. Compare the resulting peak against the standard curve to determine the nicotine content.

Carbon Monoxide: The gaseous phase collected in the plastic bag is passed through an infrared detector for the determination of carbon monoxide.

Tar: Tar is the TPM minus the water and nicotine. This is sometimes referred to as "FTC tar" to distinguish it from other definitions of "tar".

Discussion

The Filtrona smoking machine can be modified by installing a collection funnel at each port to collect sidestream smoke, which may have different constituent levels (Johnson, 1973; see also discussion in Dr. Harris' Overview chapter). Filtrona'a 8-port smoking machine is more easily modified than the 20-port model. The filter assembly can be replaced with a cold trap if this technique for collecting condensate is desired.

There may be a wide range of variability in smoking behaviors due to cigarette design, physiological, psychological, and pharmacological factors (see Dr. Burns' Topography chapter; Guyatt, 1989a,b; Kolonen, 1991, 1992; Nil, 1989; Zacny, 1988). Although the present testing methods are designed to produce comparative results, the smoking machine could be set up as closely as technically feasible to reflect future data on smoking behavior. The machine has sufficient range to acommodate possible changes, for example, puff frequency from one puff per minute to six puffs per minute or volume from 20 mL to 50 mL puffs. The draw velocity would also change since it is related to the frequency and volume.

A high degree of replicability for tar, nicotine, and carbon monoxide was found in parallel testing between FTC and most other laboratories over a period of 20 years. Unfortunately, all the data from these tests were destroyed when FTC closed the laboratory in 1987. Attached to this chapter is a typical graph from my files that illustrates the close correlation of tar levels found by the FTC and a private laboratory.

Cost

The approximate cost of machine smoking and analyses for tar, nicotine, and carbon monoxide for one run with a 20-port machine would be $3,000-$4,000 (4 monitor ports + 16 test ports = 20 monitor cigarettes + 80 test cigarettes, minimum). The current capacity of the Tobacco Institute Laboratory is six runs per day.

Recommendations

The FTC method should be used as the basis for the smoking machine setup in the collection of gases and total particulate matter for low ignition-potential cigarette testing. The apparatus and methodology is adaptable to changes that may be indicated by new and future data on human smoking behavior and smoke exposure. The FTC method is replicable and well-established among the US industry.

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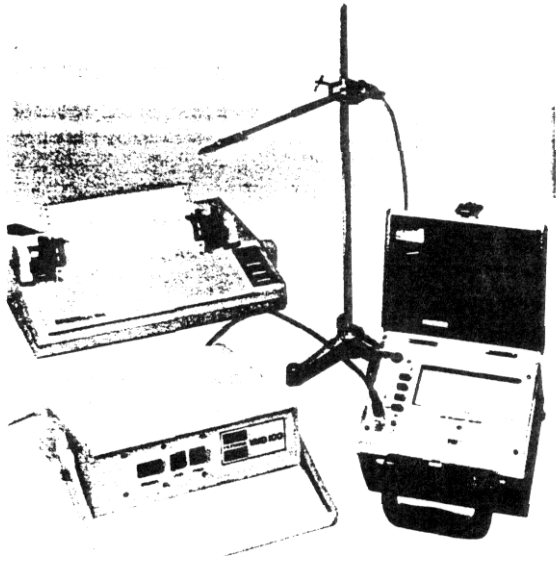
AIR FLOW VELOCITY MEASUREMENT

FOR HARMONISED SMOKING MACHINES



TSI 1640 and VMD100
(Serial Printer is optional)

- The **VMD100** provides a clear digital display enabling easy measurement of time averaged *air* flow velocity.
- Printed velocity measurements and graphical plot of values against time.
- **TSI 1640** measurement probe specially designed for maximum accuracy of low velocity *air* flows.
- Omnidirectional probe measures velocity as independently of direction as possible.
- Traceable calibration.
- Calibration matched units.

The combination of the TSI 1640 and VMD100 completely supports the measurement requirement detailed in ISO 3308 (1991) Annex A, and CORESTA recommended Method No.25.

The **TSI 1640 was** selected as the most suitable measurement device available for the measurement of air flow velocities in the smoke hood and extraction/ducting systems of smoking equipment.

The VMD100 Digitiser **has** been specially designed to be programmed with the calibration coordinates of the **TSI 1640** providing accurate digital measurements of air flow velocity.

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TSI 1640 Omnidirectional Air Flow Velocity Meter

The Model TSI 1640 Omnidirectional *Air* Velocity Meter was selected for the measurement of *air* flow velocities in the smoke hood and extraction/ducting system for the following reasons:

- It is a battery powered, portable unit, with mains battery charger.
- The TSI 1640 scale ranges are:

  0.00 - 300 mms^{-1} For Smoke Hood Velocities.

  250 - 1200 mms^{-1} } For Extracion/Ducting velocities.
  1000 - **3000** mms^{-1} }

- The probe is specially designed for maximum accuracy of low velocity flows.
- Omnidirectional probe designed to measure velocity as independently of direction as possible.
- Provides signal averaging. The thermal capacitance of the copper sensor ball approximates a lime constant of about 2s.
- The accuracy of measurement is +/- 2% of full scale deflection.
- Each sensor is individually culibrated with a system that has been verified using Laser Doppler Velocimetry.
- A certificate of traceability to the National Institute of Standards & Technology, Maryland, (USA), is supplied with each probe.

VMD 100 Airflow Measurement Digitiser/Results Plotter.

- *purpose* designed to interface directly with the analogue output of the TSI 1640.
- Calibration coordinates *can* be re-programmed by user.
- The TSI 1640 is specially calibrated in a TSI wind tunnel. The calibration coordinates obtained are programmed into the VMD 100 which provides a clear and simple display of flow velocity in mms^{-1}.
- The flow integration time in the VMD 100 is selectable from 10 to 120s in 10s steps.
- A serial output printer port is provided which gives a formatted report of elapsed time. average velocity and a graphical plot of flow variation.
- Mains powered 110V; 60HZ & 220V; 50HZ

Product Ordering Description/Code:

TSI 1640 (110V; 60HZ)	—	Stock Code: 64054
TSI 1640 (220V; 50HZ)	—	Stock Code: 64053
VMD 100	—	Stock Code: 91580

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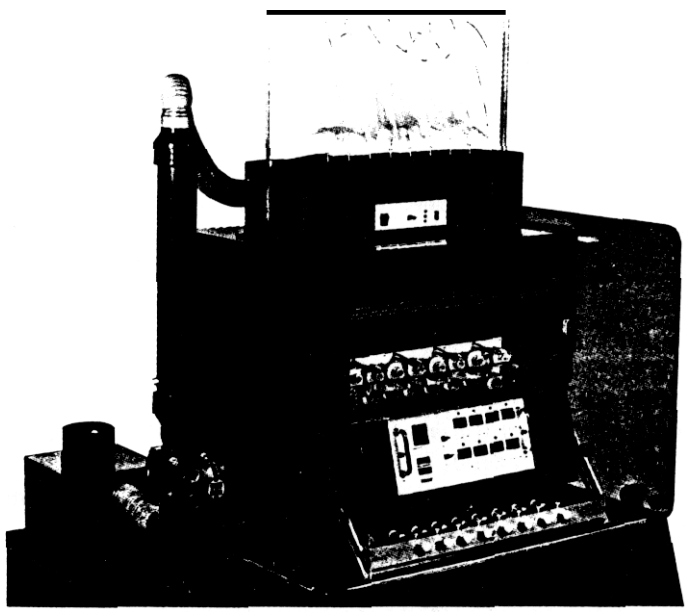
SM342 Eight Channel Harmonised Smoking Machine



SM342 shown with vapour phase attachment (COM302) mounted on top of the unit.

- Conforms to ISO 3308: 1991 and Coresta Methods.
- Simple adjustment of flow at cigarette level with single ball valve.
- Easy installation and operation — no need for special laboratory enclosures.
- Extended butt length adjustment.
- Automatic lighting sequence — ideal for routine smoking.

The Filtrona Model SM342 is an eight channel smoking machine designed for the collection of particulate matter from cigarette or cigar smoke and the collection of *vapour* phase.

This instrument is designed to have minimum dead volume for enhanced vapour phase measurement; i.e. total or puff by puff measurement of CO, or puff by puff measurement of CO and/or NO

Volume, duration and frequency of puff can be varied to suit individual requirements. It has a motor driven lighter ignition system.

SM304 Up-Grade Packages are available to convert existing SM302's in the field to the new harmonised standard.

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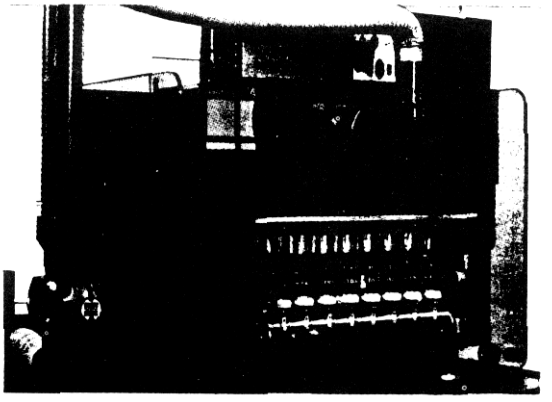
SM342 Smoking Machine



A special feature of the SM342 Smoking Machine and SM304 Up-Grade Package is the new base assembly, which enables the Smoking Machine to rotate, providing ease of access to the rear of the unit for the adjustment of the positive displacement pistons and for routine maintenance.

Specification for SM342 (and SM302 up-grade to SM3[illegible]

Number of smoking channels:	8
Type:	Restricted smoking circuit comprising separate volumetric displacement pump and change-over solenoid.
Collection media:	
Particulate matter:	Glass fibre pad (Cambridge filter)
Vapour phase:	Collection **bags** (one per channel using COM 302) and suitable analysers connected to sampling **system.**
Dead volume:	Not more than 5ml per channel. **measured** from the front face **of** the inlet **port** to the top **of** the syringe.
Puff volume:	Normally set to 35 ml. (variable **over** the range 20—40 ml)
Puff duration:	Normally set to **2.0s** (variable from **1.6** to 6.0s).
Puff frequency:	Normally set at 1 per 60s (variable from 10 to 999s).
Sample Range:	
Cigarettes:	
Length:	66—120 mm (85 mm **burn** length).
Diameter:	4.5—9.5 mm
Cigars:	
Length:	66—120 mm (85 mm burn length).
Diameter:	With alternative holder, any diameter up to 19 mm.
Mains services (operating voltages):	110/115/220/240 V; 50/60HZ.
Dimensions (bench **area required):**	
Width:	1500 mm
Depth:	850 mm
Net weight **installed:**	100 Kg.

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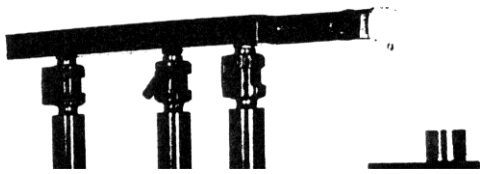
SM400 Twenty Channel Harmonised Smoking Machine



- Conforms to ISO3308-1991 and CORESTA Methods.
- Improved operator access by the use of a smoking bar which moves forward for easy cottoning and loading.
- Computer controlled, high torque motor drive system produces excellent puff profiles.

  With the new design of hood and versatile ducting arrangement, the *air* flow at the cigarette position is adjustable to meet the new ISO standards.
- SM400 upgrade packages are available for the earlier SM350 and SM300 Smoking Machines.

The Harmonisation Task Force of the **CORESTA** Smoke Study Group has been working to develop one set of standard methods which may be used worldwide.

Part of the work has been concerned with the control and standardisation of the air flow at the cigarette smoking position, and **FILTRONA'S** participation in this work has led to the introduction of a new smoking machine — Model SM400.

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SM400 Smoking Machine

PRODUCT CODE	*DESCRIPTION*
SM400	Harmonised smoking machine complete with new style (SMK401) 'operator friendly' CF Adjustors.
SM435	Up-grade package for SM350
SM430	①Up-grade package for SM300

Both up-grade packages consist of:

- SM400 Harmonised Smoke Hood.
- Extraction Ducting System.
- First Stage Extraction Fan.
- CF Adjustor (old style) Retaining Kit
- Air Velocity Setting Jig.
- Installation Instructions.
- *Air* Flow Setting Procedures.

OPTIONS:

SMK401	New butt length and eccentricity adjustors (set of 20). for SM350 smoking bars only (93mm cotton pillars/micro switch arms.)
TSI 1640	Air Velocity Meter.
VMD 100	Air Velocity Digitiser and Plotter

Note 1: The installation of a new smoking bar is recommended.

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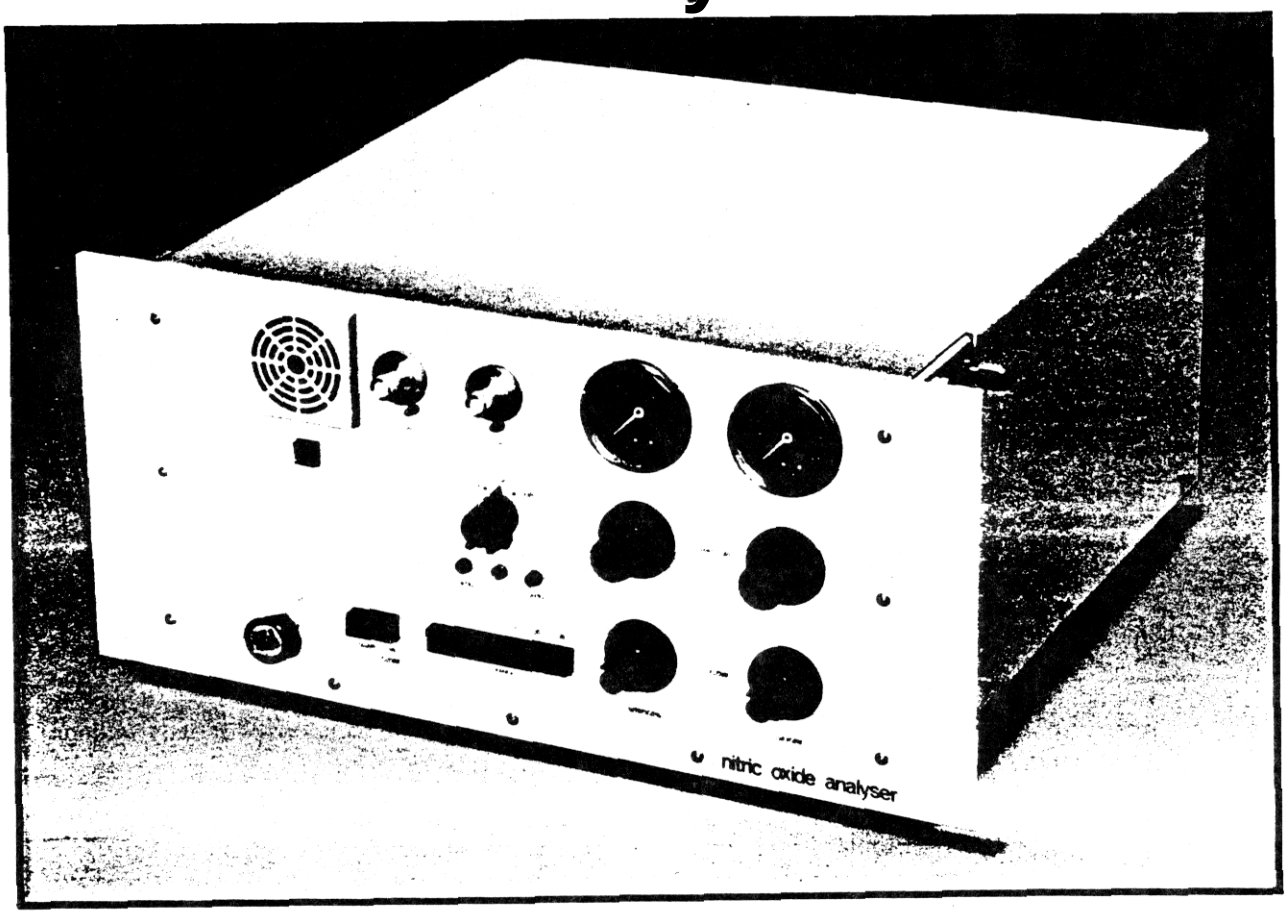
Nitric Oxide Analyser



Nitric Oxide Analyser (NOA 100)

Cigarette Smoke

Nitric oxide is a physiologically important constituent of the vapour phase of cigarette smoke, and its measurement may become the subject of future legislation. However, one of the problems of isolating and analysing nitric oxide in cigarette smoke is that it *reacts* rapidly with other smoke constituents and atmospheric oxygen to give other oxides of nitrogen. It is therefore essential that samples are analysed on a puff-by-puff basis and are diluted with an inert gas in order to prevent secondary reactions and interference from other smoke constituents.

The Filtrona Nitric Oxide Analyser is connected to a suitable smoking machine and automatically measures the amount of nitric oxide on a puff-by-puff basis.

- Designed to work with Filtrona Model 302 Smoking Machine
- Can be used with other piston-operated smoking machines with low dead volume
- Puff-by-puff dilution with nitrogen prevents secondary reactions
- Measurement by chemiluminescence technique which is specific for NO and does not require skilled operators
- High-sensitivity photomultiplier measures a wide range of NO concentrations
- Automatic operation
- Built–in ozone generator
- High-quality flow controllers with clear vernier dials
- Easy calibration

Principle of operation

The measurement technique used is chemiluminescence, which is a widely accepted method for the analysis of nitric oxide in cigarette smoke. The principle is to react the sample of cigarette smoke with ozone and to observe the photoemission using a photo-multiplier tube behind a dark-red optical filter. This reaction has a direct relationship to the quantity of nitric oxide present in the smoke. The photo-multiplier signal is processed and displayed on a chart recorder.

Interference from other smoke constituents, and secondary reactions, are avoided by dilution of the smoke sample with a large volume of nitrogen before reaction with the ozone.

Method of operation

NOA 100, developed by British-American Tobacco Company in consultation with other UK tobacco companies, is designed for automatic sampling of vapour phase with Filtrona Model 302 8-channel Smoking Machine. It can also be used manually with some other piston-operated smoking machines.

After each puff, the vapour phase is exhausted from the smoking machine into a gas sampling valve in the analyser. Nitrogen is fed into the sampling valve and sweeps the sample into the reaction cell. Ozone is there added to the sample, and the photo-multiplier tube measures the resulting photo-emission. Results are displayed on a chart recorder, and the readings are compared with those obtained from standard mixtures of nitric oxide in nitrogen. This gives the concentration (volumetric parts per million) of nitric oxide, and a simple calculation is used to convert to delivery in μg per cigarette.

By attachment of a simple valving system, that part of the vapour phase not required for NO analysis can be transferred to a Filtrona CO analyser, for simultaneous analysis of NO and CO of the same sample.

Note that this instrument must be used with a chart recorder. This must have a response time of better than 0.6 seconds for full-scale deflection.

Specifications

Range	up to 5000 volumetric parts per million (VPM)
Attenuation settings	1, 2, 5, 10, 20, 50 (low, medium and high range for each setting)
Sensitivity	1 VPM NO
Linearity	± 1%
Accuracy	± 1% of full Scale after calibration
Reproducibility	± 1%
Nitrogen supply pressure (external)	4 bar (60 psi) (Nitrogen to be *supplied* by *user)*
Oxygen supply pressure (external)	1.4 bar (20 psi) *(Oxygen to be supplied by user)*
Gas connections	1/8 inch Swageiok
Electrical supply	220-240V 50Hz supply (standard); versions for other supplies at extra cost
Dimensions (mm)	Width 483 (rack or case mounting) Depth 495 Height 223
Weight	20kg
output	An external socket is provided for connecting to a 1mV fast-response chart recorder. This can be supplied as an optional extra (see below)
Standard Equipment	All electrical and mechanical fittings needed to connect NOA 100 to a Filtrona 8-channel Smoking Machine (SM 302) are supplied.

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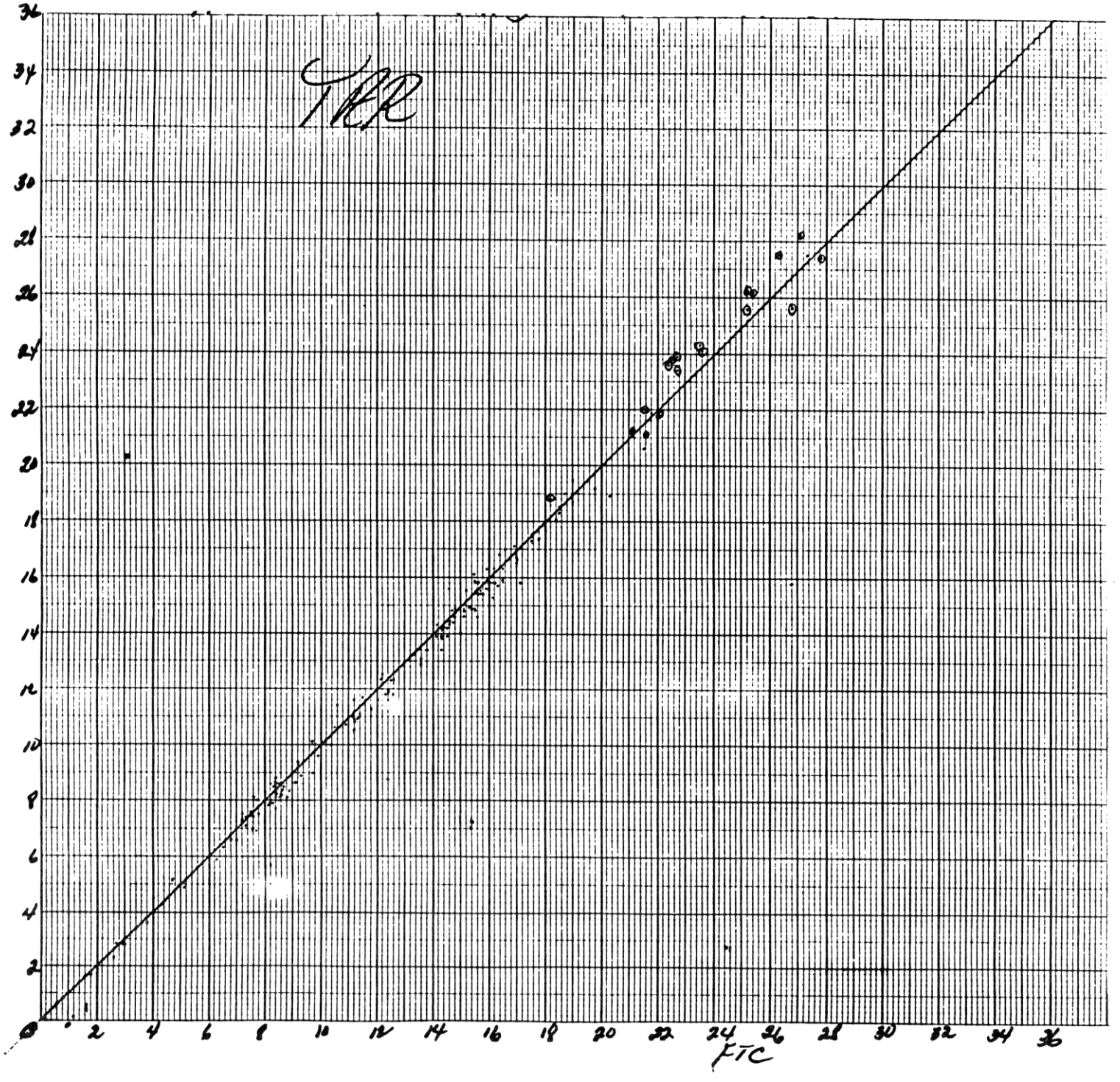

Correlation Between Tar Levels Recorded in FTC Test and a Private Laboratory

Chapter C

ASSESSING CHANGES IN TOPOGRAPHY (INHALATION PROFILE) AND BIOLOGICAL EFFECTS OF TOBACCO SMOKE IN HUMANS

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INTRODUCTION

The most useful approaches in the evaluation of differences in risks between presently marketed and low-ignition-potential cigarettes focus on chemical analyses of the cigarette smoke, measures of smoke absorption, and assays in biological systems. Because of practical constraints, human epidemiologic studies would be of very limited practical value. Although the ideal database would derive from prospective human epidemiologic studies, at least twenty years of observation would be needed to collect sufficient data on seriously adverse health effects. Also, if small differences in toxicity are expected, then large sample populations would be needed.

The toxicity of cigarette smoke is a function of the toxic constituents present in the smoke, the levels of the constituents in the smoke, and the dosage of the constituents to the smoker. Thus these data must also be collected for the evaluation of the relative risks of low ignition-potential cigarettes in comparison to current brands.

Smoke production, as quantified by tar yield, varies substantially among the current cigarette brands. It also varies for a single brand when different patterns of inhalation are used. Moreover, the relative concentrations of toxic constituents also vary with brand of cigarette and pattern of inhalation, at least as measured by tar and nicotine yield. These differences are related to the health risks among different brands of cigarettes (DHHS 1981). Since it is possible that ignition-potential reducing designs in cigarette manufacturing might quantitatively and qualitatively alter the smoke produced, there is concern that the health risks might be increased.

The inhalation profile of a smoker as he or she smokes a cigarette is termed "topography". Ignition-potential reducing designs may alter the topography in ways that lead to greater inhalation and retention of the smoke. Differences in the depth and pattern of inhalation may change the amount of smoke that is deposited and retained in the airway. Therefore, machine generation of smoke from low ignition-potential cigarettes for testing should reflect the human patterns of inhalation for the specific brand of cigarette. This will ensure that the smoke being tested is similar in composition to that being inhaled by human smokers.

Assessment of differences in the risks of smoking low ignition-potential cigarettes in comparison to current brands of cigarettes should address:

- Differences in chemical composition of mainstream and sidestream smoke produced by these cigarettes

- Differences in the amount of the mainstream and sidestream smoke produced by these cigarettes

- Differences in the amount of smoke inhaled and retained by smokers, and

- Toxicity of the smoke produced by these cigarettes, as tested in biological systems.

FACTORS INFLUENCING SMOKE YIELD AND COMPOSITION

Different brands of cigarettes currently manufactured in the US vary markedly in yields of tar and nicotine (DHHS **1981)** when smoked using the standard puff profile developed by the Federal Trade Commission (Chapter B). When the same brand of cigarette is smoked using different puff profiles, the yields of tar and nicotine also vary substantially (Zacny **1992).** Individuals smoking the same brand of cigarette may inhale using markedly different patterns (Nil **1989).** When smokers of a high yield cigarette switch to a lower yield cigarette, their inhalation pattern often changes (Kolonen **1991;** Woodman **1987;** Guyatt **1989).** Interactions have been demonstrated between the yield of a cigarette and the pattern with which the smoker smokes the cigarette (DHHS **1988;** Benowitz **1983;** Kolonen **1991;** Hofer **1992).** These changes in yields and smoking patterns should be considered during the evaluation of the health effects.

A number of the manufacturing changes under consideration in the effort to reduce the ignition potential of cigarettes (e.g., higher porosity paper, less densely packed tobacco, different tobacco blends) may alter the amount and chemical composition of the smoke produced (Gann 1991), potentially changing its toxicity. The same changes in cigarette manufacturing processes may also alter the pattern of inhalation of the cigarette (Bridges **1990;** Kolonen **1991;** Armitage **1988).** This, in turn, may change the chemical composition of the smoke (Kozlowski **1988;** Fischer 1989), influence the retention of toxic and carcinogenic compounds from the smoke in the lungs of smokers (Zacny **1992;** Hofer **1991;** Battig **1982;** Bridges 1986), and alter the composition and toxicity of the environmental tobacco smoke (Adams et al **1985).**

A single set of machine smoking parameters, such as the current FTC protocol (Chapter B), could be followed for the generation of cigarette smoke for testing. However, this single set would ignore possible differences in patterns of smoking (and resultant constituent yield) of low-ignition potential cigarettes. For example, a drop in the draw resistance of a cigarette may lead to a puff volume greater than that specified in the FTC protocol. The larger puff volume could then lead to a deeper inhalation of the smoke and a greater fraction of the total particulate matter being deposited in the lung.

The complexity of the interaction of smoke yield and pattern of inhalation suggests that chemical and biological approaches are needed. Chemical analyses of the differences in whole smoke exposure of the smoker can be assessed from measures of the amount and composition of the smoke produced when cigarettes are smoked by machine using a variety of inhalation patterns, and these quantitative estimates can be compared to measures of the absorption of smoke constituents obtained from human smokers of these brands of cigarettes.

Biological assays of relative carcinogenicity and toxicity of the smoke produced by low ignition-potential cigarettes can be accomplished using a combination of chemical analytic techniques to measure the relative yields of individual compounds produced by different cigarettes and bioassay techniques to assess the relative toxicity of the smoke produced.

The toxicity of cigarette additives is of concern. A new additive, or its pyrolysis products, could increase the known toxicity of the smoke. It might also cause toxicities that are qualitatively different than those presently associated with cigarette smoke. Direct toxicity testing of additives and their combustion products should also be required.

The following section describes how smoking patterns can be measured and explores what is known about the variation in topography of smoking among smokers of the same type of cigarette, among smokers of cigarettes with different yields and among those who switch to cigarettes with different yields. A subsequent section defines what is known about the absorption of smoke constituents; and finally, an approach will be recommended for use in assessing the changes in risks and exposures that may occur with implementation of the proposed technologies to reduce the ignition potential of cigarettes.

PATTERN OF SMOKING

The first step in the process of assessing the relative risk of low ignition-potential cigarettes would be to establish how the patterns of smoking differ for low ignition-potential cigarettes compared to current cigarette brands. The major determinants of disease risks from smoking are the duration of smoking and the intensity of smoke exposure (DHHS **1982; 1983; 1984).** Several measures of the intensity of smoke exposure correlate with increased disease risks, including: number of cigarettes smoked per day (DHHS **1982; 1983; 1984),** depth of smoke inhalation (DHHS **1982),** and tar and nicotine content of the cigarette (DHHS **1981).** Each of these measures of intensity of exposure might change when a smoker switches from smoking conventional brands of cigarettes to low ignition-potential cigarettes.

Topographical characteristics, such as puff volume, draw rate, puff duration, and draw pressure differ between smokers and may alter the composition of the smoke. Smokers differ in the number of puffs per cigarette, length of time between puffs, depth of inhalation, and holding of the puff in the mouth before inhaling (Nil **1986;** Guyatt **1989;** Bridges **1990;** Russell **1982;** Battig **1982;** DHHS **1988).** Ignition potential-reducing changes in the blend or amount of tobacco in a cigarette, packing density of the cigarette, and porosity of the paper wrapper are changes under consideration (Gann **1988)** and may also alter the topography.

The major purpose of examining topography when smoking low ignition-potential cigarettes is to determine appropriate smoking profiles for machine smoking of these cigarettes. Once the range of smoking topography is established for each brand of low ignition-potential cigarette, then the yields and chemical composition of mainstream and sidestream smoke likely to be generated by human smokers can be estimated.

Measurement of Smoking Pattern

Number of cigarettes smoked per day is typically estimated by self-report (by the smoker) through an interview or a questionnaire. This measure has been shown to be closely correlated with risks of serious disease (DHHS **1982;** DHHS **1983,** DHHS **1984).**

Inhalation depth has also been assessed by self-report and is associated with disease risk in most, but not all, epidemiologic studies (DHHS **1982).** A number of other methods have also been used to estimate depth of inhalation including measures of chest wall motion with strain gauges, impedance, magnetometers and whole body or inductance plethysmography (DHHS **1988).** Depth of inhalation has also been assessed by measurement of blood carboxyhemoglobin to estimate exposure of the lung alveolar surface to the carbon monoxide in smoke (Herling **1988).**

Draw characteristics of the topography have been measured using a variety of techniques including self-report and third person observation (Hofer **1991).** The most common approach has been to use a flowmeter attached to the butt end of the cigarette as it is smoked (Creighton **1978;** Puustinen **1987).** This device allows direct measurement of the flow of smoke drawn into the mouth, draw pressure, and flow duration. The flow rate is integrated to calculate puff volume. The inter-puff interval is calculated from the time between periods of flow. The limitation of using a flowmeter is that it is placed between the smoker and the cigarette and may affect the pattern of smoking.

Variation in the Pattern of Smoking with Existing Cigarettes

There is a marked variation in the number of cigarettes smoked per day by different smokers. Some individuals smoke less than one cigarette per day and others smoke 60 or more cigarettes per day (Pierce and Hatziandreu **1989**; Burns and Pierce **1992**). The day-to-day variation in number of cigarettes smoked per day by an individual smoker is much smaller than the variation among different smokers in the population, particularly for those who smoke every day. The number of cigarettes smoked per day varies somewhat with age, gender, and socioeconomic factors. There is also some variation according to racial grouping. Black smokers tend to report fewer cigarettes smoked per day than white smokers, and Hispanic smokers are more likely to be occasional smokers.

An extensive presentation of the variation in smoking topography is presented in the Surgeon General's report on nicotine dependence (DHHS 1988), and the variation in the topography of smoking as measured in published studies is presented in Table 1. There is relative uniformity in the mean values for the measures of smoking topography across these studies; but there is a substantial variation in the measures of smoking topography among individual smokers (Nil **1986**; Guyatt **1989**; Bridges **1990**; Russell et al.; Battig **1982**). This variation among individual smokers is defined by the standard deviation of the measured values presented in Table 1, and it is evident that there is a wide variation in the pattern of inhalation among individual smokers. The variation in pattern of smoking is much less for two cigarettes smoked by the same smoker (Battig 1982), suggesting that it was differences between smokers in the way that they smoked, rather than differences in the way a specific smoker smoked sequential cigarettes, that produced the variation in smoking topography found by these studies.

The pattern of smoking also varies with the tar and nicotine yield of the cigarette smoked. Some smokers of lower yield cigarettes clearly compensate for the lower yield of these cigarettes by altering the way they smoke these cigarettes to increase the absorption of nicotine. Their nicotine absorption levels are substantially higher than would be predicetd from the nicotine yields generated from machine smoking (Benowitz **1983**; Kolonen **1991**; Hofer **1992**). This compensation is largely accomplished by changes in puff volume and frequency, rather than by increasing the number of cigarettes smoked per day (Kolonen **1991**; Battig **1982**; Bridges 1990).

A number of investigators have examined changes in the topography when smokers switch to lower yield cigarettes, both immediately and after a period of regular smoking of these cigarettes. When smokers switch to a cigarette that has a lower yield than their regular brand, there is an immediate compensation in the smoking topography with an increase in puff volume and puff frequency (Kolonen **1991**; Woodman **1987**; Guyatt

1989). However, the change in puff frequency can revert to the original rate within a few weeks or months (Guyatt 1989). In the studies that measured nicotine absorption, there was a fall in amount of nicotine absorbed when the smoker switched to a lower yield cigarette, but the magnitude of the fall was much smaller than would be predicted from the machine smoking results.

Existing data clearly indicate that differences in cigarette manufacturing processes which affect the yield of the cigarette can influence smoking topography. Many of the changes appear to be a compensatory repsonses intended maintain nicotine absorption from cigarettes with a lower nicotine yield, such as increased puff volume and inhalation depth. Topography alterations may also result from changes in draw resistance (Guyatt 1988), filter perforations (Kozlowski 1988), and possibly other differences in manufacturing technology.

MARKERS OF THE DOSE ABSORBED BY THE SMOKER

Chemical analyses can quantify the several thousand constituents present in tobacco smoke (Chapters B and D), but it is neither practical nor technically possible to measure human absorption of each of the several thousand smoke constituents. Therefore, evaluation of the relative toxicities of the smoke produced by low ignition-potential cigarettes will need to rely heavily on selected chemical analyses and biological (in vitro and in vivo) toxicity tests of the whole smoke and its components. Measures of absorption can be used predominantly as markers of whole smoke exposure.

Biochemical markers, such as nicotine and carbon monoxide, can quantitatively estimate the amount of smoke absorbed by the smoker and effectively integrate the sometimes competing effects of differences in cigarette manufacturing and resultant changes in smoking topography. Biochemical markers produce a unifying estimate of the differences in exposure/dose resulting from smoking different brands of cigarettes. If switching to low ignition brands leads to a greater number of cigarettes being smoked per day, a deeper inhalation, or a change in the topography of smoking, it may lead to greater smoke absorption; these would increase the risk of those adverse health effects summarized in Chapter A. A number of biochemical markers have been used to quantify the amount of smoke inhaled and retained by the smoker (IARC 1986, DHHS 1988).

The accuracy and utility of using a single biochemical marker to estimate the absorption of whole smoke is influenced by several factors. First is the quantitative relationship between an increase in the level of the marker and the amount of the smoke absorbed by the smoker. Second is the specificity of the marker for the absorption of tobacco smoke as compared to other

sources of the marker. Third is the direct quantitative relationship between the absorption of the marker and the absorption of other toxic constituents in the smoke. And finally, there is the precision of the measurements.

Biochemical markers of absorption can be used under non-experimental conditions that represent more typical smoking behavior. Biochemical markers are advantageous since they can be sampled after a period of smoking under non-experimental conditions approximating normal behavior. This may be less disruptive to the smoking behavior than topographical measures taken during smoking by attaching instrumentation to the cigarette or to the human smoker.

Carbon Monoxide

Carbon monoxide (CO) is a toxic gas phase constituent of tobacco smoke and is a useful measure of the depth of inhalation of cigarette smoke. The CO produced is dependent on the amount of oxygen available to the burning tobacco. However, other factors, such as density of the tobacco, paper porosity, puff volume, and draw rate, may influence the amount of CO produced and reduce the correlation between CO and particulate matter (IARC **1986).** Therefore, CO can not be predicted on the basis of particulate matter (or tar) yield.

CO binds reversibly to hemoglobin (the oxygen-carrying protein in the blood), and is cleared from the body primarily by exhalation. The concentration of carbon monoxide in the blood can be determined as the partial pressure of CO in the expired air or as the level of carboxyhemoglobin in the blood (DHHS **1988;** IARC **1986).** The blood and breath levels of CO vary markedly during a smoker's day due to the intensity of smoking in the hours immediately before the measurement and the short 4-hour half-life of CO in the blood.

The single point-in-time measurement of blood or breath CO is very precise, but may not represent the total daily smoke absorption due to its short half-life, the background sources of CO and endogenous production of CO (Woodward **1991).** However, the change in blood or breath CO level after smoking a single cigarette can represent the smoke absorbed and may be a better measure of the depth of inhalation of the cigarette smoke than serum nicotine or cotinine levels. Since CO is absorbed almost exclusively by the lung, it is a more specific measure of smoke inhalation than nicotine, which is also absorbed through the oral mucosa (Herling **1988).** CO is also a marker for exposure to the gas phase constituents of smoke since it is present in only the gas phase.

Nicotine and Cotinine Levels

Concentrations of nicotine and its longer half-life metabolite, cotinine, can be measured in the blood, saliva and urine to very low levels (DHHS **1988;** IARC **1986;** Anderson **1991;** Benkirane **1992;** Etzel, **1990;** Feyerabend **1990;** Weinhold **1987).** The major non-tobacco sources of nicotine are nicotine patches and gums used as adjuncts to smoking cessation. Nicotine is not produced by the body and is found in only trace amounts in plants or foods other than tobacco (DHHS **1990).** The contribution **of** environmental tobacco smoke to the cotinine level of most regular smokers is less than one percent (DHHS **1986;** Watts **1990).** **As** a result, nicotine and cotinine levels are highly specific to tobacco smoke exposure.

Tar and nicotine yields are well correlated for most current brands of cigarettes (DHHS **1981).** Therefore, nicotine or cotinine levels can be used to estimate the amount of particulate matter ("tar") absorbed and retained by a smoker. Cotinine is the preferred measure of nicotine absorption because of its longer **20-30** hour half-life in the blood (Benowitz **1983;** IARC **1986).** Cotinine is also less influenced by active smoking or environmental tobacco smoke than salivary nicotine levels.

Salivary, blood, or urinary levels of cotinine can represent the average daily dose of particulate phase constituents absorbed. This is important because the bulk of the carcinogenic activity in tobacco smoke is in the particulate phase (IARC **1986).** Cotinine may also be useful for determining the net effect of changes that have opposing effects on smoke absorption, for example a cigarette with a lower tar and nicotine yield that is inhaled more deeply.

Thiocyanate

Hydrogen cyanide is found in the gas phase of cigarette smoke and is metabolized to thiocyanate by the body. Thiocyanate can be measured in plasma, urine, and saliva (IARC **1986).** Although thiocyanate has an extremely long half-life (approximately 2 weeks), there are a number of foods that influence thiocyanate levels, including leafy vegetables and nuts. Dietary sources make the independent contribution of tobacco smoke to the thiocyanate level difficult to determine. Therefore, thiocyanate is an imprecise and poorly reproducible quantitative marker for smoke absorption.

Other Compounds

A number of other constituents of tobacco smoke, or metabolic products of tobacco smoke constituents, have been used as measures of smoke absorption including N-nitrosoproline and thioethers (IARC **1986).** However, none of these measures appear to offer any advantages in estimating smoke absorption over the three measures described above.

TESTING FOR TOXIC EFFECTS OF THE SMOKE ABSORBED BY THE SMOKER

A number of short-term toxic effects of human tobacco smoke inhalation and retention can be measured. Some are postulated to be early or intermediate steps leading toward seriously adverse health effects (Chapter A). These effects include reduced serum high-density to low-density lipoprotein ratios, increased platelet aggregation, acute bronchial reactivity to smoke inhalation, small airway dysfunction in the lung, and greater mutagenicity of the urine. Measurement of these effects might possibly define the degree of damage being done by the smoke in a way that would integrate the effects of dose of exposure and biologic susceptibility. In addition, they might also detect pathophysiological toxicities that would not be evident from chemical analyses or markers of whole smoke exposure.

The limitations of these tests include their variability of measurement, the relatively long period of time required for smoking behavior to produce measurable change, and the degree of scientific uncertainty concerning their role in the pathophysiology of smoking-related disease. In addition, the general population varies widely in the levels of most of these effects. Due to the large variation and possibly small relative changes, a large group of volunteers may be needed to attain statistical significance.

COMPARISON OF LOW IGNITION-POTENTIAL CIGARETTES

The ideal comparison "standard" for the low ignition-potential candidate brands would be existing cigarette brands tested under the same protocol. This would allow comparison of the toxicity of the candidate brands with the range of toxicities for the currently marketed brands.

One approach is to compare the risk estimates for the candidate brand to the range of risk estimates for brands currently on the market. For example, low ignition-potential cigarettes with risk estimates that are within the range of those for currently marketed brands might be assumed to be no more hazardous than current brands of cigarettes. Risk estimates for current brands of cigarettes would be obtained by performing the same tests used on the low ignition-potential candidates. This approach avoids the problem of defining an acceptable standard for low ignition-potential cigarettes.

A variant of this approach would be to require no increase in the sales-weighted average risk for all of a given cigarette manufacturer's products when a low ignition brand of cigarettes is marketed. This allows the manufacturer to adjust the mix of their brands to avoid an overall increase in risk. These

approaches assume that existing brands of cigarettes will be allowed in the market even though they may have higher ignition potential.

Another approach, which does not assume that existing brands will continue to be marketed, compares a candidate brand with the one it was intended to replace or duplicate in the marketplace or the one from which it was developed. This approach would ensure that a new cigarette brand would not increase the average risk of cigarette smoking. However, it may not always be possible to pair a candidate brand with an existing brand.

SOURCES OF INCREASED HAZARDS

The major measurable increases in the hazard of smoking low ignition-potential cigarettes compared to current cigarettes are likely to come from differences in the:

1. cigarette manufacturing process or tobacco used that lead to a greater total yield of mainstream or sidestream smoke,

2. pattern of smoking that lead to a greater total yield of mainstream or sidestream smoke,

3. chemical composition of mainstream and sidestream smoke produced that lead to more toxic or carcinogenic smoke,

4. pattern of smoking that lead to greater absorption of smoke, and

5. additives to the cigarette that increase the toxicity or add new toxicities to the smoke.

Changes in toxicity could be evaluated for each of the many different possible designs that could reduce the ignition potential of cigarettes. However, this approach would be both impractical and wasteful since the many different ignition-potential reducing strategies would generate a prohibitively large number of combinations to be tested. The vast majority of the combinations would also be unlikely to be used in commercial cigarettes. Thus, it is only necessary to examine those combinations of low-ignition-potential changes that cigarette manufacturers present as potential commercial brands.

RECOMMENDATIONS

Generation of information to assess the risks of low ignition-potential cigarettes is product research and toxicity testing, a responsibility most appropriately belonging to the

cigarette manufacturers. The data should be generated by the manufacturer when contemplating the introduction of a new brand or replacement of an existing one. In addition, a new low ignition-potential cigarette is likely to combine designs to reduce the ignition potential with those to improve the taste or other marketing characteristics of the cigarette. It is the combination of all changes in a low ignition-potential cigarette, not just those that reduce the ignition potential, that determine its relative toxicity. Therefore, these cigarettes will need to be examined on a brand-by-brand (product performance) basis rather than on a manufacturing change-by-change (product design) basis. The following data should be collected on a candidate cigarette brand:

1. Smokina topography
   The number of cigarettes smoked per day, puff volume, puff duration, puff interval, maximal puff inspiratory pressure and flow, and number of puffs per cigarette should be measured in an experimental group of smokers of the brand after they have acclimatized to smoking the low ignition-potential brand.

2. Smoke yield and composition
   The range of mainstream and sidestream concentrations of the compounds listed in Table 3 of Chapter D should be assessed for smoke produced by machine smoking the brand using a range of smoking topographies that correspond to those observed for that specific brand.

3. Additives
   A complete list of additives and the concentrations used, as well as their likely pyrolysis products, should be disclosed for each brand. Confidential Business Information status may be requested to protect proprietary information.

4. Inhalation and retention of smoke
   The change in CO level that results from smoking a single cigarette of the brand should be measured in acclimitized smokers as a marker for acute exposure. Cotinine levels should be measured in acclimatized smokers of the brand as a marker for average daily exposure.

5. Toxicity of the smoke produced
   The toxicity of the mainstream and sidestream smokes produced by machine smoking each brand of cigarettes should be evaluated using the approaches described in Chapters E and F of this report.

Testing Sequence

The sequence of testing for the evaluation of toxicity and carcinogenicity of low-ignition-potential brands of cigarettes should include the following stages for mainstream and sidestream smokes:

I. Initial Evaluation

1. Machine testing

Initial evaluation of each candidate brand before testing in humans, should include analyses of CO, nicotine, tar, and each of the other constituents of tobacco smoke listed in Table 3 in Chapter D using the FTC method (Chapter B) to generate the smoke.

If the smoke produced using the FTC method did not yield substantially greater amounts of the compounds listed in Table 3 of Chapter D, then testing would proceed using a range of smoking topographies observed for current brands of cigarettes. Topographies reported by several studies for current cigarettes (Table 1) are graphically presented in a puff volume / puff duration matrix (Table 2 of this chapter). The central cells in this matrix are the most commonly reported values from the studies in table 1 and the values for the FTC method. Tar, nicotine and CO yields should be measured under the smoking conditions specified by each shaded and unshaded cell (15 ml/1 sec through 90 ml/3 sec) to reflect the range of observed human topographies.

The toxic chemical constituents in Table 3 of Chapter D should then be analyzed using smoke generated according to the topographies indicated by the shaded cells of Table 2 in this chapter (except for the FTC protocol, which has already been conducted). These represent the most common and the extremes of reported topographies. The reason for testing under conditions reflecting the extremes is to examine the effects on yield of using rapid and slow puffing, and large and small puff volumes. It is under these conditions that unexpected changes in smoke yield and composition are most likely to occur. The smoke consitutents should also be analyzed for any cell that resulted in a tar level that was substantially higher than the average for the entire matrix. Smoke constituents should be reported as a fraction of the tar generated, for example nicotine/g of tar.

If the ranges of the constituents generated using the smoking profiles defined by this matrix is within or below the range for existing commercial brands of cigarettes, the candidate cigarette may proceed to the biological testing stage. If one or more of the constituents exceeded the range for current brands of cigarettes, a risk benefit analysis which includes the other constituents and the reduction in ignition potential may decide whether to reject the cigarette or to proceed with biological testing.

2. Biological testing

Biological testing, described in Chapters E and F, can be conducted utilizing mainstream and sidestream smoke generated using the FTC method and the topographies specified by any cell in the matrix in Table 2 where the ratio of toxic constituents to tar content is statistically significantly greater than the mean value for all of the tested cells (see section of machine testing above). The results from the tests of the low ignition brands can be compared with the range of the results available from testing of existing commercial brands. An increase in the toxicity of the candidate brand over the range of results for currently marketed brands may be considered an increase in the health hazard attributable to the candidate brand.

3. Additives

A complete list of the levels of additives in the candidate cigarette brand and the probable identities of associated pyrolysis products should be reported. If the toxicity data on these additives and pyrolysis products are not available, additional toxicity testing should be performed.

11. Characterization of topography in humans

Topography studies in humans may be conducted when the initial evaluation of the candidate brand indicates it may be no more hazardous than current commercial brands of cigarettes (considering both disease risks and ignition potential). Human studies would define the actual topography of smoking that occurs with a new low ignition-potential cigarette. It would also collect data on the associated markers of smoke absorption, which are needed for determination of human risk.

Volunteers should be selected to represent a balanced gender, socioeconomic, and ethnic/racial distribution. Additionally, the group should be selected to equally represent smokers who smoke the full range of the number of cigarettes per day of cigarette brands with nicotine yields (FTC method) in the top, middle and lowest thirds of the current brands of cigarettes by market share. In order to be confident that there is adequate representation of each of these factors in the study, at least 200 smokers should be selected for each study group.

Data collected before and after switching to the candidate brand include smoking topography, cigarettes smoked per day, urinary cotinine, and co levels in the breath before and after smoking a candidate cigarette. The volunteers would be allowed to acclimatize to the candidate brand for two to three weeks. Mean values of the markers of smoke absorption that exceed the range reported for current cigarettes or increases in mean levels

that result from switching to the prototype brand would be evaluated for toxicity concerns.

The range of smoking topography observed in the groups should then be compared to the matrix in Table 2 of this chapter. If the results are significantly above or below the ranges in the table, then chemical analysis and toxicity testing must be repeated using the topographies that were outside the matrix.

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Table 1

Smoking Topography		Number of Subjects	Cigarettes Per Day	Puff Volume (ml)	Puff Duration (sec)	Peak Flow (ml/sec)	Puffs Per Cigarettes	Inter-Puff Interval (sec)	Cigarette Duration (sec)	Inhalation Volume (ml)
Rawbone (1978) Low Tar	mean s.e.m.	5			1.74 ± 0.28		9.8 ± 1.07	43.6 ± 5.430		
Rawbone (1978) Middle Tar	mean s.e.m.	7			1.92 ± 0.205		10.9 ± 0.77	38.8 ± 4.873		
Jarvik (1978)	mean	9	18.5				9.7			
Guillerm (1978)	mean range	8		38.5 15-80	1.85 0.8-2.9	35 15-50	12 5-16	40.7 23-115	390	452
Ashton (1978)	mean	14	10-25 (range)		1.47			24.0		
Schultz (1978) 1971 Group	mean	100			1.41		10.5	50.3		
Schultz (1978) 1974 Group	mean	218			1.34		11.8	41.5		
Moody (1980)	mean s.d.	517	20.7 ± 12.5	43.5 ± 21.9	2.12 ± 0.88		9.26 ± 3.79	25.8 ± 17.1	[1]232.2 ± 103.8	
Russell (1980b)	mean	10	26.7				10.7			
Henningfield (1981)	mean 95% c.i.	8	26 [2]15-35		.99 [2]0.70-1.19		9.9 ± 0.30	39.4 ± 1.54	351 ± 9.0	
Stepney (1981)	mean s.d.	19	[3]18.4 ± 8.1				12.9		400	485
Battig (1982) [4]male	mean s.e.m.	67	22.3 ± 9.0	43.7 ± 14.0	2.28 ± 0.5		12.1 ± 4.2	24.8 ± 10.7		527 ± 196.1
Battig (1982) [4]Female	mean s.e.m.	43	20.9 ± 8.6	37.1 ± 10.1	1.99 ± 0.42		13.0 ± 4.5	24.7 ± 11.6		481 ± 208.2
Epstein (1982) Male	mean s.e.m.	33	20.1 ± 1.9		[5]33.1 ± 1.61		12.7 ± 0.6			278.5 ± 20.8
Epstein (1982) Female	mean s.e.m.	30	14.9 ± 1.5		[5]27.84 ± 1.55		13.3 ± 0.6			263.0 ± 22.3
Russell (1982) Usual Brand	mean s.d.	12	23.8 ± 8.1	39.8	2.3		14.7	25.6	[1]324	547

		Number of Subjects (ml)	Cigarettes Per Day	Puff Volume (ml)	Puff Duration (sec)	Peak Flow (ml/sec)	Puffs Per Cigarettes	Inter-Puff Interval (sec)	Cigarette Duration (sec)	Inhalation Volume (ml)
Gritz	mean	8	27.5 ± 10.4	65.6 ± 27.6	2.2 ± 0.5	78.1 ± 23.3	9.5 ± 2.1	± 16.2		± 269.8
[6]Ossip-Klein (1982)	mean s.d.	9	30.0 ± 14.1		1.4 ± 0.1		7.2 ± 1.5		378 ± 108	
[7]Ossip-Klein (1982)	mean s.d.	9	29.2 ± 11.1		1.7 ± 0.6		10.3 ± 4.6		402 ± 84	
[8]Ossip-Klein (1982)	mean s.d.	6	45.0 ± 8.4		1.3 ± 0.5		13.6 ± 6.7		310 ± 90	
Gust (1982)	mean	8	16	43.7	1.6		9.0	48.4	393	
Adams	mean	10		44.2 ± 12.7	1.88 ± 0.91			25.9 ± 14.5		± 358
Nil (1984) [9]Group 1	mean s.e.m.	10	19.5 ± 6.8	41.1 ± 13.6	1.67 ± 0.50	41.1 ± 13.7	14.8 ± 3.8	25.9 ± 14.8		619 ± 329.3
Nil (1984) [7]Group 2	mean s.e.m.	10	20.5 ± 11.3	39.5 ± 8.9	1.57 ± 0.41	39.5 ± 8.9	14.7 ± 4.7	25.7 ± 11.9		579 ± 244.8
McBride (1984) Own Brand	mean s.d.	9		42.1 ± 7.5	2.14 ± 0.56		15.7 ± 3.8	25.3 ± 8.4	352 ± 69	
Medici	mean	17		43.1 ± 3.8	2.2 ± 0.2	31.4 ± 1.6	13.7 ± 0.7	18.7 ± 2.6		
Burling (1985) Low CO	mean s.d.	12	21.4 ± 11.5		1.65 ± 0.3		12.6 ± 3.7	25.1 ± 3.7	342.5 ± 80	
Burling (1985) High CO	mean s.d.	12	19.4 ± 7.6		1.65 ± 0.3		11.0 ± 2.2	30.4 ± 8.5	342.5 ± 85.0	
Nil, Buzzi, Battig Male	mean s.d.	69	25.2 ± 14.7	42.3 ± 14.5	2.18 ± 0.71	34.8 ± 10.6	12.6 ± 4.6	22.9 ± 12.8		511.6 ± 220.1
Nil, Buzzi, Battig (1986) Female	mean s.d.	48	24.2 ± 14.1	41.4 ± 13.3	2.02 ± 0.52	36.2 ± 8.4		21.7 ± 10.7		± 224.4
Hughes Type A	mean s.e.m.	19	23.7 ± 2.0		1.58 ± 0.1		11.9 ± 0.8			
Hughes Type B	mean s.e.m.	27	21.0 ± 1.4		1.56 ± 0.1		10.4 ± 0.4			
Bridges (1986) Group 1	mean s.e.m.	5	19.4 ± 5.4	85.4 ± 10.6			13.2 ± 2.8			

		Number of Subjects (ml)	Cigarettes Per Day	Puff Volume (ml)	Puff Duration (sec)	Peak Flow (ml/sec)	Puffs Per Cigarettes	Inter-Puff Interval (sec)	Cigarette Duration (sec)	Inhalation Volume (ml)
Bridges (1986) Group 2	mean s.e.m.	16	[9]23.1 ± 2.6	63.7 ± 8.0			11.4 ± 1.4			
Bridges (1986) Group 3	mean s.e.m.	22	[9]24.7 ± 6.6				11.1 ± 0.9			
Bridges (1986) Group 4	mean s.e.m.	65	[9]25.6 ± 1.6	52.2 ± 1.8			10.6 ± 0.5			
[10]Woodman (1986)	median range	9	22 15-30	47.9 32.0-59.0	1.6 1.1-1.3		13.0 8-19	18.0 6.7-36.6	255 127-376	639 284-1006
Nemeth-Coslett (1986a) Placebo	mean range	8	33 20-50				8.2	64	414	
Nemeth-Coslett (1986b) Placebo	mean ± s.e.m.	8	[2]30 (20-40)		1.4 ± 0.2			47 ± 6.4	362 ± 28.6	
Nil, Woodson, Battig (1986) High CO Absorbers	mean s.d.	19	32.3 ± 7.8	34.2 ± 10.8	2.2 ± 0.6	33.2 ± 13.0	14.6 ± 4.3	21.3 ± 9.5		485.2 ± 171.0
Nil, Woodson, Battig (1986) Low CO Absorbers	mean s.d.	20	19.7 ± 16.6	25.4 ± 8.1	1.9 ± 0.6	22.8 ± 6.4	15.0 ± 3.8	22.0 ± 7.7		372.6 ± 180.0
Nil, Woodson, Battig (1986) Nonselected Smokers	mean s.d.	132	28.5 ± 12.4	30.3 ± 11.2	2.2 ±0.7	27.6 ± 10.5	12.7 ± 5.3	28.0 ± 15.4		375.7 ± 210.4

1.Units changed from minutes to seconds

2.Range

3.Units changed from weeks to days

4.First cigarette smoked data

5.Total puff time

6.Experiment 1, naturalistic setting Data

7.Experiment 2, naturalistic setting Data

8.Experiment 3, naturalistic setting Data

9.Number cigarettes in past 24 hours

10.Data from first visit

Table 2

Matrix for Machine Measurement of Tobacco Smoke Yield					
	Puff Volume (ml)				
Puff Duration (sec)	15	30	45	60	90
1					
2					
3					

Chapter D

Analysis of Toxic **Smoke** Constituents

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Contents

Introduction

The chemical analytical evaluation of several key constituents of cigarette smoke is a useful predictor of potential toxic and/or tumorigenic activity of the combustion products of cigarettes. This chapter describes the methodology for the determination of select known toxic and tumorigenic agents in tobacco smoke as well as the standardized analytical procedures that can be applied to the evaluation of the vapor phase and particulate matter of cigarette smoke.

Although sidestream smoke (*ss*) of cigarettes is a major contributor to environmental tobacco smoke, and the emission of SS constituents from prototype cigarettes may raise some health concerns, this chapter does not specifically address the overall systematic analytical elucidation of sidestream smoke composition, yet it highlights some compounds in *ss* that are relevant to health concerns.

The burning of cigarettes generates mainstream smoke (MS) during puff drawing and SS during smoldering between puffs. The physicochemical nature of these smoke types is dependent upon factors such as the type of tobacco, the temperatures prevailing during puff-drawing (860-900°C) and smoldering (500-650°C), the reducing atmosphere that is characteristic of the burning cone, and the physical design of the cigarette (e.g., length, diameter, filter tip, and type of cigarette paper). These different parameters also influence the ignition propensity of a cigarette.

The **400-500** mg of mainstream smoke that are freshly emerging from the mouthpiece of a cigarette are an aerosol that contains about $1x10^{10}$ particles per milliliter in the vapor phase (1). The range in diameter is 0.1-1.0 μm with a mean of about 0.2 μm. About **95%** of the MS effluent of a nonfilter cigarette is comprised of **400-500** individual gaseous compounds with nitrogen, oxygen and carbon dioxide as major constituents. Until now, at least 3500 individual compounds have been identified in the particulate matter (Figure 1; 2, 3).

For chemical analysis, MS is arbitrarily separated into vapor phase and particulate phase. Individual compounds of which more than **50%** appear in the vapor phase of fresh MS are considered volatile smoke constituents; all others are particulate phase components (Figure 1). Tables 1 and 2 list the major types of compounds identified and their estimated concentrations in the smoke of one nonfilter cigarette (4). All data were derived from machine-smoking of cigarettes under standardized laboratory conditions **(5).** [Machine smoking does not necessarily reflect the range of human smoking behavior characteristics (6-8, Topography chapter).]

Tables 1 and 2 do not contain information about the presence of agricultural chemicals and pesticides that originate from the residue of such compounds on the tobacco **(9)**. These residues differ in respect to chemical nature, concentrations, and type of agricultural chemicals used in the various tobacco-growing countries, and they may vary from season to season (10). The list also lacks information on flavor additives, because the nature and composition of such agents remain trade secrets. One exception among flavor additives is menthol of which up to 500 ug/cigarette may be found in MS (11).

Tobacco is known to contain at least 30 metals (12). In general, less than 1% of the metals is transferred from the tobacco into the MS of a cigarette (13). The levels of these elements are very low and thus are not listed in Table 2. However, the formulation of cigarettes with lower ignition propensity may include metallic additives. Therefore, it may be advisable to consider the analysis of added metals if they are suspected of having biological significance.

I. Toxic Agents in Tobacco Smoke

Hundreds of studies have been concerned with the chemical nature and quantitative aspects of toxic and tumorigenic agents in the mainstream smoke of cigarettes. Table 3 lists those toxic agents that have been most extensively studied and are considered to be major contributors to the toxicity of the smoke (14-16). This listing is not complete; however, the concentrations of all of these compounds give sufficient indication of the overall toxicity and tumorigenicity of the MS of a particular cigarette relative to a control cigarette, or to other commercial cigarettes. The possible presence and effects of additives to the tobacco or paper and their corresponding combustion products in the MS should be considered. The determination of such additives and their combustion products in the MS and **ss** may require specific methods.

The yields of particulate matter in undiluted **ss** are 1.3-1.9 times higher than those in the MS of cigarettes. About 30-40 compounds have been identified and quantified in the particulate matter of ***ss***. Nicotine levels in undiluted SS are 2-4 times higher than in MS. However, **ss** yields of aromatic amines exceed those in MS 20-to-30-fold. Undiluted ***ss*** also contains remarkably high levels of ammonia and of N-nitrosodimethylamine and N-nitrosopyrrolidine (Table 4). The greater release of the trace metals into SS might point toward increased releases of metallic additives into environmental smoke.

ss which is generated during smoldering of the cigarette, is the major contributor to environmental tobacco smoke (ETS) (or secondhand smoke). Minor contributions to ETS are made by vapors

diffusing through the cigarette paper, smoke escaping from the mouthpiece or burning cone, and exhaled smoke. Table **5** presents some of the data for toxic agents reported in indoor environments.

11. Smoking Conditions

The analysis of most of the individual smoke compounds requires 20 cigarettes or less. Cigarettes should be selected, prepared, and smoked individually by the standard smoking conditions established by the Federal Trade Commission in 1969 as modified in 1979 (22), described in Chapter B, unless human data (Chapter **C**) indicates otherwise. Additionally, cigarettes within ± **20** mg of the average weight of **200** cigarettes should be selected for analysis. In the case of filter cigarettes, draw resistance must be within ± **5%** of the average of the weight-selected cigarettes **(14)**. Quantitative assessment of most of the individual smoke compounds requires **20** cigarettes or even less.

When analyses require **20** cigarettes or less, piston-type smoking machines (Chapter B), such as the 20-channel Phipps and Bird smoker, should be employed **(23)**. When the analysis requires more than **20** cigarettes, a constant-volume-constant-time smoking machine, such as the Borgwaldt-20 smoker with rotating head, may be preferred (Chapter B). [The latter is available in the US through International Planters Corp., Borgwaldt Division, P.O. Box **24505**, Richmond VA, **23224**; phone 804-230-0011]. The machines with rotating head are preferred because they allow flushing with nitrogen after each puff in procedures where avoidance of artifacts is important. They also require less space and are easy to operate. The determination of each smoke constituent should be completed in duplicate or triplicate.

111. Analysis of Individual Smoke Constituents

The undesirable effects of cigarette smoking relate to the exposure to toxic, ciliatoxic, tumor-initiating and tumor-promoting agents and to organ-specific carcinogens. The selection of key compounds in cigarette smoke that are to be determined analytically for the evaluation of the toxic and tumorigenic potential of cigarette smoke is guided by knowledge on the proven toxic and/or tumorigenic effects in bioassays (Tables **3** and **4)**. The extent to which these same agents are toxic and/or tumorigenic to humans is not always known but can be deduced from interpretation of their probable activities made by the International Agency for Cancer Research (IARC Monograph Series, Vols. **1-53**; 1972-1991).

The carcinogenicities of tobacco and tobacco smoke in bioassays as well as the tobacco-related cancers in human epidemiologic studies are dose-related. Human risk assessment therefore depends not only on the relative biologic potency of individual or total tobacco carcinogens, but also on the quantitative aspects of exposure as these relate to personal smoking habits. Since smoking of cigarettes by machines mimics yet never completely duplicates the smoking patterns of individuals, the analytical evaluation of key components of tobacco smoke by standardized methods provides primarily valid comparisons of the relative toxic and tumorigenic potential of one cigarette brand over another.

It is proposed that MS should be analyzed for 14 parameters. These are listed below. The cited analytical methods are known to be reproducible within a given laboratory. However, only methods for total particulate matter ("tar"), carbon monoxide, and nicotine are standardized. Methods for these three were compared in collaborative studies between laboratories.

Measures should be taken to avoid artifacts that might occur during cigarette smoke analysis, e.g. aging of smoke resulting in the conversion of NO to NO_2, nitrosamine formation during trapping, degradation of carbonyl chemicals by secondary reactions, etc. Where practical, at least two methods are suggested for each parameter.

1. Total Particulate Matter (TPM-dry)

The major carcinogenic activity of tobacco smoke resides in the particulate matter. Therefore, the total yield of particulate matter in the smoke of a given cigarette is a key determinant of its carcinogenic potential. However, the vapor phase does contain additional toxins and tumorigenic agents which must be determined for a complete assessment of inherent risk.

Macro-methods of assessment, such as fluorescence of TPM solutions or elemental analysis are not recommended. Those methods are not sufficiently specific to be associated with the carcinogenic activity of TPM.

The method for TPM determination, including its moisture content should be based on the FTC-method (22), described in detail in Chapter B. Reproducibility of the data will be confirmed by including a University of Kentucky standard reference cigarette during the analyses.

2. pH of Mainstream Smoke

Nicotine, the major habituating agent and an important toxic compound in tobacco smoke (19, 24), is protonated in the MS of U.S. blended cigarettes and of cigarettes that are made entirely with bright tobacco, because the smoke of these cigarettes rarely exceeds pH 6.2 (18). Higher smoke pH increases the toxicity. At pH above 8.0, which occurs in smoke from cigarettes made entirely with black or burley tobacco, more unprotonated nicotine is present in the vapor phase. This tends to raise blood pressure far more rapidly and to a greater extent than does the protonated (bound) nicotine in the smoke (24, 25). Thus, the pH of the mainstream smoke should be measured.

Two methods are usually followed for determining smoke pH. The method developed by Sensabaugh and Cundiff (26) and applied by others (18) provides for measurement of the pH of individual puffs. In this case, single cigarettes are smoked by a piston-type machine. Each puff is led over a modified electrode; the latter is connected to a pH-meter (Beckman Model SS-2) and the signals are recorded by a strip-chart recorder (18). The test is run in triplicate to obtain representative values. The advantage of this method lies in its ability to record the pH of each puff from the first to the last puff. This method has been utilized primarily for research; it requires constant recalibration and maintenance.

Grob describes another method in which three cigarettes are machine-smoked under standard conditions through a 40-ml glass tube that is loosely filled with cotton (27). After the smoking, the cotton is washed with 40 ml CO_2-free distilled water and subsequently twice more with 10 ml water. The pH of the combined water extracts is measured with a pH meter resulting in an average value from all puffs of a cigarette.

The average pH of a cigarette can also be crudely measured by smoking a cigarette puff by puff through 2 interconnected impingers filled with 10 ml CO_2-free distilled water each (total free volume 40 ml). After 2 or 3 cigarettes have been smoked, a clearing puff is taken, the contents of the impingers are combined with 20 ml of water used for rinsing the impingers and, after filtration, the pH of the whole smoke is measured. To obtain representative pH values this test has to be done in triplicate.

A smoking machine-extractor device has been developed for large-scale determinations of the smoke pH of cigarettes. The device is reported to deliver reproducible, average pH values within 20 minutes (28).

3. Redox Potential of Cigarette Smoke

It has been demonstrated that the reducing property of cigarette smoke adversely affects the respiration of the mammalian cell. Specifically, the redox potential of the smoke of a given tobacco product is correlated with the degree of cytochrome C reduction (29, 30). It is known that the redox potential in each puff of cigarette smoke gradually declines from the 230-240 mV initially measured for air with the reference calomel electrode to the 140-160 mV in the smoke of the last puff. Smoking a cigarette through a glass fiber filter demonstrates that the gaseous phase is practically free of reducing agents and that the latter reside primarily in the particulate phase. Ammonia and hydroquinone do not contribute to the redox potential of cigarette smoke, and nicotine does so only to a minor extent. 1,4-Benzoquinone, naphthoquinones and anthraquinones appear to have a stabilizing effect on the redox potential of cigarette smoke (31, 32), thus diminishing the reducing effect of cigarette smoke on subcellular components of the mammalian cell.

To determine the redox potential of individual puffs, a piston-type smoking machine is connected with an apparatus containing a platinum (Pt) electrode which operates in conjunction with a reference calomel electrode (Figure 2; 32). The smoke is led over the Pt electrode only. Figure 3 shows the redox potential of 85-mm US blended non-filter cigarettes for puffs 1 through 8 (32).

Vapor Phase

4. Carbon Monoxide

The toxic burden of environmental carbon monoxide exposure is of special significance to cardiovascular health. The methodology for the determination of carbon monoxide in cigarette smoke is described in Chapter B.

5. Nitrogen Oxides (NO_x)

It is suspected that nitrogen oxides (NO_x) in cigarette smoke contribute to the development of pulmonary emphysema (30, 33) and the acceleration of platelet aggregation (34). They are potent inhibitors of thiol-dependent enzymes (35) and, at high concentration, they can induce bradycardia and arrhythmias (36). In addition, NO_x in cigarette smoke may prevent the activation of scavenger cells such as macrophages in the respiratory system. NO_x in cigarette smoke are also of concern because of their potential to nitrosate precursor amines and thus contribute to the formation of carcinogenic N-nitrosamines.

Cigarette smoke contains 3 forms of NO_x. These are nitrous oxide (N_2O; ≤ 1 µg/cigarette), nitrogen dioxide (NO_2; <10 µg/cigarette) and nitric oxide (NO; 6-600 pgfcigarette). The fresh smoke of a US non-filter cigarette contains 200-300 µg NO, depending on the nitrate content of the tobacco, which represents the major precursor for the nitrogen oxides in smoke (37). It is important for the analytical chemist to realize that unaged MS contains hardly any NO_2, but only NO, and that the latter oxidizes quickly to NO_2 (half-life in smoke, 6-10 minutes; 38, 39). This is of major consequence since NO, is essential for the toxicity and the N-nitrosamine formation in cigarette smoke (section III-9).

Several methods have been employed for determining NO, in cigarette smoke. These include the widely used colorimetry (40-42), gas chromatography (43), nitrate ion electrode (44), infrared (39), and chemiluminescence (45, 46).

The colorimetric method is based on the Saltzman procedure (41). It involves the Griess reagent which reacts only with NO_2. For cigarette smoke analysis, NO must first undergo oxidation to NO_2. Sloan and Morie have discussed the shortcomings of the Saltzman method for the analysis of NO, in cigarette smoke in great detail (44).

The preferred method for the determination of NO, in cigarette smoke is by chemiluminescence. In this technique nitric oxide is measured by photoelectric amplification the chemiluminescent reaction of NO with ozone. NO_x ($NO+NO_2$) are measured by photoelectric amplification of the chemiluminescent reaction of NO, and atomic oxygen, which is derived from thermal decomposition of O_3.

For chemilumniscent analysis, cigarettes are selected by weight and draw resistance and are smoked by a piston-type smoking machine through a Cambridge filter. The gas phase of each individual puff is directed through a gas sampling valve. An aliquot of the puff is injected into a GC column filled with a 100/200 mesh porous polymer (Chromosorb 104). The column temperature is set at 45°C. Argon, the carrier gas, is adjusted to a flow rate of 15 ml/min. The column exit is connected with a chemiluminescence detector (Figure 4). For each NO_x analysis, 4 cigarettes have to be smoked individually; this results in an experimental deviation for commercial US blended cigarettes of ±6% (detection limit = 0.5-1.0 µg NO per puff). The British Tobacco Research Council, London, refined the chemiluminescence method for NO, in cigarette smoke and adopted it as a standard method (47).

6. Hydrogen Cyanide

Hydrogen cyanide (HCN) is an inhibitor of several respiratory enzymes; as such it can influence cellular metabolism in the myocardial and arterial wall. As a major ciliatoxic agent in cigarette smoke HCN greatly inhibits the clearance of tar components from the respiratory tract (19). Nitrate is a major precursor for HCN in the smoke (48), even though tobacco proteins are also precursors for HCN in smoke (49).

The methods developed for HCN analysis in cigarette smoke include ion-selective electrode titration, gas chromatography and coulometric methods (50). With one exception all methods measure cyanogen [(CN),] as part of HCN. However, (CN), in the smoke of a cigarette amounts to less than 2.5% of the total HCN (51). To separate HCN from (CN),, the smoke of individual puffs of the cigarette is directed through a Cambridge filter and subsequently through gas wash bottles containing 100 ml 0.1N NaOH. When loaded with the smoke of 1 cigarette, the Cambridge filter is extracted with 100 ml 0.1N NaOH, washed, and combined with the solution from the gas wash bottles (total volume including washings 250 ml). A 1-ml aliquot is pipetted into a mixture of 2 ml 1.0 M NaH_2PO_4 with 1 ml chloramine-T solution and 20 ml n-hexane; it is then thoroughly mixed. One μl of the n-hexane layer is analyzed by GLC with ^{63}Ni-EC detector (51). The cyanogen chloride, formed by the reaction of HCN with chloramine-T gives a distinct peak which is clearly separated in the GC. The relative standard deviation of this HCN method is less than 5&, the detection limit is 50 ng HCN per cigarette. (Modification of the method by smoking more than 1 cigarette can greatly increase the detection limit).

Coulometric analysis for hydrogen cyanide by the method of Sloan (50) is recommended. It requires machine-smoking of 2 or more cigarettes through a scrubber containing 50 ml 0.1 N NaOH and through a Cambridge filter. Aliquots of the "smoked" scrubber solution plus washings are transferred to a titration cell which contains pH 11 buffer solution and 0.001 M lead acetate solution. The generator electrodes are connected to a coulometer, and the indicator electrodes are connected to the terminals of a potentiometer. The output of the potentiometer is connected to a strip-chart recorder. HCN that is not retained in the scrubber solution but trapped by the Cambridge filter is extracted with 0.1 N NaOH and an aliquot is titrated coulometrically as discussed for the aqueous scrubber solution. The two sets of data are combined and compared with data obtained from the other more involved methods. The standard deviation for this simple and rapid method for HCN in cigarette smoke was less than 6% (50).

7. Volatile Hydrocarbons

The gaseous phase of cigarette smoke has been shown to contain about 20-25 alkanes, up to 20 alkenes, some alkynes such as acetylene, a number of dienes, especially 1,3-butadiene and isoprene, and in addition to benzene, up to 30 volatile aromatic hydrocarbons (2, 3, 59). Together these hydrocarbons constitute 0.5-1.0% of the weight of the total mainstream smoke effluent of a cigarette. Despite this, few of them have been discussed as possible contributors to the toxicity of the smoke.

The exceptions are 1,3-butadiene and benzene. In inhalation studies in mice and rats, 1,3-butadiene is carcinogenic; however, the International Agency for Research on Cancer considers "the evidence for carcinogenicity of 1,3-butadiene to humans as inadequate" (17). Benzene, on the other hand, is a recognized occupational carcinogen, which upon long-term exposure increases the risk of workers for various types of leukemia (17). Case control studies and large-scale prospective follow-up studies have shown an association between cigarette smoking and leukemia especially myeloid leukemia (52-54). There is a correlation in cigarette smokers between urinary cotinine, a major metabolite of nicotine, and urinary *trans,trans*-muconic acid, a metabolite of benzene (55).

Rapid advances in chemical-analytical instrumentation have led to capillary GC-MS methods which enable the investigator to determine quantitatively within minutes dozens of volatile components in the vapor phase of only a fraction of individual puffs of a cigarette (56-58). These instruments are primarily helpful for research, they require great expertise; the methods are time consuming and generally not suitable for routine analyses of toxic hydrocarbons in the gaseous phase of cigarette smoke.

The following GC-MSD method is suggested for routine analysis of 1,3-butadiene, isoprene, benzene, and toluene. The gas phase of individual puffs from freshly generated mainstream smoke of selected cigarettes (section II) is led through a 1.0 ml sample loop. Upon reaching atmospheric conditions the sample loop is switched in line with gas chromatographic (GC) columns, first passing through a 5 m X 0.53 mm HP-1 precolumn and then onto a 30 m X 0.25 mm DB-5 analytical column. The effluent of the analytical column is diluted by the carrier gas, helium 1:25, and the emerging peaks representing individual volatile smoke components are determined by mass selective detection (GC-MSD).

The advantage of GC-MSD is that volatiles that were masked by the GC peak of a hydrocarbon in a regular GC-MS trace can be eliminated by selective ion monitoring and thus allow accurate quantification. The disadvantage of this method lies in the fact, that the hydrocarbons to be assayed have to be determined

for each individual puff separately in order to avoid losses during aging of the smoke. This is especially so for 1,3-butadiene (conventional smoking of a cigarette requires in general **8-12** puffs or **7-11** minutes before an aliquot of all puffs can be analyzed). For routine analysis, especially when comparing different cigarettes, the seventh puff is chosen as a representative puff of the cigarette (59).

8. Aldehydes

Formaldehyde, acrolein, and acetaldehyde together with hydrogen cyanide are the major ciliatoxic agents in cigarette smoke and are known irritants to the mucous membranes of the upper respiratory system and to the eyes. Upon inhalation they clearly contribute to the inhibition of lung clearance mechanisms, thus allowing extrinsic particles, such as tobacco smoke particulates, to settle **(21,** 60). Formaldehyde and acetaldehyde are known animal carcinogens. The International Agency for Research on Cancer regards formaldehyde as "probably carcinogenic to humans", acetaldehyde as "possibly carcinogenic to humans" and acrolein as "not classifiable as to its carcinogenicity to humans" **(17).**

Formaldehyde is determined by smoking **2** cigarettes individually through an 800-ml Kjeldahl flask containing a trap with **200** ml of saturated solution of 2,4-dinitrophenylhydrazine (DNPH) in **0.2** N HCl. After the smoking, the trapping solutions are extracted repeatedly with chloroform and triphenylene is added as an internal standard. The combined chloroform solutions are washed twice with **2** N HCl, then twice with water, and are then concentrated under a stream of nitrogen. The dried residue is dissolved in 5 ml methylene chloride. Aliquots are injected into a HPLC system which is described in great detail (61). The recovery rate is reported as better than 90% and the reproducibility better than 5%.

Acetaldehyde, acrolein, and propionaldehyde may be analyzed by the method of Manning **(62)** at Oak Ridge National Laboratory. The cigarettes are individually smoked and the volatile aldehydes are trapped by reacting them with DNPH in **2** N HCl solution. The concentrates of the 2,4-dinitrophenylhydrazones of the aldehydes are separated and analyzed by reverse phase HPLC with the absorbance detector at 365 nm. The detection limit is **10** µg aldehyde/cigarette, the relative standard deviation is about **12%.**

9. Volatile N-Nitrosamines (VNA)

Volatile N-nitrosamines in cigarette smoke originate from the tobacco by transfer into the smoke, and from thermal degradation of nitrosamino acids, as well as from pyrosynthesis during smoking. For example, during tobacco processing proline is nitrosated to N-nitrosoproline (NPRO); its yield in the

tobacco is greatly influenced by the processing of the tobacco and by its nitrate concentration. During smoking NPRO gives rise to some N-nitrosopyrrolidine (NPYR) in MS (0.1-1%) and to much higher yields of NPYR in *ss* (20). Model studies have shown that volatile secondary amines can be nitrosated to nitrosamines in the vapor phase, however, the yields are low because freshly generated smoke contains primarily NO and only traces of NO_2. The latter is essential since the nitrosating agent for the formation of nitrosamines is N_2O_3 **(64).**

All 8 VNA identified in cigarette smoke (Figure **5)** are organ-specific carcinogens in animals **(65).** For routine analyses usually only the **3** major VNA in cigarette smoke are quantitated. These are N-nitrosodimethylamine (NDMA; 0-75 ng/cigarette), N-nitrosodiethylamine (NDEA; **0-5** ng/cigarette) and N-nitrosopyrrolidine **(5-40** ng/cigarette; **20).**

The MS analysis requires 3 times 10-20 cigarettes without filter tips or **3** times **20-40** filter cigarettes; these have to be smoked individually under standard laboratory conditions (section 11). [Cellulose acetate filter tips selectively remove **VNA** by 70% and more; thus, the VNA analysis in the smoke of filter cigarettes requires 20 cigarettes] **(63, 66).** The *MS* is led through a gas wash bottle containing 100 ml citrate-phosphate buffer, pH **4.5,** with **20 mM** ascorbic acid and an internal standard ([^{14}C]NDMA or N-nitrosodipropylamine). A Cambridge filter treated with a solution of ascorbic acid, is placed between gas wash bottle and smoking machine (after smoking 10 cigarettes the loaded Cambridge filter is replaced). The loaded Cambridge filter is thoroughly washed with dichloromethane (CH_2Cl_2) and the washings are filtered. The buffer solution in the gas wash bottle is extracted **4** times with **100** ml CH_2Cl_2, all organic-extracts are washed with 2 N NaOH (to remove interfering nitroalkanes), dried (Na_2SO_4) and concentrated to 5 ml. The concentrate is chromatographed on **65** g basic alumina (Woelm, activity II-III). The VNA are eluted from the column with 200 ml CH_2Cl_2, vacuum concentrated to about **1-2** ml and measured by GC-thermal energy analysis **(63).** The recovery rate is better than 70%. The detection limit is 0.05 ng NDMA per injection; the deviation coefficient for NDMA and NPYR is ± **5%;** for NDEA it is up to ± 10%

Particulate Matter

10. Nicotine

The standard FTC-method for nicotine, the main pharmacologic agent in cigarette smoke (22), is described in Chapter B.

11. Phenols

More than 40 semivolatile phenols have been identified in cigarette smoke (3). The major precursors for these phenols in tobacco smoke are glucose, polysaccharides, pectins, rutin and other polyphenols. Minor amounts of semivolatile phenols that were formed in the tobacco during processing transfer into the smoke (14). It has been reported that the nonfilter 85-mm cigarette, made entirely from bright tobacco, delivered in the mainstream smoke 95 μg phenol, one made from Turkish tobacco yielded 120 μg, and from Maryland tobacco 60 pg; a burley tobacco cigarette produced **43** μg, and a US blend delivered 100 μg phenol (14). These findings indicate that the type of tobacco plays a major role in the yields of volatile phenols in cigarette smoke.

The volatile phenols contribute significantly to the tumor promoting activity of cigarette tar (14, 67, 68) and are active as ciliatoxic agents (21). The latter effect is one of inhibition or temporary paralysis of the mucus clearance that is normally provided by the ciliated epithelium of the respiratory tract. Ciliastasis allows foreign particles to remain in the respiratory tract where they can exert their particular activity or allow other agents to impair physiologic or biochemical functions.

The preferred analytical method for volatile phenols is gas chromatography of the weakly acidic portion of cigarette smoke condensate (69-71). Twenty to 40 cigarettes are smoked individually through a gas wash flask containing 2N NaOH with an internal standard (e.g. 2-chlorophenol, [^{14}C]phenol) and a Cambridge filter. The "loaded" Cambridge filter is extracted with 2N NaOH, filtered, combined with the NaOH solution from the gas wash bottle and the washings. This combined NaOH solution is extracted **3** times with ether to liberate the phenol concentrate obtained by solvent extractions without the need for concentrating by water steam distillation (72).

Cellulose acetate filter types, and especially those with specific plasticizers and with perforated filter tips reduce volatile phenols highly selectively (up to 85%); therefore, in some cases more than **40** or even 60 cigarettes are required for each analysis.

12. Catechols

The most abundant phenolic component in cigarette smoke is catechol (1,2-dihydroxybenzene; 80-400 μg/cigarette). Although 1 g of processed tobacco contains microgram levels of catechol, most of the catechol in the smoke is formed during the burning of tobacco from cellulose, monosaccharides, chlorogenic acid and pectins as precursors **(73)**. Cigarette smoke also contains small

amounts of alkylated catechols including 3-methylcatechol (<20 pgjcigarette), 4-methylcatechol (~20 pgjcigarette) and 4-ethylcatechol (<25 pgjcigarette; 74).

Catechol is not a carcinogen but a very effective cocarcinogen in tobacco smoke. Upon co-application with benzo(a)pyrene or with other carcinogenic polynuclear aromatic hydrocarbons (PAH), it greatly enhances the carcinogenic activity of these agents (75, 76).

Two methods are primarily employed in the analysis of catechol, 1) enrichment of catechol from tar by distribution between solvent pairs, followed by a spectrophotometric method (77), and **2)** enrichment of the catechols from the acidic fraction of cigarette tar by extraction with boric acid, followed by GLC (74). Both methods are simple, require only 20-40 cigarettes per analysis and are reproducible, when an internal standard is used (e.g. [^{14}C]catechol), within ± **6%.**

Schlotzhauer (72) enriched the dihydroxybenzenes, catechols, resorcinols and hydroquinones from the weakly acidic fraction by gel filtration chromatography and analyzed the catechol concentrate by GC–MS. This method found catechol and six alkylcatechols, as well as other dihydroxybenzenes. It is used for the profile analysis of these types of chemicals in cigarette smoke.

13. Polynuclear Aromatic Hydrocarbons (PAH)

Inhalation studies with laboratory animals have demonstrated that the particulate matter of tobacco smoke induces malignant tumors of the respiratory tract, most notably in the larynx of the Syrian golden hamster **(16,** 79, **80).** The particulate phase is much more carcinogenic than the gas phase (79). Fractions and subfractions of the particulate matter have been extensively assayed for tumorigenicity on rabbit skin and on mouse skin. It has been clearly demonstrated that the most tumorigenic fractions in these assays are those with highly concentrated PAH (14, 68).

However, the carcinogenic activity of cigarette smoke particulates cannot be explained by the presence of carcinogenic PAH alone. When PAH concentrates of the neutral fraction (<1% of whole tar) are combined with the tumor-promoting weakly acidic fraction, which by itself does not induce tumors, the tumor yield on mouse skin reaches 70-90% of the carcinogenic activity observed with the whole tar (68, **81).** Thus, the PAH serve as tumor initiators. To date about 80-100 PAHs have been identified in cigarette smoke (most <10 ng cigarette). A PAH concentrate of the neutral fraction of cigarette smoke condensate was the only portion that induced squamous tumors in the lung of rats upon intratracheal instillation (82). Benzo(a)pyrene (BaP), a major

carcinogenic PAH in smoke (20-40 ng/cigarette), induced tumors in the hamster lung upon inhalation (83).

The International Agency for Research on Cancer regards 11 PAH and 3 nitrogen-containing PAH (aza-arenes) as established animal carcinogens. BaP, benz(a)anthracene and dibenz(a,h)anthracene are rated as "probably carcinogenic to humans" (Fig. 6; 17).

A great many studies were concerned with the analysis of PAH in cigarette smoke (14, 84). Often the PAH are enriched by distribution of cigarette tar between solvent pairs such as methanol-water (4:1) and cyclohexane followed by a second partition between cyclohexane and nitromethane which leads to a 10-fold enrichment of the PAHs (81). The PAHs are further concentrated by column chromatography followed by paper chromatography, or TLC; the individual PAH are then identified and quantitated by W-spectrophotometry (85).

During the last 2 decades the final step of PAH analysis relies on capillary GC *(86)*. Using an internal standard (e.g. [^{14}C] BaP) the recovery is better than 70%. The reproducibility for the major PAH (>5 ng/cigarette) is ± *8%* with at least 100 cigarettes. Several PAH profile studies of the MS of nonfilter cigarettes showed the highest PAH yields for the smoke of cigarettes made entirely with bright tobacco (BaP= 35-53 ng/cigarette) and lowest PAH yields for cigarettes made entirely with burley tobacco (BaP= 20-24 ng/cigarette; 14, 18).

Most of the carcinogenic PAH in cigarette smoke (>90%) are pyrosynthesized via highly reactive C,H-radicals (14, 84) which result from thermal degradation of nonvolatile organic tobacco components. Since BaP is one of the most abundant carcinogenic PAH in cigarette smoke and its mechanism of formation is similar to the pyrosynthesis of other PAH, BaP is often quantitated as a monitor for the entire class of compounds in the smoke of a given cigarette. For this purpose, several quick methods for the analysis of BaP were developed recently (87, 88). Five to 10 cigarettes are smoked through a Cambridge filter assembly. One ml of cyclohexane for each 1 mg of TPM is used to agitate the mixture of the filter and tar for 1 hour. After filtration, the volume is reduced to 10 ml by rotary evaporation. Following refiltration through a 0.45 µm membrane filter, a 2 ml aliquot is chromatographed through a NH_2-Sep-Pak column, pre-conditioned with 10 ml hexane. The BaP fraction is eluted with *8* ml of hexane, evaporated to dryness, redissolved in 1 ml of acetonitrile and analyzed by reverse-phase HPLC using an isocratic solvent system *(65%* acetonitrile in water) and fluorescence detector (excitation = 289 nm; emission = 412 nm); benzo(e)pyrene served as internal standard *(88)*.

14. Tobacco-Specific N-Nitrosamines (TSNA)

The TSNA are exclusively formed from nicotine and the minor Nicotiana alkaloids during tobacco processing and during smoking. So far 7 TSNA have been identified (Figure 7). Four of these are usually determined in cigarette smoke. These include the powerful organ-specific carcinogens N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). In mice, rats and hamsters these TSNA induce benign and malignant tumors of the lung, upper aerodigestive tract, pancreas and/or liver. The other two major TSNA are the weakly carcinogenic N'-nitrosoanabasine (NAB) and the non-carcinogenic N'-nitrosoanatabine (NAT; 89).

To determine the four major TSNA in cigarette mainstream smoke, 3 X 20 weight-selected cigarettes are smoked individually under standard laboratory conditions (section 11; in the case of filter cigarettes selection must also be done according to average draw-resistance). The mainstream smoke is retained on a Cambridge filter (9.0 cm diameter) which is treated with a solution of ascorbic acid (90). The filter assembly is placed between the smoking machine and two gas wash bottles in line, each containing **60** ml distilled water to which 2 ml of 20% ammonium sulfamate solution in **3.6** N sulfuric acid is added, and also containing **0.5** µg [^{14}C]NNN as an internal standard (others have used N-nitrosodibenzylamine, N-nitrosopentylpicolylamine or 2-(methylnitrosamino)-1-(2-pyridyl)ethane; 91, 92). The total particulate matter trapped on the Cambridge filters is extracted twice with 100 ml ethyl acetate and the combined buffer solutions of the wash bottles are extracted with 3 times 100 ml ethyl acetate. The ethyl acetate extracts are dried (Na_2SO_4), concentrated to about 2 ml and chromatographed on **50** g basic alumina (Woelm, activity II to III) on a 2 X 20 cm column with **150** ml dichloromethane and a 4:1 mixture of dichloromethane: acetone (200 ml). The latter solvent mixture eluates the TSNA which are concentrated to 1-2 ml (recovery rate of [^{14}C]NNN= **75-85%).**

The method of Adams (93) is recommended for the gas chromatography-thermal energy analysis (GC-TEA). The specific detector for NO-containing substances assures clear separation of NAB from NAT. The TSNA values are determined in a triplicate analysis within ± 7% (detection limit = 1 ng of a single TSNA per cigarette). Other analytical methods for TSNA were recently reported (20, 92).

IV. Postscript

It was the goal of this chapter to suggest analysis of those tobacco smoke parameters and smoke components that are considered

major contributors to the toxicity of cigarette smoke and are likely to be quantitatively affected by modifications that might reduce the ignition propensity of cigarettes. Athough this proposal is based on longstanding experience in the tobacco sciences and on a thorough study of the literature, it is not comprehensive for all toxicants which may occur in cigarette smoke. It was deemed important to keep the number of measurements practical.

For example, the determination of polonium-210 (^{210}Po) was not suggested, although the U.S. National Council on Radiation Protection and Measurement ascribed about 1% of the risk of lung cancer in long-term cigarette smokers to ^{210}Po **(94)**. Polonium-210 in the smoke originates from the tobacco by transfer; a change in the make-up of a cigarette will not greatly alter the ^{210}Po concentration in the smoke (0.03-1.0 pCi ^{210}Po/cigarette; 14).

Similarly, analysis of nickel (0.1-0.6 μg/cigarette) or cadmium **(<0.5** μg/cigarette) was not suggested even though the International Agency for Research on Cancer considers these elements as "carcinogenic to humans" and "probably carcinogenic to humans", respectively (17). **As** discussed earlier (section I), inorganic additives, including silicates, that are added to the tobacco or paper to reduce ignition propensity must be determined in the smoke in order to ascertain that the toxicity of the smoke is not increased.

Cigarette smoke also contains traces of a few known human carcinogens (17), such as 4-aminobiphenyl (2.4 ngfcigarette; **95),** 2-naphthylamine (1.0 ngfcigarette; 95) and vinyl chloride **(5-16** ng/cigarette; **96).** Because these compounds are present in minute amounts, and analytical methods required for their determination are rather involved, we have not included the determination of these three chemicals in the overall analysis of toxic constituents. However, the analytical profiling of cigarette smoke can be extended to include these and/or any other agents, deemed to be of significance in respect to human health.

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Table 1 Major constituents of the vapor phase of the mainstream smoke of nonfilter cigarettes

Compound	Concentration/cigarette (% of total effluent)
Nitrogen	280 - 320 mg (56-64%)
oxygen	50 - 70 mg (11-14%)
Carbon dioxide	45 - 65 mg (9-13%)
Carbon monoxide	14 - 23 mg (2.8-4.6%)
Water	7 - 12 mg (1.4-2.4%)
Argon	5 mg (1.0%)
Hydrogen	0.5 - 1.0 mg
Ammonia	10 - 130 μg
Nitrogen oxides (NOx)	100 - 600 μg
Hydrogen cyanide	400 - 500 μg
Hydrogen sulfide	20 - 90 μg
Methane	1.0 - 2.0 mg
Other volatile alkanes (20)[a]	1.0 - 1.6 mg[b]
Volatile alkenes (16)	0.4 - 0.5 mg
Isoprene	0.2 - 0.4 mg
Butadiene	25 - 40 μg
Acetylene	20 - 35 μg
Benzene	12 - 50 μg
Toluene	20 - 60 μg
Styrene	10 μg
Other vol. aromatic hydrocarbons (29)	15 - 30 μg
Formic acid	200 - 600 μg
Acetic acid	300 - 1700 μg
Propionic acid	100 - 300 μg
Methyl formate	20 - 30 μg
Other volatile acids (6)	5 - 10 μg[b]
Formaldehyde	20 - 100 μg
Acetaldehyde	400 -1400 μg
Acrolein	60 - 140 μg
Other volatile aldehydes (6)	80 - 140 μg
Acetone	100 - 650 μg
Other volatile ketones (3)	50 - 100 μg
Methanol	80 - 180 μg
Other volatile alcohols (7)	10 - 30 μg[b]
Acetonitrile	100 - 150 μg
Other volatile nitriles (10)	50 - 80 μg[b]
Furan	20 - 40 μg
Other volatile furans (4)	45 - 125 μg[b]
Pyridine	20 - 200 μg
Picolines (3)	15 - 80 μg
3-Vinylpyridine	10 - 30 μg
Other volatile pyridines (25)	20 - 50 μg[b]
Pyrrole	0.1 - 10 μg
Pyrrolidine	10 - 18 μg
N-Methylpyrrolidine	2.0 - 3.0 μg

Volatile pyrazines (18)	3.0 - *8.0* μg
Methylamine	4 - 10 μg
Other aliphatic amines (32)	3 - 10 μg

[a] Numbers in parentheses represent the individual compounds identified in a given group.

[b] Estimate

Table 2 Major constituents of the particulate matter of the mainstream smoke of nonfilter cigarettes

Compound	µg/Cigarette
Nicotine	1000 - 3000
Nornicotine	50 - 150
Anatabine	5 - 15
Anabasine	5 - 12
Other tobacco alkaloids (17)[a]	n.a.
Bipyridyls (4)	10 - 30
n-Hentriacotane [$n\text{-}C_{31}H_{64}$]	100
Total nonvolatile hydrocarbons (45)[c]	300 - 400[c]
Napthalene	2 - 4
Napthalenes (23)	3 - 6[c]
Phenanthrenes (7)	0.2 - 0.4[c]
Anthracenes (5)	0.05 - 0.1[c]
Fluorenes (7)	0.6 - 1.0[c]
Pyrenes (6)	0.3 - 0.5[c]
Fluoranthenes (5)	0.3 - 0.45[c]
Carcinogenic polynuclear aromatic hydrocarbons (11)[b]	0.1 - 0.25
Phenol	80 - 160
Other phenols (45)[c]	60 - 180[c]
Catechol	200 - 400
Other catechols (4)	100 - 200[c]
Other dihydroxybenzenes (10)	200 - 400[c]
Scopoletin	15 - 30
Other polyphenols (8)[c]	n.a.
Cyclotenes (10)[c]	40 - 70[c]
Quinones (7)	0.5
Solanesol	600 - 1000
Neophytadines (4)	200 - 350
Limonene	30 - 60
Other terpenes (200-250)'	n.a.
Palmitic acid	100 - 150
Stearic acid	50 - 75
Oleic acid	40 - 110
Linoleic acid	150 - 250
Linolenic acid	150 - 250
Lactic acid	60 - 80
Indole	10 - 15
Skatole	12 - 16
Other indoles (13)	n.a.
Quinolines (7)	2 - 4
Other aza-arenes (55)	n.a.
Benzofurans (4)	200 - 300
Other 0-heterocyclic compounds (42)	n.a.
Stigmasterol	40 - 70
Sitosterol	30 - 40
Campesterol	20 - 30
Cholesterol	10 - 20

Anline	0.36
Toludines	0.23
Other aromatic amines (12)	0.25
Tobacco-specific N-nitrosamines (6)[b]	0.34 - 2.7
Glycerol	120

• Number in parentheses represent individual compounds identified.
[b] For details, see Figure 6.
[c] Estimate.
n.a. Not available.

Table 3. Compounds That Contribute To The Toxicity Of Cigarette Mainstream smoke of US Nonfilter Cigarettes [a]

Compound	Yield/Cigarette Range	Toxic Effects
1. Total Particulate Matter (TPM, dry)	12-40 mg	Carcinogen'
2. pH		Influences nicotine toxicity[b]
3. Redox Potential		Influences toxicity of whole smoke[c]
Vavor Phase		
4. Carbon Monoxide	14-23 mg	Reacts with hemoglobin, inhibits O, transport'
5. Nitrogen Oxides (NO,)	100-600 µg	Nitrosating agent[d], inhibitor of thiol dependent enzymes"
6. Hydrogen Cyanide	400-500 µg	Ciliatoxic"; inhibitor of respiratory enzymes'
7. Hydrocarbons		Suspected or known carcinogens'
benzene	12-50 µg	
1,3-butadiene	25-40 µg	
8. Aldehydes		Ciliatoxic", animal carcinogens'
formaldehyde	20-100 µg	
acrolein		
acetaldehyde	400-1400 µg	
9. Volatile N-Nitrosamines (VNA)		Strong animal carcinogens"
N-nitrosodimethylamine	13-65 ng	
N-Nitrosopyrrolidine	7-34 ng	
Particulate Matter		
10. Nicotine	1-3 mg	Associated with cardiovascular disease'
11. Phenols		Tumor promoters
phenol	80-160 µg	
other phenols	60-180 µg	
12. Catechol	200-400 µg	Major cocarcinogen
13. Polynuclear Aromatic Hydrocarbons		Major tumor initiators[a]
benzo(a)pyrene	20-60 ng	

14. Tobacco-Specific N-Nitrosamines		Strong organ-specific animal carcinogens'
NNN*	80-90 ng	
NNK*	60-470 ng	

"According to the International Agency for Cancer Research (17) TPM and benzene are human carcinogens, formaldehyde, benzo(a)pyrene and some other PAH as well as some volatile N-nitrosamines are probably carcinogenic to humans and acetaldehyde and the tobacco-specific N-nitrosamines are possibly carcinogenic to humans.

[b] Brunnemann and Hoffmann, 1974 (18).

[c] U.S. Surgeon General, 1983 (19).

[d] Brunnemann and Hoffmann, 1991 (20).

[e] Battista, 1976 (21).

* NNN - N'-Nitrosonornicotine
* NNK - 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone

Table 4. Some toxic and tumorigenic agents in undiluted cigarette sidestream smoke

Compound	Type of toxicity	Amount in sidestream smoke per cigarette	Sidestream: mainstream smoke ratio
Vapor phase			
Ammonia	T	50.0 - 130 μg	40 - 170
Carbon monoxide	T	26.8 - 61 mg	2-5 - 14.9
Carbonyl sulfide	T	2 - 3 μg	0.03 - 0.13
Benzene	C	240 - 290 μg	8 - 10
Formaldehyde	C	1500 μg	50
3-Vinylpyridine	SC	330 - 450 μg	24 - 34
Hydrogen cyanide	T	14 - 110 μg	0.06 - 0.4
Hydrazine	C	90 ng	3
Nitrogen oxides (NO_x)	T	500 - 2000 μg	3.7 - 12.8
N-Nitrosodimethylamine	C	200 - 1040 ng	20 - 130
N-Nitrosopyrrolidine	C	30 - 390 ng	6 - 120
Particulate phase			
Tar	C	14 - 30 mg	1.1 - 15.7
Nicotine	T	2.1 - 46 mg	1.3 - 21
Phenol	TP	70 - 250 μg	1.3 - 3.0
Catechol	CoC	58 - 290 μg	0.67 - 12.8
o-Toluidine	C	3 μg	18.7
2-Napthylamine	C	70 ng	39
4-Aminobiphenyl	C	140 ng	31
Benz[a]anthracene	C	40 - 200 ng	2 - 4
Benzo[a]pyrene	C	40 - 70 ng	2.5 - 20
Quinoline	C	15 - 20 μg	8 - 11
NNN	C	0.15 - 1.7 μg	0.5 - 5.0
NNK	C	0.2 - 1.4 μg	1.0 - 22
N-Nitrosodiethanolamine	C	43 ng	1.2
Cadmium	C	0.72 μg	7.2
Nickel	C	0.2 - 2.5 μg	13 - 30
Zinc	T	6.0 ng	6.7
Polonium-210	C	0.5 - 1.6 pCi	1.06 - 3.7

C, Carcinogenic; CoC, cocarcinogenic; SC, suspected carcinogen; T, toxic; TP, tumor promoter.

NNN - N'-Nitrosonornicotine
NNK - 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone

Table 5. Some toxic and tumorigenic agents in indoor environments polluted by tobacco smoke"

Pollutant	Location	Concentration/m^3
Nitric oxide	Workrooms	50 - 440 µg
	Restaurants	17 - 270 µg
	Bar	80 - 520 µg
	Cafeteria	2.5 - 48 µg
Nitrogen dioxide	Workrooms	68 - 410 µg
	Restaurants	40 - 190 µg
	Bar	2 - 116 µg
	Cafeteria	67 - 200 µg
Hydrogen cyanide	Living room	8 - 122 µg
Benzene	Public places	20 - 317 µg
Formaldehyde	Living room	23 - 50 µg
Acrolein	Public places	30 - 120 µg
Acetone	Public places	360 - 5800 µg
Phenols (volatile)	Coffee houses	7.4 - 11.5 ng
N-Nitrosodimethylamine	Restaurant, public place	0 - 240 ng
N-Nitrosodiethylamine	Restaurant, public place	0 - 200 ng
Nicotine	Public places	1 - 6 µg
	Restaurants	3 - 10 µg
	Workrooms	1- 13.8 µg
Benzo[a]pyrene	Restaurant, public place	3.3 - 23.4 ng
NNN	Public places	1.8- 22.8 pg
NNK	Public places	1.4- 29.3 pg

'References: Klus and Kuhn (97); IARC (17); US National Research Council (98); Klus et al. (99); Brunnemann (100).

NNN - N'-Nitrosonornicotine
NNK - 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone

Legends to Figures

1. Total Cigarette Smoke Compostition [% w/w] (2)

2. Approaches for Measuring Redox Potential of Cigarette Smoke (30)

3. Redox Potential of Puffs 1 to *8* of an *85* mm Plain U.S. Cigarette (30)

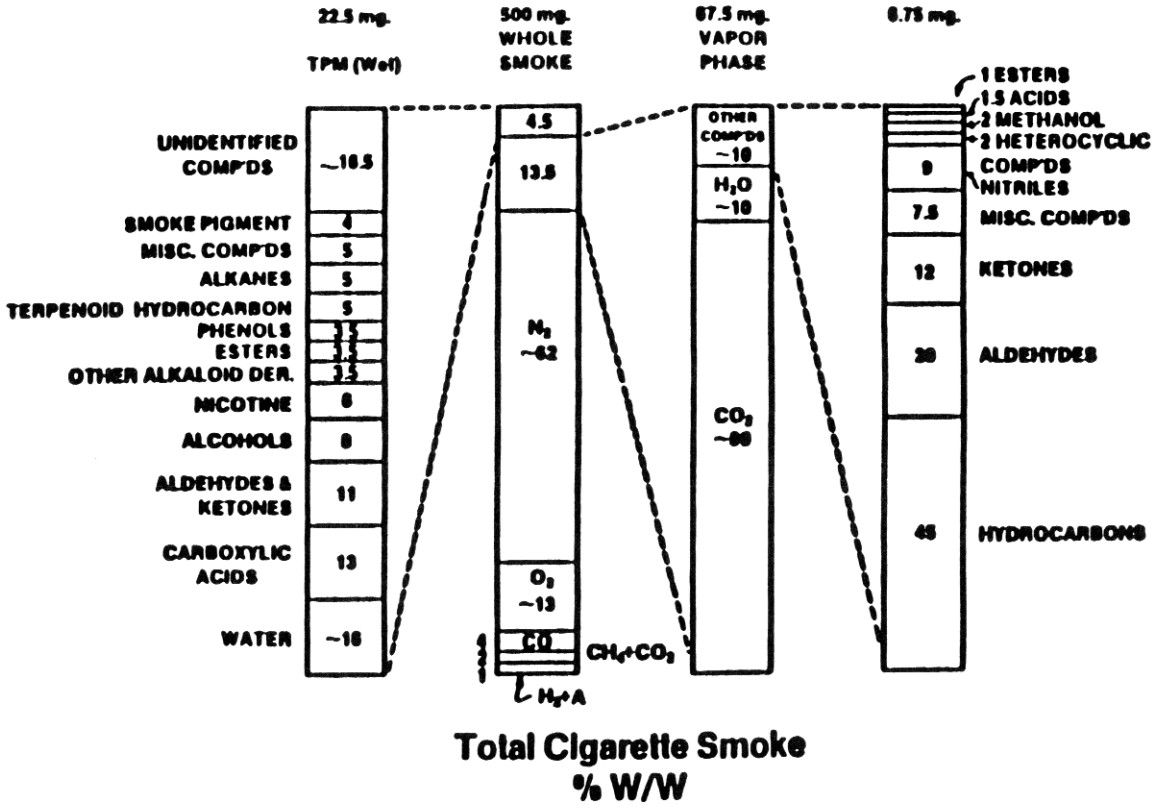
4. Diagram of Thermal Energy Analyzer (20)

5. Structures of Volatile N-Nitrosamines in Cigarette Smoke (20)

6. Carcinogenic Polynuclear Aromatic Hydrocarbons in Cigarette Smoke (16, 17)

7. Formation of Tobacco-Specific N-Nitrosamines (20)

Figure 1 Total Cigarette Smoke (% W/W; 2)



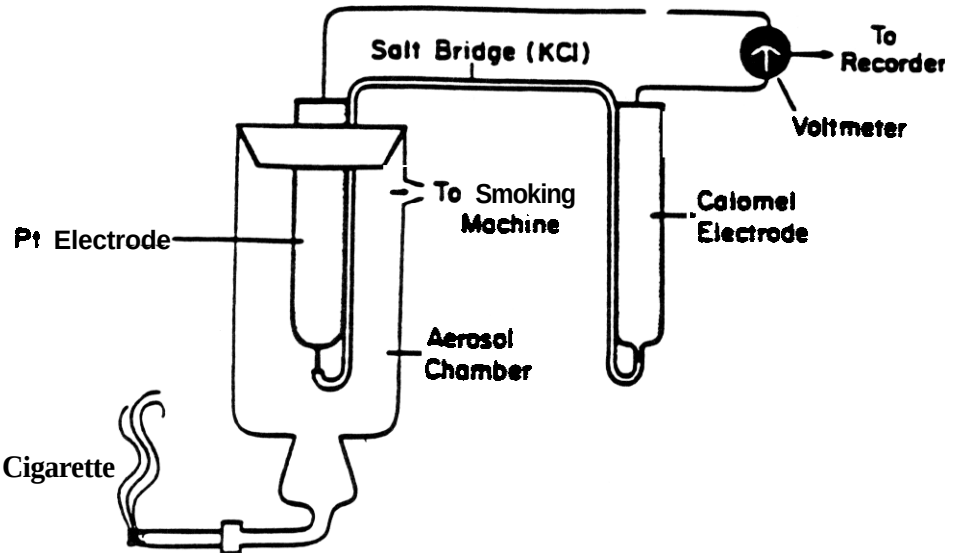


Figure 2 Apparatus for Measuring Redox Potential *of* Cigarette Smoke (30)

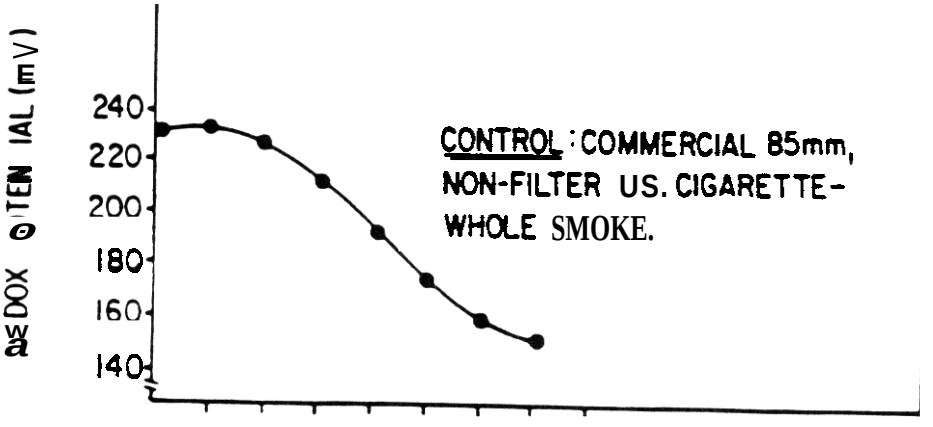
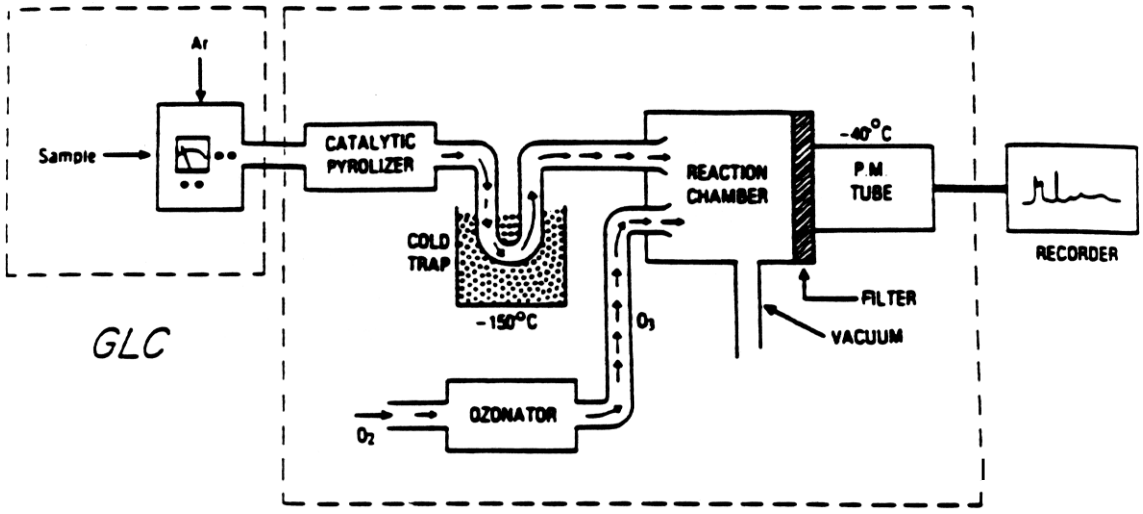


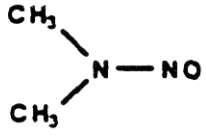
Figure 3 Redox Potential *of* Puffs **1 to 8** *of* **an 85 mm** Plain **US. Cigarette (30)**

Figure 4 Diagram of the Thermal Energy Analyzer (20)

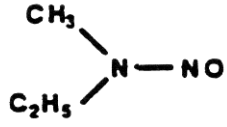


$$NO \xrightarrow{O_3} NO_2^* \longrightarrow NO_2 + h\nu$$

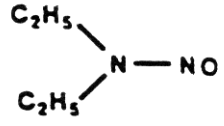
Figure 5 Volatile N-Nitrosamines (20)



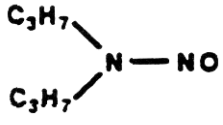
N-Nitrosodimethylamine (NDMA)



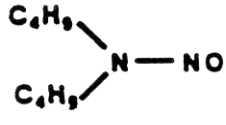
N-Nitrosoethylmethylamine (NEMA)



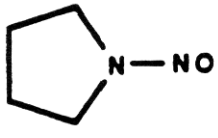
N-Nitrosodiethylamine (NDEA)



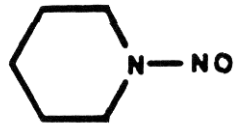
N-Nitrosodipropylamine (NDPA)



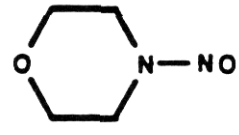
N-Nitrosodibutylamine (NDBA)



N-Nitrosopyrrolidine (NPYR)

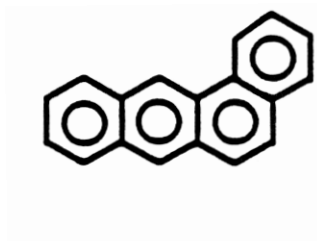


N-Nitrosopiperidine (NPIP)

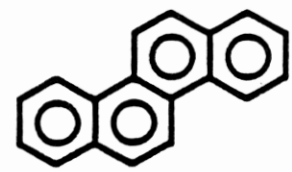


N-Nitrosomorpholine (NMOR)

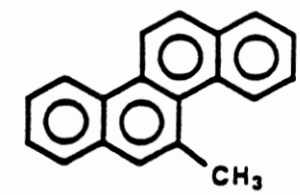
Figure 6 Carcinogenic Polynuclear Aromatic Hydrocarbons and Aza-Arenes in Cigarette Smoke (16, 17)



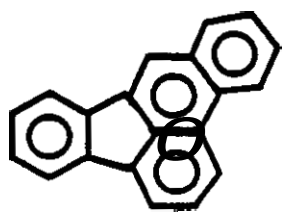
Benz(a)anthracenes



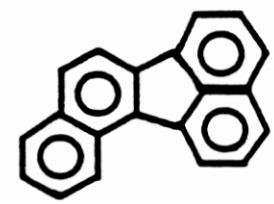
Chrysene



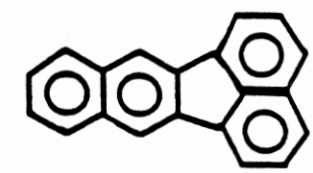
5-Methylchrysene



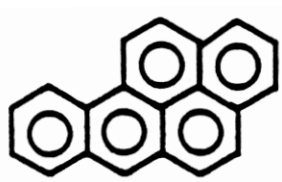
Benzo(b)fluoranthene



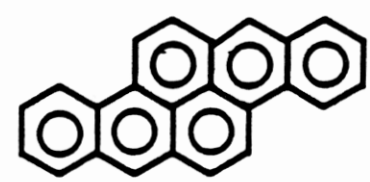
Benzo(j)fluoranthene



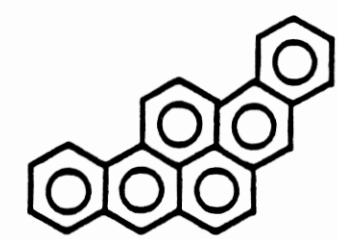
Benzo(k)fluoranthene



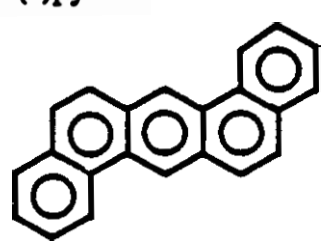
Benzo(a)pyrene



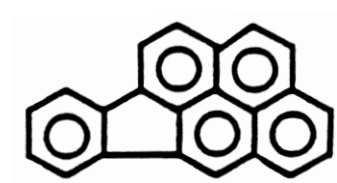
Dibenzo(a,h)pyrene



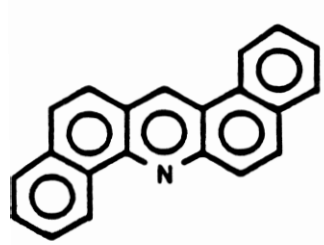
Dibenzo(a,i)pyrene



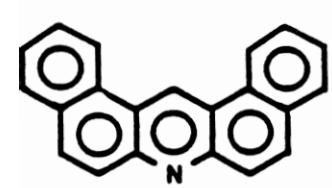
Dibenz(a,h)anthracene



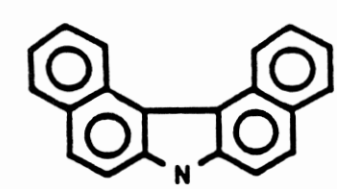
Indeno(1,2,3-cd)pyrene



Dibenz(a,h)aeridine

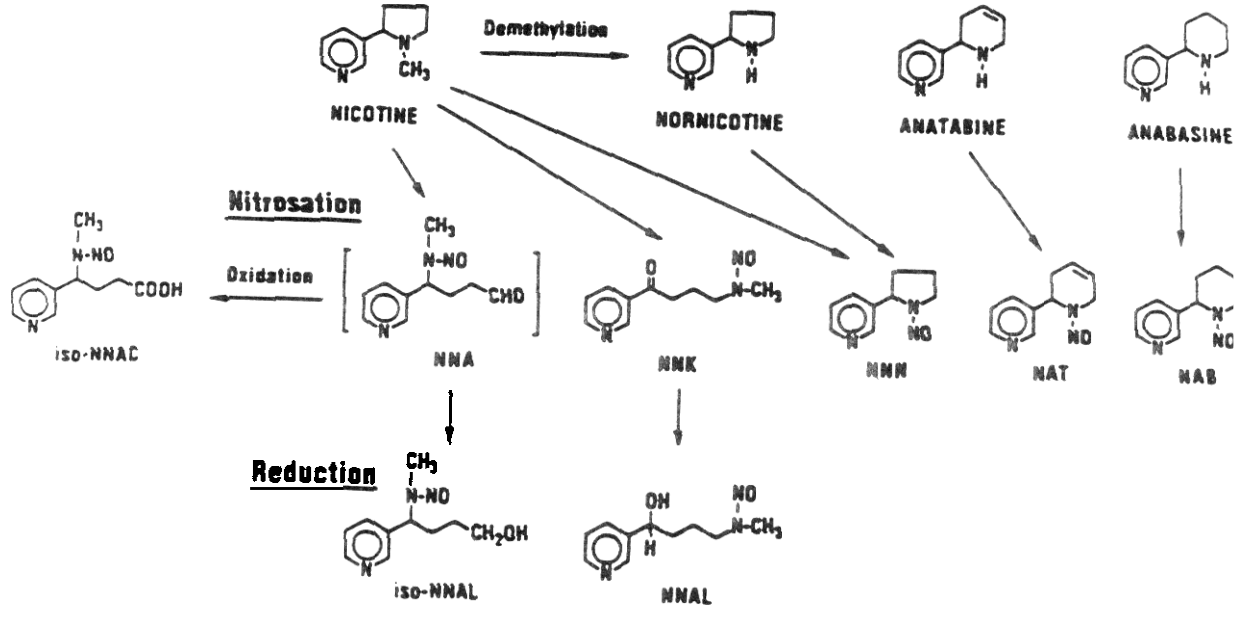


Dibenz(a,j)acridine



7H-Dibenzo(c,g)carbazole

Figure 7 Formation of Tobacco-Specific N-Nitrosamines (20)



Glossary of Terms

carcinogen	Substance that results in the production of tumors
ciliatoxic agent	Substance that inhibits the movement of cilia in the mucus-secreting respiratory epithelium. Total inhibition of such movement ciliastasis) prevents lung clearance and leads to accumulation of foreign substances in the respiratory airways.
cocarcinogen	Not a carcinogen by itself but potentiates the activity of a carcinogen when co-administered.
complete carcinogen	A carcinogen with both tumor initiating and tumor promoting activity.
draw resistance	Pressure developed by the full length of a cigarette when air is drawn through at a rate of 17.5 ml/sec (20^{0}C, 760 torr). Expressed as inches (or mm) of water column.
flavor additives	Plant extracts or synthesized chemicals that are added to the tobacco to impart flavor to the smoke.
mainstream smoke	For analytical purposes and for collecting the smoke in a closed system, mainstream is the smoke issuing from the mouth end of a cigarette. For the smoker, mainstream is the smoke that is drawn from the mouth end of a cigarette during puffing.
nonvolatile smoke constituents	All particulate phase constituents.
organ-specific carcinogen	Exerts carcinogenic activity in host tissues that have appropriate

	activating enzymes; acts on specific organs regardless of the route of application.
overwrap	Tipping paper, opaque-white paper, or cork wrapped around the filter and the cigarette rod to join both.
perforation	Tiny holes in cigarette paper or filter wrapper increases air permeability. Can be made by mechanical or electrostatic methods or by laser beams.
redox potential	Potential for electrobiochemical reduction/oxidation.
sidestream smoke	For analytical purposes, sidestream is that part of the smoke of a cigarette that emits from the burning end during puff intervals and that diffuses through the paper. In free-smoking situations, some sidestream smoke exits from the mouthpiece during puff intervals.
total particulate matter (TPM)	Fraction of smoke collected on a Cambridge (glass fiber) filter in machine smoking.
TPM dry	Total particulate matter minus water and minus nicotine.
toxic agent	Adversely pharmacoactive substance, for example, nicotine.
tumorigenic agent	Substance known to elicit neoplasms in animal assays.
tumor initiator	A substance or an active metabolite that forms chemical lesions with DNA having potential to develop into benign and malignant neoplasms.
tumor promoter	An agent that facilitates tumor development in an initiated cell when applied subsequent to initiation.

vapor phase (gas phase)	**The fraction of tobacco smoke that passes through a Cambridge filter. Contains some condensable smoke particulates that have not been retained on the glass fiber filter.**
volatile smoke constituent	**Chemical having more than 50% in the vapor phase.**

Estimated Coats For the Analysis of Individual Cigarette Smoke Components[1,2]

Smoke Component	Eatimated Coat
Total Particulate Matter, dry	$350
pH	$250
Redox Potential	$500
Carbon Monoxide	$250
Nitrogen Oxides	**$400**
Hydrogen Cyanide	$350
Volatile Hydrocarbons (esp. 1,3-Butadiene, Isoprene, Benzene, Toluene)	$600
Volatile Aldehydes	$700
Volatile N-Nitrosamines	$800
Nicotine (by GC)	$250
Phenols	$500
Catechols	$350
Polynuclar Aromatic Hydrocarbons (GC)	$1,500
Benzo(a)pyrene only	$500
Tobacco-Specific N-Nitrosamines	$800

'These estimates pertain to the direct cost of each determination (duplicate analyses) for one brand or prototype of cigarette. They exclude overhead as approved for the individual institute by the U.S. Dept. of Health and Human Services.

[2] For practical purposes, we recommend that analytical profiles of the smoke of cigarettes are done for at least two different brands or prototypes and one reference cigarette at any time.

Chapter E

SHORT-TERM TESTS FOR THE EVALUATION OF CIGARETTE SMOKE TOXICITY

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March 16, 1993

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Chapter Contents

1. Introduction:

Several epidemiological and experimental studies have implicated cigarette smoking with the increased incidence of a variety of human diseases. Major health consequences of smoking have been discussed in Chapter A. Also the physicochemical complexity of tobacco smoke and its major toxic constituents have been described in Chapter D. The main aim of this Chapter is to describe a testing strategy for comparing the toxicity of low ignition propensity cigarette prototypes using short-term tests.

Cigarette smoke is a complex aerosol which is composed of gaseous and particulate phases and contains thousands of different types of compounds. Any alteration in cigarette design and/or composition is likely to alter the physical and chemical characteristics of smoke and thereby its potential to cause toxicity. Some examples of such alterations have been described in Chapter A. To provide a comparison of toxicity of different cigarette prototypes, it is important that the tests selected for their toxicological evaluation are quantitative and have relevance to smoking-related health consequences described in Chapter A.

During the past several years, a number of short-term tests have been developed for routine assessment of the toxicity of chemicals. The main focus of these tests has been the identification of potential carcinogens in the environment. As a result of such testing, large databases of chemical genotoxicity and carcinogenicity have been developed by the National Toxicity Program, US Environmental Protection Agency, and IPCS (Parodi and Waters **1991).** Analyses of these data have demonstrated a relationship between genotoxicity and carcinogenicity of chemicals (Huff and Haseman **1991,** Tennant **1991,** Parodi et al. **1991).**

Currently, there are several genotoxicity tests that are used for identification of chemical carcinogens. These tests can be generally classified into four categories;tests for detecting (i) gene mutations, (ii) chromosomal aberrations, (iii) primary DNA damage, and (iv) induction of mammalian cell transformation (Brusick **1987).** Many of the tests from these four categories have been applied to genotoxic assessment of cigarette smoke and its condensates. These tests demonstrate that tobacco smoke possesses cytotoxic, mutagenic and carcinogenic activities (DeMarini **1983,** Hoffmann et al. **1987).**

A plan for the evaluation of toxicity of low ignition potential cigarettes is presented. Two types of tests are proposed. The prokaryotic Ames' Salmonella mutagenicity assay and a eukaryotic mammalian cell transformation assay are included to assess the genotoxic potential of the test cigarette smoke condensates. An inflammatory lung cell response bioassay is

proposed to assess the general pulmonary toxicity of the whole smoke from cigarette prototypes. These three tests should be included in a reasonable evaluation of test cigarette smoke toxicity.

2. General Considerations:

There are three types of smoke preparations that are used for in vitro testing of cigarette smoke: i) condensate collected by freezing smoke in a cold trap, ii) smoke particulates collected at room temperature on Cambridge filters, and iii) freshly generated whole smoke or its gas phase. The first two preparations have been used quite often as a solution/suspension in DMSO or acetone for testing in various short-term tests (DeMarini **1983).** The use of fresh whole smoke for in vitro testing has been limited.

Since most biological assays have baseline noise, it is important that the toxicity of test cigarettes be compared to known reference compounds and cigarettes that serve as positive control. Compounds like benzo(a)pyrene or 3-methylcholanthrene and the University of Kentucky reference research cigarettes are included in each experiment to serve as references for comparison of test cigarettes. In our hands, the condensates from the University of Kentucky reference cigarettes have given fairly consistent response in the Ames' Salmonella assay and has allowed identification of condensates of low and high mutagenic activity (Gairola **1979).**

There are several research cigarettes of different tar and nicotine delivery that are available from the University of Kentucky Tobacco and Health Research Institute (Davis et al. 1984). These cigarettes were developed to minimize the experimental variability introduced by the use of commercial cigarettes in analytical and biological studies of smoke (Benner **1970)** and have been used worldwide for experimental studies of tobacco smoke. Two newer research cigarettes, designated 1R4F and 1R5F, were manufactured in the **1980s** and contain **0.8** and **0.16** mg nicotine/cigarette, respectively. These two cigarettes probably represent low and ultralow nicotine cigarettes currently sold on the market.

Standard reference materials for complex environmental mixtures developed by the National Institute of Standards and Technology (May et al. 1992) may also be useful as reference materials, but have not been tested in conjunction with tobacco smoke studies.

3. Recommended Tests:

The Salmonella mutagenicity assay and a mammalian cell transformation assay (C3H/10T1/2 or BALB/3T3) are proposed for routine testing of genotoxicity of smoke condensates. To evaluate general pulmonary toxicity of whole smoke, a lung inflammatory cell response assay in mice has been proposed. Gene mutation and cell transformation assays are selected because of the high relevance of these two genotoxic endpoints in the overall carcinogenic process and tobacco smoke carcinogenesis in particular, which is known to involve the action of both tumor initiators and tumor promoters (Hoffmann and Wynder 1971, Melikian et al. 1989).

The Ames Salmonella assay is a gene mutation assay which quantitatively measures the ability of the test condensates to induce specific point mutations in a prokaryotic bacterial system. Since mutational events are generally believed to be essential for the initiation phase of carcinogenesis, this test may provide an estimation of the potential tumor initiators in tobacco smoke preparations.

Mammalian cell transformation assays use eukaryotic mammalian cell culture systems to measure the potential of smoke to induce malignant cell transformation. Cell transformation is a multistep process and is considered a close approximation of events occurring during in vivo oncogenesis. This assay may, therefore, measure the activity of both tumor initiators and promoters. Past studies have shown that cigarette smoke condensates give a positive response in both of these tests (Kier et a1.1974, Benedict et al. 1975). A good dose response relationship between the amount of test preparation and genotoxic response is generally observed in at least the Salmonella assay.

Selection of the bronchoalveolar lavage (BAL) cell response assay for pulmonary toxicity is based on the observation that chronic inflammatory conditions exist in the lungs of smokers and the BAL cells obtained from smokers exhibit altered characterstics which play an important role in the development of chronic obstructive pulmonary diseases associated with cigarette smoking (Hunninghake et al. 1979, Niewoehner 1988). Studies in the animal models have also demonstrated several alterations in the lungs of mice that resemble those reported in human smokers (Matulionis 1984, Gairola 1986).
This test, however, has never been employed to differentiate the toxicity of different types of cigarettes and may, therefore, need developmental work before routine use in toxicity evaluation.

3.a. Tests for Evaluatina Genotoxicity of Smoke Condensates:

The Ames Salmonella assay can be performed on dimethylsulfoxide solutions of freshly collected particulates from 1-5 test cigarettes. The particulates are collected on 0.3

μm Cambridge filters (Wartman et al. 1959), as noted in Chapter B. Larger quantities of condensates, needed for transformation assays, are collected in cold traps. The condensates are suspended in acetone for testing in mammalian cell assays.

Test 1: AMES' SALMONELLA TYPHIMURIUM MUTAGENICITY ASSAY:

Ames' Salmonella assay is the most widely used mutagenicity assay which has been used to predict the carcinogenicity of pure chemicals and complex mixtures (Ashby and Tennant 1991, Claxton et al. 1992). This is a simple assay which is easy to perform and provides results within a relatively short period of time. The test employs genetically constructed strains of a bacterium, Salmonella typhimurium, to detect the ability of chemicals to induce gene mutations. The tester strains possess defined mutations which prevent them from growing in a medium that does not contain an essential aminoacid, histidine. Upon treatment with a mutagenic chemical, some of these mutants revert back to their wild type form and regain capacity to grow in the absence of histidine (Ames et al. 1975). This simple feature of the test allows detection of chemicals that interact with bacterial DNA to cause reverse mutations. Up to five strains of S.typhimurium have been used for routine testing of chemicals.

The test is performed by mixing the low-histidine top agar, tester strain, test compound, and the S-9 mix in a sterile tube. The mixture is poured into petridishes containing bottom agar and the dishes are incubated at 37^0 C for 48-72 hrs. Revertant colonies are scored and the data are analysed. The assay procedure has been described in detail by Ames and coworkers (Ames, McCann and Yamasaki, 1975, Maron and Ames, 1983).

Past studies indicate that two strains of S.typhimurium, TA98 and TA100, provide an adequate evaluation of condensate mutagenicity (Mizusaki et al. 1977, Sato et al. 1977, Gairola 1979, Yoshida and Matsumoto 1980). The most sensitive strains for condensate evaluation are strains TA1538 and TA98 (DeMarini 1983), which detect those substances that induce frameshift types of mutations. Since genetic backgrounds of these two strains are basically the same and TA98 is somewhat more sensitive due to the presence of a plasmid, use of the latter strain is recommended. Strain TA1535 and TA100 which detect chemicals inducing base substitution types of mutations also give a positive response for smoke condensates and are especially useful for assaying the levels of direct mutagens eg.,nitrate-rich tobacco smoke condensates (Kier et al. 1974, Sugimura et a1.1977). In view of increased concern for the role of oxy-radicals in tobacco carcinogenesis (Church and Pryor 1985), a newer tester strain TA 102 may also be considered in the evaluation of condensate mutagenicity. This strain has been successfully used to detect the mutagenicity of oxidizing agents (Levin et al. 1982) and some

metal species that act via oxidative mechanisms, e.g., selenite, (Kramer and Ames 1988). However, data on the mutagenic evaluation of smoke condensates using strain TA102 are presently not available. Also this strain has a high spontaneous reversion rate which may necessitate a feasibility study of this strain for evaluation of smoke condensate mutagenicity before its use for routine genotoxicity evalation.

Tests are performed in the absence and presence of Aroclor-induced rat liver S-9 fraction to evaluate the presence of direct mutagens and those that require metabolic activation. In our experience, freshly prepared condensates give a near linear dose response curve when tested at concentrations of 100-400 μg/plate. If the condensates are stored for more than a week or two, a flattening effect is observed at higher concentrations, possibly due to cytotoxicity.

A general description of the procedure is provided below:

a) Culture Maintenance and Growing Conditions: Bacterial cultures are maintained as frozen stocks and are grown in Oxoid nutrient broth No. 2 to a density of 1-2 X 10^9 cells/ml. This is achieved by inoculating media flask with a tester strain and incubating at 37°C overnight as a stationary culture. Early next morning the cultures are placed in a gyrorotary incubator running at approximately 200 rpm at 37°C for 4-6 hrs. Once the cultures are ready they are stored on ice before use.

b) Preparation of Rat Liver S-9: Rat liver enzymes are induced with Aroclor 1254 suspension in corn oil (200 mg/ml). A single intraperitoneal injection of 500 mg/kg body wt of rat is given 5 days prior to sacrifice. Rats are sacrificed by cervical dislocation and their livers are excised under sterile conditions for placing in ice cold sterile PBS. All the steps are performed at 0-4°C under sterile conditions. The liver is chopped into small pieces and homogenized in chilled 0.15 M KCl using 3 ml of solution for each gm wet liver weight. The homogenate is centrifuged at 9,000G for 10 minutes and the supernatants are collected for storage in sterile plastic tubes at -80°C. **S-9** preparations are standardized with respect to their cytochrome P-450 content and mixed function oxidase activity.

c) Preparation of S-9 mix: S-9 mix is composed of 100 mM phosphate buffer, pH 7.4, 8 mM magnesium chloride, 33 mM potassium chloride, 4 mM NADP and 0.05-0.1 ml of S-9/ml of mix. The mix is made fresh for each experiment and can be stored on ice for the day without significant loss of activity.

d) Assay Procedure: Bottom agar plates and top agar are prepared in advance as described by Moran and Ames (1983). On the day of the experiment, to the 100 ml of melted top agar, 10 ml of a solution containing 0.5 mM histidine and 0.5 mM biotin

are added and thoroughly mixed. This low histidine top agar is then dispensed at 2 mls per sterile 13 x 100 mm tube and maintained at 45°C in heating blocks. To each tube, the test sample and 0.1 ml of the tester strain culture with and without 0.5 ml of S-9 mix are added and mixed before pouring onto plates containing 25 ml of bottom agar. The plates are incubated at 37°C for 48 to 72 hours and the revertant colonies are counted to obtain the number of induced revertants/plate.

e) Experimental Design: Each experiment should include negative (spontaneous revertants) and positive controls (one compound which is a direct mutagen and one which requires metabolic activation). A solvent control is also necessary. Four to 5 concentrations of each condensate sample with three replicates for each concentration are generally tested in each experiment. For reference cigarettes, 50 to 300 μg and 100 to 500 μg condensate/plate are tested in the absence and presence of S-9 mix, respectively. Numbers of revertants/plate for each concentration are obtained by taking an average of three replicates for that concentration of the sample. The number of revertants vs. concentration of condensate/plate are plotted to obtain a dose-response curve. If necessary, log transformations of the response can be plotted against condensate concentration to obtain a dose-response curve.

f) Data Evaluation and Interpretation: The data are generally presented as the number of induced revertants per plate, which are obtained by subtracting the number of spontaneous revertants from the total revertants/plate for each concentration of the test sample. When plots are developed for induced revertants vs. condensate concentration, a near linear dose-response curve is obtained; this is particularly common at lower concentrations. Specific activity of each condensate can be calculated from these plots. Specific activity is defined as the number of induced revertants/mg of condensate in the absence of metabolic activation, or the number of revertants/mg condensate, when metabolically activated with S-9 containing a given amount of cyt. P-450 or S-9 capable of metabolizing a given amount of benzo(a)pyrene/min/mg protein. Once the data have been generated for each of the test condensates, direct comparisons can be made to a reference or with each other to determine their mutagenic potential. Given the delivery of total particulate matter for each cigarette, the total number of revertants/cigarette can also be calculated to compare cigarette types.

In the mutagenic evaluation of tobacco smoke condensates, it has been our experience and that of other's (Matsumoto et al. 1977) that condensates generally do not show significant activity for direct mutagens, except for some experimental cigarettes, e.g., high nitrate tobacco cigarettes. Therefore, it is difficult

to obtain a dose-response curve when condensates are tested in the absence of S-9s.

g) Limitations of the Assay: Because of the high toxicity of fresh whole smoke to bacterial strains, the assay is limited to providing a genotoxic evaluation of only the particulate phase of cigarette smoke. Any changes in the constituents of gas phase resulting from low ignition modifications of cigarettes are, therefore, not evaluated in the plate incorporation version of this assay.

Certain mutagens and carcinogens, e.g., metallic salts, some organometallics, halogenated compounds, give negative results in the Ames' test (Ashby and Tennant 1988). If such chemicals are present in the low ignition potential cigarettes, their mutagenic activity will not be detected by the Ames' test.

Test-2: MAMMALIAN CELL TRANSFORMATION ASSAY:

A number of mammalian cell transformation assays have been used in the evaluation of genotoxicity of chemicals. C3H/10T1/2 mouse embryo, BALB/c 3T3 mouse fibroblast and Syrian hamster embryo (SHE) cell assay systems are the most commonly used assays for which reasonable baseline data exist (IARC/NCI/EPA 1985, IARC 1985, Dunkel et a1.1991). Cell transformation has been defined as the induction of certain neoplasia-related phenotypic changes in the cultured cells.

The most commonly examined endpoints in cell transformation assays have been the morphological alteration of cell colonies. Normally, cells in culture grow to form a confluent monolayer and then stop dividing when surrounded by the cells (contact inhibition). However, if the cells have been treated with a carcinogen, some cells continue to grow and form foci of transformed cells. These foci exhibit dense, haphazard overgrowth on the monolayer. When injected into appropriate host animals, the transformed cells form tumors, but the normal cells generally do not give rise to tumors. Because of their ability to form a tumor in the host, the cells from such foci are considered malignantly transformed (Landolph 1985).

The selection of the C3H/10T1/2 cell assay for condensate evaluation is based on a very low rate of spontaneous transformation of these cells in culture and positive results with smoke condensates (Benedict et al. 1975). Furthermore, C3H/10T1/2 and BALB/c 3T3 cells are continuous cell lines which give relatively more reproducible results than SHE cells, which are primary culture cells and are by nature variable. Since the main purpose of this testing program is to provide a quantitative comparison of the activity of different smoke condensates, it is desirable that the selected assays are reproducible.

The theory that tumor initiator and tumor promoter activities of chemicals play important roles in the carcinogenic process reinforces the relevance of the cell transformation assay. Cigarette smoke condensates are known to contain both tumor initiators and promoters (Hoffman and Wynder 1971).

Mammalian cell transformation assays can be performed with either C3H/10T1/2 or BALB/c 3T3 cells. Detail test protocols for these assays have been described (IARC 1985). The basic features of the C3H/10T1/2 assay procedure are described below which also apply to BALB/c 3T3 cell assay.

Cell Transformation Assay: Transformation assays are performed essentially as described by Reznikoff et al., (1973) with some later modifications (Landolph 1985, Dunkel et al. 1991). For each concentration of the test condensate, 30-40 60-mm petri dishes are seeded with 2 x 10^3 cells. In all experiments a set of dishes for positive control (3-methylcholanthrene) and another set for solvent control are run in parallel. After 24 hr of incubation at 37°C in a 5% CO_2 atmosphere, appropriate concentrations of the test condensates in dimethylsulfoxide (DMSO) or acetone are added to the cultures. After another 24 hr incubation, the old medium is changed with fresh medium. Thereafter, the medium is replaced every fourth day until the cultures are confluent. Once the cultures have reached confluency, the medium is changed every seventh day with Eagle's basal medium (BME) containing 5% heat-inactivated fetal calf serum (FCS). The experiments are terminated after 6 weeks of incubation. The medium is removed and the cultures are washed with phosphate buffer solution (PBS), fixed with methanol, stained with Giemsa and scored for type 2 and type 3 foci of transformants (Reznikoff et al. 1973).

Cell Culture Methods: Mouse embryo fibroblast cell line (C3H/10T1/2), clone 8 is grown in BME supplemented with 10% heat inactivated FCS and 5 mg/ml gentamycin (Kennedy, 1985). It is important that several batches of sera are pretested for plating efficiency, cell attachment and transformation with 1 mg/ml 3-methylcholanthrene prior to purchase to insure the consistency of the transformation assay. Only those lots that give a plating efficiency of over 20% are purchased for use in the assays. The C3H/10T1/2 stock cultures are seeded at a density of 2 X 10^4 cells per 25 cm^2 flask and grown in a humidified incubator in a 5% CO_2/air atmosphere at 37°C. Every 7 days, the stock cultures are passaged using trypsin (0.1% in PBS for 3 min) to detach the cells from the flask. After detachment, the cells are resuspended in complete BME, counted, and reseeded into 25 cm^2 flasks.

It is important that the cultures used for the assay are in early passage. For storage, the cells from the log phase of growth are harvested by trypsinization and suspended in antibiotic-free medium containing 10% FCS and 10% DMSO. The cell suspensions are frozen and stored in liquid nitrogen.

Cytotoxicity Assay: Cytotoxicity assays are performed in advance and in parallel with the cell transformation assays. The information derived from the cytotoxicity experiments is necessary for running the transformation experiments and calculating the transformation frequencies. The toxicity of the test solution is assessed by determining the plating efficiency of untreated and treated cells in each experiment. Five 60-mm Petri dishes/group are seeded with 200 cells and treated with different concentrations of the test solution or the solvent in the same manner as described above for the cell transformation assay. After 10-12 days of culture, the cell monolayers are washed and stained. Colonies are counted and the plating efficiency, which is defined as the number of colonies formed as a percentage of the number of cells seeded per dish, is calculated.

This assay is capable of detecting the cell transformation activity of 3-methylcholanthrene in the absence of a metabolic activation system (Reznikoff et al. 1973) and has also been reported positive for condensates in the absence of S-9 (Benedict et al. 1975). Presence of some metabolic activation capacity in C3H/10T1/2 cells for polycyclic aromatic hydrocarbons has been demonstrated (Gehly and Heidelberger 1982).

Data Evaluation and Interpretation: Data from these experiments are generally expressed as transformation frequencies, defined as the percentage of type 2 and type 3 foci, based on the number of survivors that form colonies in the dishes. The data generated can be plotted as % transformed colonies on a log scale vs the concentration of test condensate. Cytotoxicity data can also be plotted on the same graph to express percent cell survival in treated cultures.

Transformation frequencies depend heavily on the number of survivors. The frequencies decrease if the surviving number of cells/dish is too high or too low. In view of this problem, tabulation is currently preferred of the number of type 2 and type 3 foci per total number of treated dishes and the total number of dishes containing type 2 and type 3 foci out of the total number of dishes treated. Both formats of data expression allow the development of a dose-response curve.

When polycyclic aromatic hydrocarbons are tested in the assay, a dose response is usually obtained. Comparison of the transformation potential of smoke condensates from different test cigarettes will require tightly controlled conditions. In each

experiment a reference compound or a reference condensate or both are run in parallel. The data from the test condensate can then be compared to the values for the reference substance in each experiment and a potency value assigned to each test condensate. These manipulations are necessary to minimize the impact of variability of response in the assay from experiment to experiment.

Limitations of the Assay: Because of the high toxicity of fresh cigarette smoke to mammalian cells, C3H/10T1/2 assay may also be limited to assessing the genotoxicity of cigarette smoke condensates. Any changes in gas phase constituents resulting from low ignition modifications of cigarettes will, therefore, not be evaluated in this assay. Very few studies of smoke condensates in cell transformation assays have been performed. It will be necessary to further develop assay conditions for routine evaluation of condensate activity.

Other limitations of the cell transformation assay exist (Landolph 1985, IARC 1985, Dunkel et al. 1991). First, C3H/10T1/2 cells have a maximum plating efficiency of about 30%. In the standard transformation assay when toxic concentrations of the smoke condensates are tested the plating efficiency will be further reduced and the number of survivors may be too few for a valid transformation assay. Second, too few or too many cells per dish can affect the number of transformed colonies thus making the test somewhat semiquantitative even under tightly controlled conditions.

Third, the spontaneous transformation rate of C3H/10T1/2 cells is extremely low, which makes it difficult to obtain a baseline transformation frequency in each experiment. As many as 500 control plates may be needed to obtain the true spontaneous transformation rate of C3H/10T1/2 cells. However, this characteristic of C3H/10T1/2 cells also makes the test attractive, because the induction of even a few transformed colonies on the plates can be attributed to the action of the test condensate with confidence. In this respect, it may be noted that the BALB/c 3T3 cell system, which has a low but still easily obtainable basal transformation rate, has been often utilized for routine testing of various chemicals but sufficient information about smoke condensate activity in this system is presently not available. In contrast, the C3H/10T1/2 system has been successfully used to detect cell transformation activity of smoke condensate and its fraction (Benedict et al. 1975).

3.b. Tests for Evaluating Genotoxicity of Fresh Whole Cigarette Smoke:

While microgram quantities (50 to 500 µg/plate) of smoke condensates can be tested in the bacterial and mammalian cell assays, direct exposure of tester cells to fresh whole cigarette

smoke can cause high cell mortality due to the presence of highly toxic constituents, such as carbon monoxide. Therefore, the above assays are generally difficult to use in their standard format for genotoxic evaluation of fresh cigarette smoke. A yeast cell system which tolerates cigarette smoke much better than do bacterial or mammalian cell systems may be used for fresh smoke evaluation, if necessary.

In cases where chemical and physical analyses indicate a significant alteration in smoke composition, especially that of the gas phase constituents, the induction of mutations and other types of genetic damage by fresh whole smoke may be assessed by using the D-7 strain of Saccharromvces cerevisiae. This tester organism is a diploid strain of yeast which is used to identify chemicals that induce mitotic crossing-over, mitotic gene conversion, and gene mutations (Zimmermann et al. 1975). An exposure system has been developed to directly expose tester yeast cells to fresh smoke and has been found useful in quantitating various types of genetic damage by fresh smoke from different types of tobacco cigarettes (Gairola 1982).

3.c. Tests for Evaluating Pulmonary Inflammatory Response in Animals

i) Rationale: Cigarette smoking is a major risk factor in the development of chronic obstructive pulmonary disease, as discussed in Chapter A. Considerable evidence exists to suggest that chronic pulmonary inflammation plays an important role in the development of various pulmonary diseases, including those associated with cigarette smoking (Hunninghake et al. 1979, Neiwoehner 1988, Costabel and Guzman 1992). A test that evaluates the ability of test smokes to induce pulmonary inflammation will therefore be a useful indicator of the in vivo biological activity of cigarette smoke. The assay described below utilizes an analysis of the **BAL** cells from smoke-exposed mice for toxicological evaluation of fresh cigarette smoke.

ii) Bioassay procedure

Animal model: Rodents (mice, rats, guinea pigs and hamsters) have been generally used as animal models for studying the inhalation toxicity of cigarette smoke. Past studies have shown that exposure of rodents to cigarette smoke induces an inflammatory cell response in their lungs (Rylander 1974, Hoidal and Niewoehner 1982, Matulionis 1984, Gairola 1986). The studies have further shown that the pulmonary response of mice to cigarette smoke inhalation, as monitored by bronchoalveolar lavage **(BAL),** is significantly more pronounced than that of rats (Gairola 1986). Furthermore, it has been found that, as in human smokers, the **BAL** cells recovered from smoke-exposed mice show an infiltration of inflammatory cells including polymorphonuclear neutrophils (PMN) into the lungs, while those recovered from

smoke-exposed rats hardly show any PMNs. Among other changes induced in BAL cells of smoke-exposed mice are increased oxidant production and lysosomal enzyme content of pulmonary alveolar macrophage (Gairola **1986)** which resemble those reported in human smokers (Hunninghake et al. **1979,** Finch et al. **1982,** Fisher et al. **1982).**

In view of the observations described above, mouse is proposed as an animal model for this assay. An additional advantage of using mouse is its smaller size which allows for increasing the number of animals in treatment and control groups at relatively lower costs.

Smoke exposure protocol: Male or female C57Bl mice are exposed to smoke in a nose-only exposure system (Griffith and Standafer **1985)** to fresh smoke from one cigarette in the morning and one in the afternoon. Since nose-only exposures require the use of restrainers for animals during exposures, it is important that a group of animals be given the same treatment as smoke-exposed animals but in the absence of smoke (sham control) to simulate stress conditions similar to those of the smoke-exposed group. The first week of the experiment is a "break in" period during which the animals are gradually acclimatized to treatments by exposing them to **3, 6,** and **9** puffs of smoke each session for two days at a time. Thereafter, the animals receive exposure to **10** puffs of smoke per session, twice a day, seven days a week. Groups of animals **(6-8)** are sacrificed at different exposure points and the free lung cells are obtained by bronchoalveolar lavage (BAL) for further study.

Markers of smoke exposure: A number of markers are monitored to ascertain the inhalation of smoke by the animals. Total particulate matter **(TPM)** intake, blood carboxyhemoglobin, and urinary cotinine excretion are generally measured, (Griffith and Standafer 1985, Gairola **1986, 1987,** Stanley et al. **1991).** Animal TPM intake values are determined by measuring the removal of smoke particulates by the animals from the exposure chamber during each exposure session (Griffith and Hancock **1985).** Some of the exposure markers, e.g., urinary cotinine and blood carboxyhemoglobin levels, used in animal studies are the same as those described for human studies in Chapter C.

Bronchoalveolar lavage: The morning after the last treatment, animals are anesthetized by an ip injection of pentobarbital and are exsanguinated by severing the abdominal aorta. The lungs are lavaged and the BAL cells are obtained for analyses (Gairola **1986).**

Assessment of BAL cells: Total cell counts and viable cell counts by trypan blue exclusion are made with a hemocytometer. Small aliquots of BAL cell suspension are used to prepare Diff-Quik stained slides for differential leukocyte

counts. These data allow an assessment of the degree of macrophage, lymphocyte and polymorphonuclear neutrophil (PMN) infiltration into the lungs.

Differential counts for each batch of lavage cells at different exposure points indicate the time course of PMN infiltration into the lungs and can be plotted against exposure duration to determine the rate and extent of their influx. Since neutrophilic alveolitis has been implicated in the development of cigarette smoke-induced pulmonary diseases, such plots prepared for animals exposed to various test cigarette smokes are likely to provide a reasonable indication of their toxicity and the data can be used to differentiate the biological activity of test smokes.

The cells remaining after total, viable, and differential counts are cultured for one hour at 37°C to isolate macrophages as monolayer. 5'-nucleotidase activity of the cell lysates is measured by a radiometric procedure, which is a good indicator of smoke-induced macrophage activation in mice (Gairola 1986).

Data Evaluation and Interpretation: Graphs of the BAL cell data obtained from the study can be used to determine the time course of inflammatory cell influx into the bronchoalveolar lumen, which will indicate the toxicity of smoke. A useful endpoint is the time of PMN infiltration which may reflect the potency of the test smoke toxicity. Macrophage enzyme activities can also be used to assess the potential biological activity of test cigarette smoke.

iii) Limitation of the Bioassay: Even though markers of smoke exposure are used, it is difficult to ascertain the dose of smoke inhaled by the animals. Also, interanimal variation may complicate interpretation of data, but this problem can be controlled by increasing the number of animals per group. The use of this assay has been limited for comparative assessment of smoke from different cigarette types and therefore may require further development of standardized assay conditions before use in any routine evaluation program. Also the expense of performing this assay may be a deterent for its routine use.

The presence of particulates in the ambient atmosphere, respiratory infections, etc., can also induce an inflammatory response in the lungs of animals, thus complicating an assessment of smoke effects. Therefore, it is very important to house the animals in Bioclean rooms equipped with HEPA filters and maintained at higher (30 to 40) air changes/hour to minimize the exposure of animals to particulates and/or any infectious agents.

4. Estimates of Cost and Duration of Tests:

Ames' Test: The length of time for testing five concentrations of one reference and four unknown condensate samples, in the

absence and presence of S-9s, is estimated at about 4-6 weeks. The current cost of testing five concentrations of one compound in the absence and presence of S-9, in two to five strains of the test bacteria, varies between $1,500-2,200/condensate sample.

Cell Transformation Assay: The length of time for testing 5 concentrations of one condensate in the absence of S-9 in cell transformation assay is about 6 months and costs range from $7,000-8,000/condensate sample.

Inflammatory Cell Response Bioassay: The length of time for testing smoke from one type of cigarette in this bioassay will take 8-10 months. The cost of testing one cigarette type in this bioassay is estimated at about $50,000. If more than one cigarette type is tested at the same time, the cost will be reduced.

5. Other Toxicological Endpoints Associated with Smoke Exposure-related Health Problems:

In addition to genotoxicity and pulmonary toxicity, cigarette smoke has been implicated in other health effects discussed in Chapter A. Smoking-induced immunosuppression in human smokers may be one of the mechanisms contributing to the development of smoking-associated health effects. Although the incidence of immunosuppression in smokers is well documented (Holt 1987, Johnson et al. 1990), the exact nature of immunotoxicants in smoke is not known. It may be useful to include some tests for evaluating the immunotoxic activity of low-ignition potential cigarette smoke. Unfortunately very few studies addressing immunotoxicity of smoke have been performed in animal models. Only recently some experimental evidence suggesting an impairment of thymic-dependent and independent functions of lymphocytes from long-term smoke-exposed mice and rats has been reported (Sopori et al. 1989, Chang et al. 1990, Goud et al. 1992).

Reproductive toxicity and atherogenesis are two other health problems associated with cigarette smoking. A number of epidemiological studies have suggested that chronic smoking is deleterious to female reproductive health and fetal development (Abel 1980, Mattison et al. 1989, Werler 1986). Relatively fewer data regarding the effects of smoke on reproductive system are available in animal models (Mattison 1982). Preliminary studies in mice have shown that long-term exposure to cigarette smoke lengthens the murine estrous cycle and causes accelerated loss of oocytes from the ovaries (Gulati et al. 1989, Gairola and Gulati 1991).

Still fewer studies have examined the formation of atherosclerotic plaques following exposure to cigarette smoke in animal models. One study reported the formation of aortic

lesions in Syrian hamsters following a 12-15 month exposure to cigarette smoke (Haley and Axelrad 1982). Recently, enhanced development of atherosclerosis has also been reported in rabbits which had been fed high cholesterol diets and were exposed to environmental tobacco smoke (Zhu et a1.1993).

The long duration of exposures required to induce many of the above described immunological, reproductive, and atherogenic toxicities in animals would make bioassays based on these endpoints prohibitively expensive for routine use in the evaluation of low-ignition cigarette prototypes.

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GLOSSARY

carcinogenesis: or Oncogenesis:	Processes through which tumors are produced.
Genotoxicity:	Any adverse effect on structure, function, or expression of genetic material in a living cell.
Initiators:	Agents that begin but do not necessarily promote the carcinogenic process.
Malignant Cell Transformation:	Change in the phenotypic characterstics of cells in culture that indicates their capability to induce tumors when injected into animals.
Mutagenesis:	Processes by which heritable alterations in genetic material or functions are produced.
Mutations:	Any heritable alterations in the expression of genetic material or functions.
Promoters:	Agents that encourage but do not necessarily initiate cancer development.

Chapter F

In Vivo Bioassays for Carcinogenicity

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Chavter Contents

Introduction

In the 1989 Report on the Health Consequences of Smoking, the Surgeon General of the U.S. Public Health Service, in evaluating the health effects of smoking, concluded that "smoking is responsible for more than one of every six deaths in the United States" (1). Cigarette smoking alone increases the risk for coronary heart disease from 23/1000 to 54/1000, together with hypercholesterolemia cigarette smoking raises the risk to 103/1000, and together with high blood pressure, to 92/1000. All three risk factors, smoking, hypercholesterolemia, and high blood pressure, synergistically increase the risk for coronary heart disease to 189/1000 (1).

Cigarette smoking is also a major risk factor for chronic obstructive pulmonary disease, and here primarily for emphysema and chronic bronchitis. Eighty to 90% of the morbidity from chronic obstructive lung disease in the United States has been attributed to cigarette smoking (2).

Smoking of cigarettes is causally associated with cancer of the lung, larynx, oral cavity, esophagus, pancreas, renal pelvis and urinary bladder and is also linked with an increased risk for cancer of the nasal cavity, liver and the uterine cervix and possibly, related to cancer of the stomach (1). In 1992, the National Cancer Institute published a population-based case-control study that provided evidence for the association of cigarette smoking with several types of leukemia and thereby confirmed earlier prospective and case control studies (3). The National Cancer Institute estimated that in 1991 of the 514,000 cancer deaths at the seven sites causally associated with cigarette smoking, 30.6% are due to smoking (4).

Chemical analyses for the major known carcinogens offer a meaningful indication of the carcinogenic potential of cigarette smoke, especially in conjunction with chemical analytical data for the smoke of cigarettes already bioassayed for carcinogenic activity. In vitro assays for genotoxicity such as the Ames test with various bacterial strains, the DNA repair assay with primary rat liver cells, and the sister chromatid exchange assay have remained inconclusive in regard to the quantitative aspects of the genotoxic potencies of cigarette smokes (5). At present, conclusive data on the carcinogenicity of the smoke of new cigarettes can only be ascertained with long-term bioassays with laboratory animals (5-7).

Three animal species are primarily utilized for bioassays of whole cigarette smoke in inhalation experiments. These are mice, rats, and Syrian golden hamsters. All of the inhalation studies have the inherent shortcoming that the animals are obligated to breathe through the nose and that their inhalation of tobacco

smoke is shallow. They do not inhale smoke through the mouth as human smokers do. Nevertheless, cigarette smoke inhalation studies with mice, rats and Syrian golden hamsters have led to the induction of significant numbers of benign and malignant tumors in the respiratory tract of these animals.

The data from cigarette smoke inhalation studies with mice have not been fully accepted since only lung adenoma and lung adenocarcinoma have been elicited and not squamous cell tumors in the lung. Furthermore, most strains of mice have a fairly high rate of spontaneous lung adenoma. The rates of both adenocarcinoma and squamous cell carcinoma of the lung have increased in cigarette smokers and the current ratio of lung adenocarcinoma to squamous cell carcinoma in male smokers is 1:2-3 *(8)*.

As will be discussed under "Inhalation Bioassays", the critique on the data from smoke inhalation studies with mice is no longer fully justified. A large-scale inhalation study with rats using highly advanced methodology presents encouraging data (9). However, until additional long-term inhalation bioassays have been completed with this exposure system, the database is too limited to recommend this rat bioassay for routine studies.

The largest database from cigarette smoke inhalation studies stems from assays with Syrian golden hamsters. As will be discussed, these long-term inhalation studies have only in a few cases led to lung tumors; however, they have induced highly significant incidences of benign and malignant tumors in the upper respiratory tract of hamsters. The tumors occurred primarily in the larynx.

Since the early 1960's, remarkable progress has been achieved in respiratory carcinogenesis. We have become well aware of the existence of carcinogens with organ-specificity for the respiratory tract of laboratory animals, and bioassays of aerosols and volatilized chemicals have also provided considerable evidence for their potential to induce tumors in the respiratory tract of mice, rats and hamsters (10).

I. Inhalation Bioassays

Three decades ago, the Leuchtenbergers (11) reported the first extensive inhalation experiments in which mice were exposed daily to air-diluted cigarette smoke in specially designed chambers. This smoke exposure led to early histological, cytological, and cytochemical changes in the major bronchi of the mice. The smoke exposure also caused various degrees of bronchitis associated with atypical proliferation of the bronchial epithelium. The investigators observed extracellular deposition of a brown pigment in the lungs of all the mice that

underwent long-term exposure to cigarette smoke aerosols. After about **12-15** months, the smoke-exposed mice began to develop lung adenoma and lung adenocarcinoma in significantly higher numbers than did the control mice. In inhalation studies with the gas phase of cigarette smoke, lung adenomas have also been observed, though to a significantly lesser extent than with the whole smoke **(12).** The findings of the Leuchtenbergers (11, **12)** were confirmed by Otto **(13)** who exposed inbred albino mice to cigarette smoke daily. After at least **12** months of smoke exposure, **23** of 60 mice developed lung adenomas, while only **3** of 60 control mice were found with such tumors. One mouse in the exposed group developed a squamous cell carcinoma of the lung after 16 months **(13).**

Several criticisms have been voiced in regard to the induction of lung adenoma and lung adenocarcinoma in mice by exposure to cigarette smoke. Concerns include the fact that such exposures caused tumors in the peripheral lung, and not in the bronchi, and that some of the tested strains of mice had a relatively high rate of spontaneous lung adenomas. It has been observed that not only the carcinoma in the bronchi, but also the incidence of lung adenocarcinoma, has significantly increased in cigarette smokers and that such tumors are now even seen in nonsmokers who have been exposed to environmental tobacco smoke, to carcinogenic chemicals, or to radiation (1).

In the past, it was not understood how the topical application of tobacco "tar" to the skin of mice could lead to the development of lung adenoma and adenocarcinoma. Today, we are aware that tobacco smoke contains also organ-specific carcinogens such as the tobacco-specific N-nitrosamines, which can induce adenoma and adenocarcinoma in the lung upon application to the skin **(14)** and other sites in mice.

A major breakthrough in inhalation assays came with the development of new smoke-inhalation devices that facilitate the exposure to diluted tobacco smoke aerosols **(15-17).** When **80** rats were exposed seven times daily for intermittent periods **(8.4** x **30** seconds) to **10%** cigarette smoke aerosol for up to **2.5** years, most animals developed hyperplastic and metaplastic changes in the nasal turbinals, larynges and tracheas. Seven of the 80 smoke-exposed **F344** female rats developed tumors in the respiratory tract, including 1 adenocarcinoma and 1 squamous cell carcinoma in the lung, compared to 1 alveologenic carcinoma only in the **93** control rats **(9).**

In another study, rats were exposed to diluted cigarette smoke twice a day for 10 minutes, **5** days a week for up to **40** weeks **(18).** Subsequently, DNA from nasal, lung and liver tissues was extracted and analyzed by the ^{32}P-postlabeling procedure. In the nasal mucosa at least four new DNA-adducts were seen; the

amount of these adducts increased with the duration of smoke exposure. In the lung, one new DNA-adduct was detected; it also accumulated as smoke exposure progressed. It appears that the DNA adducts were aromatic and/or hydrophobic in nature (18). In a similar assay, rats were exposed for 22 days to diluted cigarette smoke. In the nose-only intermittent exposure and nose-only continuous exposure 14 ± 0.9 and 9.9 ± 0.7 DNA adducts per 10^9 bases were determined in the lung (19). These studies demonstrate that minute amounts of genotoxic smoke components reach the lungs of rats in inhalation assays.

Dontenwill and associates developed the "Hamburg II" smoke inhalation device in which small animals can be exposed to air-diluted smoke (Figure 1). Eighteen groups, each consisting of 80 female and 80 male random-bred Syrian golden hamsters, comprised this cigarette smoke inhalation lifetime assay. Animals in group 1 were exposed once daily for about 10 minutes, seven times each week to air-diluted smoke (7:1); those in group 2 had twice daily exposures to diluted smoke, hamsters in group 3 had 3 exposures to diluted smoke; and those in group 4 were exposed twice daily to the gas phase of diluted smoke, while group 5 consisted of sham-treated controls. In group 1, 38 animals developed papilloma and one animal had a carcinoma of the larynx (total 24%), hamsters in group 2 developed 69 papilloma and 17 carcinoma of the larynx (total 54%), corresponding tumor yields in group 3 were 77 papilloma and 11 carcinoma of the larynx (total 55%). Laryngeal tumors were not observed in group 4 (gas phase only) nor in group 5 (controls). Three hamsters in group 2 developed papilloma of the pharynx; tumors of the lung were not seen in any of the hamsters in this study (20).

In another assay, male Syrian golden hamsters from 2 inbred lines were exposed five times a week for up to 100 weeks to air-diluted smoke (21). In one inbred strain, 7 of 84 hamsters developed papilloma in the larynx, 9 had microinvasive cancer; in the second inbred strain, 11 of 87 animals had papilloma and 2 microinvasive cancers occurred in the larynx; none of the control hamsters developed laryngeal tumors (21).

In a dose-response lifetime study with a hamster strain susceptible to the induction of laryngeal tumors, twice daily exposures to 22% cigarette smoke resulted in 70% with papilloma and 47% with carcinoma of the larynx (22). The corresponding incidences in the hamsters exposed twice daily to 11% cigarette smoke were 27% and 7%. Those in the control group were 6% and 0%, respectively. In the high-dose group, 3 of 62 hamsters also developed tracheal papilloma (22).

These studies demonstrated the dose-repsonse carcinogenic effect of cigarettes. The Syrian golden hamsters are less susceptible than other laboratory animals to the toxicity of

nicotine and of carbon monoxide and are therefore preferred for inhalation studies with tobacco smoke.

11. Bioassays with Cigarette Smoke Particulate Matter

Inhalation assays with Syrian golden hamsters have demonstrated that only whole smoke induces benign and malignant tumors of the respiratory tract in a dose-dependent fashion. However, inhalation of smoke which is free of particulate matter ("tar") does not lead to tumors. This indicates that the dose of carcinogens in the gas phase by itself is not sufficient to induce tumors and that the majority of the carcinogens reside in the particulate matter of tobacco smoke. This consideration has led to in-depth fractionation studies and bioassays with tobacco smoke condensate in mice, rats and rabbits (6, 23, 24). The neutral subfractions B and BI that contain a concentrate of the polynuclear aromatic hydrocarbons (PAH), harbor the major tumor initiators (Figure 2). The PAH subfraction is also the only portion of the tar that, upon repeated intratracheal instillation, elicits tumors in the respiratory tract of rats (25).

Assays of the PAH concentrate explain only a small fraction of the total carcinogenicity of the tar. Results from bioassays of the PAH-subfraction in combination with the weakly acidic, non-carcinogenic fraction explain 70-90% of the carcinogenicity of the whole tar (23, 24). The weakly acidic fraction contains the major tumor promoters, volatile phenols, and the major cocarcinogens, catechols. In addition to tumor initiators, tumor promoters and cocarcinogens, tobacco smoke also contains carcinogens with organ-specificity. These act independently of the mode of exposure or site of application, by inducing benign and malignant tumors in specific organs. Table 1 presents a list of the known tumorigenic agents in tobacco smoke, their concentrations in the smoke of one cigarette, and the evaluation of evidence of their carcinogenicity by the International Agency for Research on Cancer (26 27). Table 2 is a listing of the likely causative agents for tobacco smoke-related cancers on the basis of organ-specificity of carcinogens and their various biological activities and concentrations in cigarette smoke.

The agents in tobacco smoke most likely to cause induction of cancer of the respiratory tract are PAH, the tobacco-specific N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the volatile aldehydes, acetylaldehyde and formaldehyde and, to a minor extent, polonium-210 (from agricultural and environmental sources).

111. Bioassay on Mouse Skin

Inhalation studies with Syrian golden hamsters have clearly demonstrated that the major carcinogenic activity of whole cigarette smoke resides in its particulate matter (tar), as discussed earlier. This has led to extensive bioassays of cigarette tar in both the connective tissue of rats and the skin of mice (6). Since the induction of sarcoma in the connective tissue of rats can be influenced by the physical form of the tar, by the presence of insoluble particles (Oppenheimer-Nothdurft effect; 28, 29) the mouse skin bioassay is now the preferred method for estimating the tumor potency of smoke condensates especially when comparing tars of experimental cigarettes that vary from the control cigarette only in a few aspects.

The tars obtained from a smoking machine (Chapter B), such as a Borgwaldt-30 cigarette smoker (30) or other devices (6), are stored in the dark at refrigerator temperature until needed for biological testing, but should not be older than 3 weeks when applied. Before use, the suspensions in acetone are thoroughly mixed in a mechanical shaker for at least 3 hours, a sample is poured into a 60-ml glass-stoppered reagent bottle. Since tars are not always fully dissolved in the acetone (1:1), vigorous shaking of the bottle is essential before each use.

Anywhere from 30 to 100, usually 50 but preferably 100, Ha/ICR/Mil (Swiss albino) female mice are used for each tar to be tested. The random bred Ha/ICR/Mil (Swiss albino) mice are sturdy animals, and they are quite resistant to nicotine toxicity. Compared to two inbred strains of mice often used in skin carcinogenesis (CAF,, C57BL), they are more susceptible to the carcinogenic activity of tobacco tars (31). Female mice are used for the bioassay since they do not fight as do the males, which results in skin scratches. Thus, females can be housed 5 to a cage, while males require one cage for each mouse. The maintenance of female mice is therefore significantly more cost effective (6). At the onset of bioassays the mice are 5 to 7 weeks of age and weigh 22 to 25 g. They receive feed and water ad libitum. Their cages are cleaned twice weekly.

Before each tar application, the dorsal hair of the mice is shaved with a Model A2 (size 40) Oster animal clipper. The tar is then applied in 0.1 ml of an acetone suspension containing 50 mg tar with a full No. 5 camel hair brush, or by pipette. The treatment is repeated three times weekly, allowing at least one day between applications for absorption of the tar before the next application. It is sometimes necessary, especially at the onset of the experiment, to skip a painting if the mice exhibit poor absorption or low tolerance of the tar.

Mice that survive the first month usually tolerate the toxic effects of the tar solutions (LD_5). All mice lost during the first month in an experiment are replaced by mice of the same age. Therefore, the initial number of animals to be scheduled for each assay must exceed the requirements for the control and experimental groups by about 10%. If the toxicity persists, even though the number of applications is cut down, the tar must be applied at a lower concentration with the necessary revision of the protocol. In recent years, however, such modifications have usually not been necessary, owing to the generally lower levels of nicotine in tobaccos. The bioassay is not terminated until 90% of the mice in the tar group with the longest survival rate have died or were moribund and had to be killed; this takes usually 18-20 months of tar application.

Average weights of the mice are recorded at the onset of the experiment and again at 2 weeks, 4 weeks, and thereafter at monthly intervals as an indicator of the general health of the mice. A reduction in weight could be associated with a reduced tumor yield, particularly if the weight loss takes place in the tumor promotion phase. It is essential that weight records be kept in experiments with tobacco carcinogenesis.

Constant observation of the animals is also essential. Any change in appearance, habit, or reaction is noted; any lesion on the back is described as to [1] type, i.e. ulcer, infection, or tumor; [2] date of appearance or change in appearance; and [3] exact location. When such a notation is first made, the animal, is marked on the head with a yellow dye (picric acid). Diagrammatic representations of the animal's back are used to facilitate the recording.

The application of the test material by painting or pipetting may lead to benign skin tumors which are recorded when they have attained a diameter of 1 mm. They enlarge by nodular growth (papilloma) or by lateral invasion (carcinoma); some may not enlarge, but regress. Those tumors that remain 1 mm or grow larger for 21 consecutive calendar days are counted and become the raw tumor yield data. Continued growth of such lesions, however, is required before they can be recorded as macroscopically observed carcinomas (raw tumor yield data). A revised count is reported after histopathological confirmation of the macroscopically observed lesions. Lateral invasion of the tumor into adjacent skin is considered as transformation into a carcinoma.

Mice with carcinomas are killed by cervical dislocation and tumors are excised for subsequent histopathological analysis. All suspicious lesions are likewise examined. The animals are autopsied for distant metastases and the occurrence of other tumors, especially pulmonary adenomas and lymphomas.

The most careful recording of experimental observations demands equally careful statistical evaluation of the final data. Therefore, sone pertinent statistical considerations will be discussed.

More than 90 mice, rats, or hamsters per group should be used in carcinogenicity bioassays with tobacco smoke condensates. We calculated that to find a difference with 80% statistical power between the carcinogenicity of condensates from an experimental cigarette [with reduced ignition propensity] vs. a control would require at least 90 animals in each group. An additional 30 are also needed for the negative control group.

The lethal toxicity of some smoke condensates require that bioassays be carried out with sufficient animals to achieve statistical soundness. Mice lost during the first month of an experiment are replaced.

Assuming, as an example, that none of the animals in a control group has a tumor and 6 or more animals in the experimental group have tumors, one may utilize the table prepared by Vos based on chi-square analysis with "Yates correction". This shows that the difference between the groups is significant at $P < 0.05$ when the number of animals in each group varies from 10 to 50 or more.

This situation merely identifies that one is dealing with a tumorigenic agent and does not allow a quantitative assay of tumor-producing agents of varying potency. If, €or example, there are as many as 4 tumor-bearing animals in a given group treated with a weak carcinogen, the number of animals in each group becomes important. If there were 10 animals in each group, all of them must bear tumors to indicate a difference at the $P < 0.05$ level of significance. In a group of 50 animals treated with a stronger tumorigenic substance, only 12 need to show tumors. Information about differences in tumor yield necessary for significance at $P < 0.05$ between two groups for groups of 10 to 50 animals may be readily extracted from tables by the Food Protection Committee of the U.S. Academy of Science (30).

IV. Inhalation Bioassay with Syrian Golden Hamsters

Inhalation bioassays with whole smoke are also strongly indicated to confirm the relative carcinogenic potencies of respective condensates in mouse skin painting bioassays. As discussed earlier, the Syrian golden hamster (SGH) is presently the animal of choice for long-term inhalation assays with whole cigarette smoke (6, 20-22). It should be noted that inhalation bioassays with whole cigarette smoke will rarely lead to lung tumors in SGH or rats (9), but it will lead to papilloma and carcinoma in the larynges of the animals. Since the larynges of

inbred strains of male SGH are apparently most susceptible to the carcinogenic effects of cigarette smoke (21, **22)** one is inclined to prefer this animal model.

Inbred strains may be difficult to obtain are not as resistant to the acute toxicity of the smoke as are random-bred SGH. Thus, the latter are generally used for inhalation studies **(7, 31).**

Three inhalation devices have been developed for exposure of SGH and rats to cigarette smoke. These are the "Hamburg II" device **(20, 34),** the "Oak Ridge" smoke inhalation exposure device **(9, 17)** and the "Walton-reverse smoker" **(21).** All **3** machines are well developed and the "Oak Ridge" device has especially favorable features in respect to forced smoke inhalation by laboratory animals. However, the "Hamburg II" device with SGH is recommended for comparing the tumorigenicity of whole smoke from various cigarettes. The device has been widely used and most data on the tumorigenicity of whole cigarette smoking, including a dose-response study, were generated with it (15, **20, 34).** In general this bioassay requires **24-26** months.

The inhalation studies with SGH generally consists of twice daily exposures to air-diluted smoke (7:1) of one cigarette each, seven times weekly, for the entire lifespan of the animals. Because of the high ***co*** concentration in undiluted smoke **(2.8 - 4.6** vol%) the maximum tolerated dose is typically a **10** minute exposure twice daily of cigarette smoke diluted by air **1:7.** Ten SGH at at time can be exposed concurrently to diluted cigarette smoke from one Hamburg II device. Since 80 male hamsters are needed for each test cigarette, the bioassay is very labor intensive and is recommended only as a last step in the cascade of assays. Details for the inhalation assay with SGH are presented in Dontenwill (15, 20). These exposure protocols are not intended to mimic human smoking behavior (see Topography chapter), but are intended to produce tumorigenic effects that can be statistically evaluated.

Inhalation assays using **F344** rats and employing the "Oak Ridge" inhalation device for exposure to cigarette smoke (9, 17) appear promising. Although it can not yet be recommended for toxicity testing, it is hoped that the methodology will be confirmed by additional studies.

Critique

Ideally one establishes the carcinogenicity of an inhalable substance by bioassays leading to the induction of benign and malignant tumors in the respiratory tract of laboratory animals. In the case of cigarette smoke this goal has been only partially reached. In the Syrian golden hamster, papilloma and carcinoma

have been induced in the larynx with cigarette smoke in a dose-related fashion. However, with a few exceptions, squamous cell tumors of the lung, which are associated with cigarette smoking in humans, are not produced by this model (15, 20-22). Even with an advanced smoke inhalation device, only a few lung carcinomas were produced in rats (9, 17).

Epidemiologists identify several hundred prospective and case-control studies demonstrating that cigarette smokers face an increased risk for lung cancer. Therefore, confirmation by inhalation bioassays is not necessary.

Simulation of human smoking behavior in terms of deep inhalation of cigarette smoke into the lungs has not been successful in laboratory rodents. However, in comparisons of the relative tumorigenicity of the whole smoke of cigarettes with reduced ignition propensity to that of a control, the inhalation bioassay with hamsters should clearly reflect possible changes in the carcinogenic potential in the number of tumors observed in the larynges.

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Table 1 Tumorigenic agents in tobacco and tobacco smoke

Compounds	In processed tobacco (per g)	In Mainstream smoke (per cigarette)	IARC evaluation of evidence of carcinogenicity[a]	
			In laboratory animals	In humans
PAH				
Benz[a]anthracene		20 - 70 ng	sufficient	
Benzo[b]fluoranthene		4 - 22 ng	sufficient	
Benzo[j]fluoranthene		6 - 21 ng	sufficient	
Benzo[k]fluoranthene		6 - 12 ng	sufficient	
Benzo[a]pyrene	0.1 - 90 ng	20-40ng	sufficient	probable
Chrysene		40 - 60 ng	sufficient	
Dibenz[a,h]anthracene		4ng	sufficient	
Dibenzo[a,i]pyrene		1.7 - 3.2 ng	sufficient	
Dibenzo[a,l]pyrene		present	sufficient	
Indeno[1,2,3-cd]pyrene		4 - 20 ng	sufficient	
5-Methylchrysene		0.6ng	sufficient	
Aza-arenes				
Quinoline	1 - 2 µg			
Dibenzo[a,h]acridine		0.1 ng	sufficient	
Dibenzo[a,j]acridine		3 - 10 ng	sufficient	
7H-Dibenzo[c,g]-carbasole		0.7 ng	sufficient	
N-Nitrosamines				
N-Nitrosodimethylamine	ND - 215	0.1 - 180 ng	sufficient	
N-Nitrosoethylmethylamine		3 - 13 ng	sufficient	
N-Nitrosodiethylamine		ND - 25 ng	sufficient	
N-Nitrosonornicotine	0.3 - 89 µg	0.12 - 3.7 µg	sufficient	
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	0.2 - 7 µg	0.08 - 0.77 µg	sufficient	
N'-Nitrosoanabasine	0.01 - 1.9 µg	0.14 - 4.6 µg	limited	
N-Nitrosomorpholine	ND - 690 ng		sufficient	
Aromatic amines				
2-Toluidine		30 - 200 ng	sufficient	inadequate
2-Napthylamine		1 - 22 ng	sufficient	sufficient
4-Aminobiphenyl		2 - 5 ng	sufficient	sufficient
Aldehydes				
Formaldehyde	1.6 - 7.4 µg	70 - 100 µg[b]	sufficient	
Acetaldehyde	1.4 - 7.4 µg	18 - 1400 µg[b]	sufficient	
Crotonaldehyde	0.2 - 2.4 µg	10 - 20 µg		
Miscellanous organic compounds				
Benzene		12 - 48 µg	sufficient	sufficient
Acrylonitrile		3.2 - 15 µg	sufficient	limited
1.1-Dimethylhydrazine	60 - 147 µg		sufficient	
2-Nitropropane		0.73 - 1.21 µg	sufficient	
Ethylcarbamate	310 - 375 ng	20 - 38 ng	sufficient	
Vinyl chloride		1 - 16 ng	sufficient	sufficient
Inorganic compounds				
Hydrazine	14-51 ng	24 - 43 ng	sufficient	inadequate
Arsenic	500 - 900 ng	40 - 120 ng	inadequate	sufficient
Nickel	2000 - 6000 ng	0 - 600 ng	sufficient	limited
Chromium	1000 - 2000 ng	4 - 70 ng	sufficient	sufficient
Cadmium	1300 - 1600 ng	41 - 62 ng	sufficient	limited
Lead	8 - 10 µg	35 - 85 ng	sufficient	inadequate
Polonium-210	0.2 - 1.2 pCi	0.03 - 1.0 pCi	sufficient	sufficient

[a] No designation indicates that on evaluation by IARC has not been carried out. [b] The 4th report of the Independent Scientific Committee on smoking and Health (1988) published values for the 14 leading British cigarettes in 1986 (51.4% of the market) of 20-105 µg/cigarette (mean 59 µg) for formaldehyde and 550-1150 µg/cigarette (mean 910 µg) for acetyladehyde. PAH, polynuclear aromatic hydrocarbons; ND. not detected.

Table 2

Likely Causative Agents for Tobacco-Related Cancers

Organ	Carcinogen	Enhancing Agents
lung, larynx	NNK acetaldehyde formaldehyde polonium^{210} (minor)	acrolein, crotonaldehyde(?)
	PAH	catechol (cocarcinogen) weakly acidic tumor promoters
esophagus	N'' NAB	ethanol, catechol
pancreas	NNK NNAL	nutrition
bladder	4-aminobiphenyl 2-naphthylamine other aromatic amines	infectious agents(?)
oral cavity	PAH NNN NNK	ethanol herpes simplex

NAB - N'-nitrosoanabasine
NNAL - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN - N'-nitrosonornicotine
PAH - polyaromatic hydrocarbons

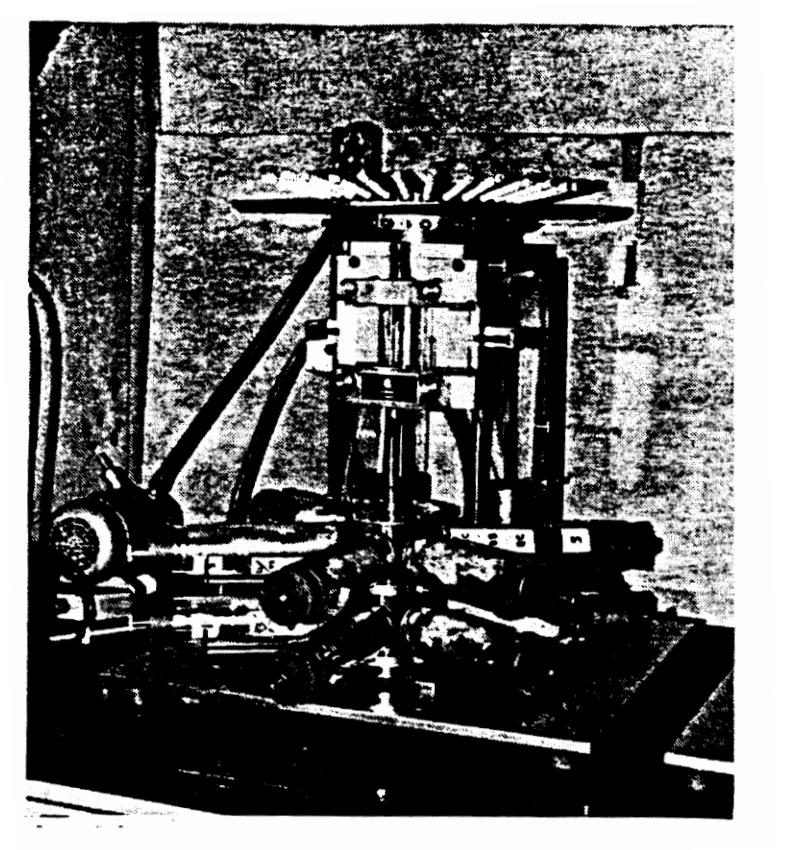

Fig.1 Hamburg II Smoke-Inhalation Device for *10* Hamsters (16).

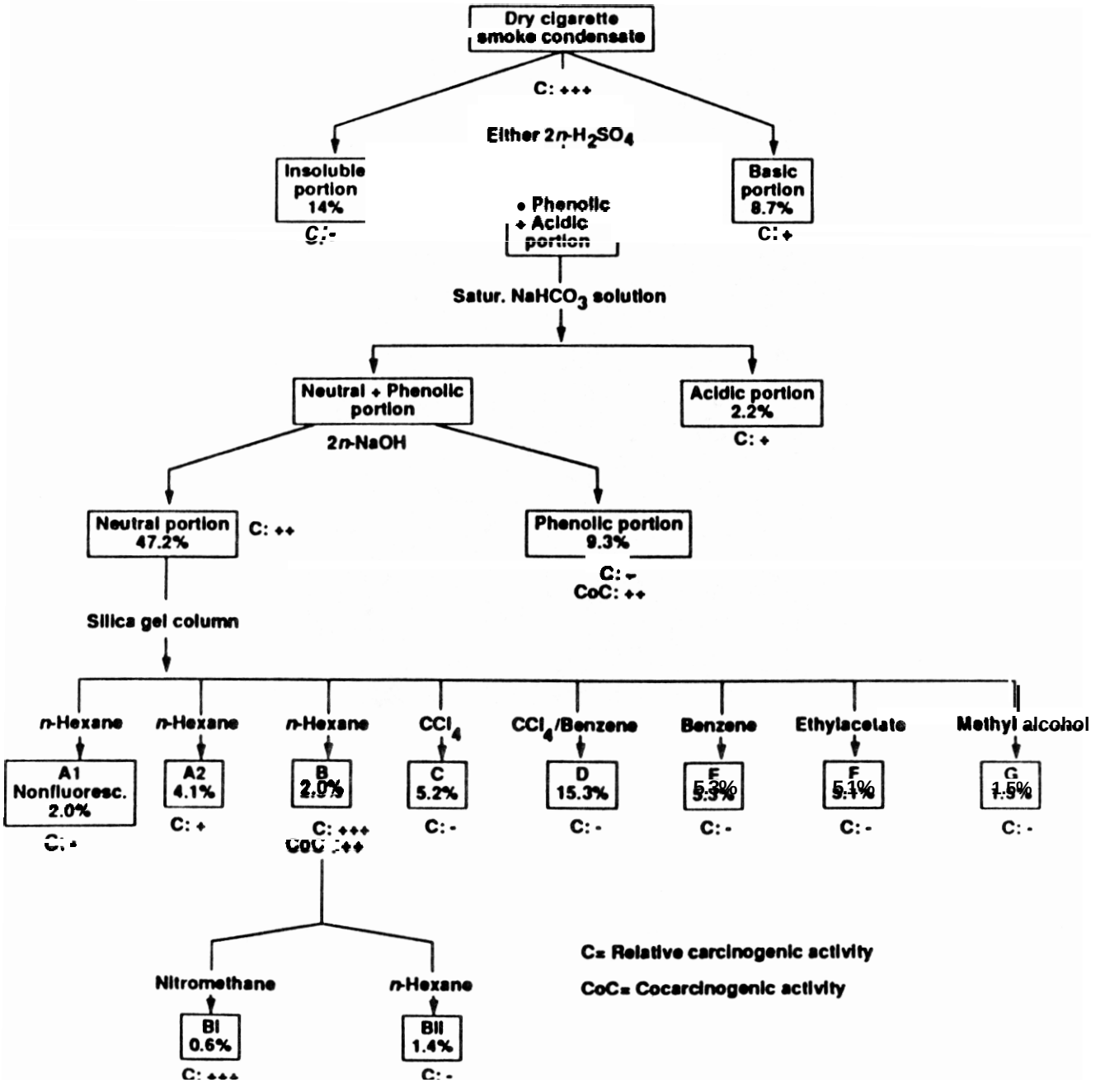

Fig.2 Fractionation of Cigarette Smoke Condensate (23).

Glossary of Terms

adenocarcinoma	malignant tumor of a glandular structure, such as in the peripheral lung
adenoma	benign tumor of a glandular structure, such as in the peripheral lung
carcinoma	malignant tumor of epithelial orgin
DNA	deoxyribonucleic acid. DNA is localized in the cell nucleus and is the molecular basis of heredity in many organisms
genotoxicity	damage to the DNA structure
in vitro	experimentation with microorganisms, isolated cells, tissues, or isolated organs in biological media
in vivo	experimentation with live animals, such as mice, rats and hamsters
papi1loma	benign tumors (warts) due to a proliferation of epithelial tissue

Cost Estimates for Carcinogenicity Bioassays

Bioassay	Animal	Groups	Number of Animals[2]	cost'
Inhalation	SG hamster (random bred)	exptl. cigarette I	60	$185,000-$250,000[3]
		exptl. cigarette II	60	
		sham control	60	
Skin	mouse (Ha/ICR/Mil)	exptl. cigarette I	90	$39,000
		exptl. cigarette II	90	
		sham control	60	

'Estimates pertain only to direct cost. The costs exclude the overhead as approved for individual institutes by the U.S. Department of Health and Human services. Direct total costs include animal purchase, health screening of the animals, maintenance, treatment (smoking of hamsters or tar application to mouse skin), weighing (first 8 weeks weekly, subsequently monthly), recording, autopsy and histology.

Estimates do not include purchase of cigarettes (inhalation study requires about 280,000 cigarettes/group; mouse skin bioassay 1.5 kg/group requires about 75,000 cigarettes, assuming one cigarette yields 20 mg tar), or the smoking of cigarettes for the preparation of the tar for the mouse skin bioassays.

[2] The number of animals per group is calculated for a difference between two groups with 80% statistical power either for the tumorigenic activity in the larynx of hamsters of whole cigarette smoke or for the tumorigenic activity on mouse skin of a tar.

[3] This includes the overtime for twice daily exposure on Saturday and Sunday.

Part II

VOLUME *5*
PART II

LIMITED TIER I AND II DEMONSTRATION OF THE TOXICITY TESTING PLAN FOR LOW IGNITION-POTENTIAL CIGARETTES

U.S. Consumer Product Safety Commission
in consultation with the
U.S. Department of Health and Human Services

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July 27, 1993

I. Introduction

The Toxicity Testing Plan for Low Ignition-Potential Cigarettes was written to fulfill part of the responsibilities given to the Consumer Product Safety Commission (CPSC) by the Fire-Safe Cigarette Act of 1990. The Plan identified various toxicological tests and associated direct cost estimates. Limited testing from Tiers I and II was recommended as a first step toward the implementation of the Plan.

CPSC staff, in consultation with the Department of Health and Human Services (HHS), directed a demonstration of this limited testing to verify its feasibility and costs. This study was designed demonstrate the ability of the testing to distinguish among and between cigarette brandsftypes. It was not designed to directly compare brands/types against each other since the prototypes were not chosen as intended replacements for the commercial cigarettes selected. All testing recommended in the first step was conducted, except for the pH test.

11. Experimental

Five cigarette brands/types were tested in this demonstration. Two commercial brands, "K" and "L", were selected from among those with the highest current sales in the U.S. The two prototypes selected, #530 and #531, showed low ignition-potential in preliminary data from the National Institute of Standards and Technology (NIST). A University of Kentucky standard reference medium tar / medium nicotine cigarette, #1R3F, was included in the testing. All cigarettes contained filters.

Except for the reference type, the cigarettes tested were randomly selected from among 300 (or 1.5 cartons) of each brand/type sent to each laboratory by NIST and CPSC staff. The average values were derived from machine smoking 100 cigarettes of each brand/type according to the Federal Trade Commission (FTC) protocol (Chapter B in the Toxicity Testing Plan).

Two contract laboratories performed the testing. The Tobacco Health and Research Institute at the University of Kentucky measured the standard FTC parameters of tar, nicotine, total particulate matter, and water. The number of puffs per cigarette was recorded. The weight of tobacco burned was estimated by weighing the amount of tobacco that would normally be burned during machine smoking in 50 cigarettes of each type. This provided sufficient data to compare results on per cigarette, per tobacco weight burned, and per tar weight bases. Per puff data are not shown, but can be calculated from the data in Tables 1-6.

The American Health Foundation conducted analyses of benzo(a)pyrene and four tobacco-specific nitrosamines, N'-nitrosonornicotine (NNN),

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosoanabasine (NAB), and N'-nitrosoanatabine (NAT), as described in Chapter D of the Plan. The Foundation also ran the Salmonella mutagenicity (Ames) assay, as described in Chapter E. Data from the Tobacco Health and Research Institute were used to express the results on per tobacco weight burned and per tar weight bases.

111. Results

Tables 1 and 2 summarize the results of testing the standard FTC parameters for cigarettes #530, #531, K, L, and the #1R3F reference. The words "soft packed cigarette", " total particulate matter" and "carbon monoxide" are abbreviated as "SPF", "TPM", and "co" respectively, in these tables. The averages are shown on a per cigarette basis in Table 1 and on a per tobacco weight burned in Table 2. The range of data for nicotine was 29-58 mg/g, "tar" 24-47 mg/g, and carbon monoxide 20-39 mg/g (Table 2). Tables 3 through 7 show the data according to run and port number, and also the estimated weight of tobacco burned for each brand/type tested.

Table 8 contains data for the levels of four tobacco-specific nitrosamines in the cigarettes. The range of data for NNN was 181-458 ng/g, NAT 210-421 ng/g, NAB 42-72 ng/g, and NNK 148-319. The range of the total tobacco-specific nitrosamine data was 582-1140 ng/g.

Results for benzopyrene levels could not generated in time for publication of this document due to laboratory technical difficulties. An addendum will contain this data.

Tables 9 through 12 summarize the mutagenicity as indicated by the number of revertant colonies in the Ames assay. A positive mutagenic effect is indicated when the response is greater than twice the spontaneous revertant rate and is denoted by an asterisk. Table 9 indicates the three tester strains are operating properly in the Ames test system.

All cigarette "tars" were mutagenic when the microsomal S-9 fraction was added (Tables 10-12). About 1.5 times more revertants occurred with the more mutagenic tars, in the presence of S-9, compared to the other tars.

IV. Discussion and Conclusions

The limited testing conducted in this demonstration can distinguish differences between and among cigarette brands/types. This indicates that the tests recommended in the Toxicity Testing Plan, at least in Tiers I and 11, can generate data that will be

useful in evaluating the potential changes in toxicity of low ignition-potential cigarettes.

Minor changes in the specifications of the Ames assay are recommended. The TA1535 strain of Salmonella gave negative results for all condensates tested in the demonstration study. However, the positive control for that strain, sodium azide, indicated that the TA1535 strain was functioning properly. Only a small response was obtained with TA1538. This suggests that these two strains may not provide useful data about differences in toxicity for the condensates. After consultation with the testing laboratories, it is recommended that the Toxicity Testing Plan use TA98 and TA1537 strains, unless it is suspected that non-frameshift type mutations might occur.

A dose-related response is typically seen in the Ames assay when testing tobacco smoke condensates. However, the lack of a dose-related response in Tables 10-12 suggests that the concentration range tested might be too high or that other toxicities, such as cell death, might be occurring. Data from the dose-related response range is more useful for comparing toxicity. Therefore, the initial test concentration should be adjusted to define the beginning and slope of this range.

The testing costs for this demonstration were within the $6,900 in direct costs per brand/type estimated in the Plan for the first step of implementation. CPSC staff contracted the testing for under $3,000 per brand/type, but the commercial rate could be somewhat higher. On the other hand, the economies of large volume testing that could be conducted by manufacturers can be expected to reduce the cost per brand/type. The estimates stated in the Plan appear to be reasonable, at least for Tiers I and 11.

TABLE 1

Summary of the Results of FTC Parameters
Average per Cigarette

CIGARETTE CODE	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
#530	28.00	1.93	3.67	22.40	18.7	8.26
sd	*1.62*	*0.06*	*0.54*	*1.50*	*0.9*	*0.32*
#531	16.97	1.27	1.84	13.86	12.1	*6.60*
sd	*1.06*	*0.06*	*0.45*	*0.65*	*1.4*	*0.29*
K	17.33	1.11	1.81	14.42	12.6	7.74
sd	*0.98*	*0.04*	*0.37*	*0.70*	*0.7*	*0.21*
L	19.80	1.50	1.68	16.61	12.7	8.97
sd	*0.63*	*0.05*	*0.27*	*0.45*	*0.6*	*0.33*
#1R3F	21.20	1.31	3.31	16.57	19.0	8.09
sd	*0.95*	*0.07*	*0.42*	*0.63*	*0.9*	*0.28*

TABLE 2

Summary of the Results of FTC Parameters
Average per Weight of Tobacco Burned

CIGARETTE CODE	TPM mg/g	NICOTINE mg/g	WATER mg/g	TAR mg/g	CO mg/g	PUFFS /g
#530	58.22	4.01	7.63	46.57	38.9	17.18
sd	*3.37*	*0.12*	*1.23*	*3.12*	*1.9*	*0.67*
#531	**35.35**	**2.64**	**3.84**	**28.86**	**25.2**	**13.75**
sd	***2.21***	***0.13***	***0.94***	***1.35***	***2.9***	***0.60***
K	**29.18**	**1.86**	**3.04**	**24.28**	**21.2**	**13.04**
sd	***1.65***	***0.07***	***0.62***	**1.18**	***1.2***	***0.35***
L	**31.19**	**2.37**	**2.65**	**26.16**	**20.0**	**14.13**
sd	***0.99***	**0.08**	**0.43**	***0.71***	***0.9***	***0.52***
#1R3F	**30.50**	**1.88**	**4.76**	**23.98**	**27.34**	**11.64**
sd	***1.37***	***0.10***	**0.60**	***0.91***	***1.29***	***0.40***

TABLE 3
Measurement of FTC Parameters
Cigarette #530

EXPERIMENTAL 92-30 FK 100'S
TIPPING LENGTH 32 MM
BUTT LENGTH 35 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	2	27.22	1.94	3.39	21.89	18.2	7.90
C06	19APR93	12	28.56	1.94	3.73	22.89	20.0	8.98
C06	19APR93	17	27.34	1.88	3.33	22.13	19.6	8.08
C07	19APR93	3	27.84	1.97	3.83	22.04	19.2	8.24
C07	19APR93	13	26.08	1.82	3.25	21.00	17.7	8.40
C07	19APR93	18	26.48	2.03	3.05	21.40	17.6	9.02
C08	20APR93	4	27.42	1.90	3.66	21.86	18.7	7.98
C08	20APR93	9	28.42	1.99	3.69	22.73	18.8	8.04
C08	20APR93	14	27.10	1.86	3.12	22.12	18.9	8.62
C08	20APR93	19	28.78	2.02	3.60	23.16	19.6	8.24
C09	20APR93	5	28.40	1.93	4.35	22.12	19.6	8.40
C09	20APR93	10	27.32	1.95	3.90	21.48	18.1	8.24
C09	20APR93	15	26.42	1.87	3.47	21.08	16.4	8.56
C09	20APR93	20	28.56	1.98	3.94	22.63	19.4	8.32
C10	21APR93	1	27.58	1.88	4.04	21.66	18.9	7.92
C10	21APR93	6	28.16	1.97	3.59	22.60	19.3	8.12
C10	21APR93	11	30.46	1.96	4.87	23.64	19.7	8.14
C10	21APR93	16	32.80	1.91	2.95	27.94	18.4	8.00
C11	21APR93	4	29.50	2.01	4.70	22.79	18.5	8.06
C11	21APR93	7	25.60	1.81	2.92	20.86	17.6	8.00
	AVERAGE		28.00	1.93	3.67	22.40	18.7	8.26
	STD DEV		1.62	0.06	0.54	1.50	0.9	0.32
AVG/GM	TOB BURNED 0.481		58.22	4.01	7.63	46.57	38.9	17.18

TABLE 4
Measurements of FTC Parameters
Cigarette #531

EXPERIMENTAL 92-31 FK 100'S
TIPPING LENGTH 32 MM
BUTT LENGTH 35 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	3	16.24	1.21	1.72	13.31	11.0	6.22
C06	19APR93	8	17.44	1.25	2.01	14.18	12.3	6.52
C06	19APR93	13	16.04	1.25	1.45	13.34	10.5	6.26
C06	19APR93	18	15.46	1.26	1.16	13.03	16.6	6.96
C07	19APR93	4	17.56	1.33	2.01	14.21	12.2	7.02
C07	19APR93	9	16.12	1.22	1.43	13.47	10.7	6.88
C07	19APR93	14	15.64	1.20	1.41	13.03	11.0	6.62
C07	19APR93	19	15.66	1.23	1.60	12.83	10.0	6.38
C08	20APR93	5	15.92	1.18	1.50	13.24	11.3	6.46
C08	20APR93	10	18.08	1.32	2.20	14.56	12.4	6.54
C08	20APR93	15	17.94	1.32	2.14	14.48	12.0	6.92
C08	20APR93	20	16.50	1.21	1.42	13.88	12.2	6.46
C09	20APR93	1	17.22	1.24	2.07	13.91	12.3	6.30
C09	20APR93	6	17.20	1.28	1.69	14.23	12.1	6.96
C09	20APR93	11	16.46	1.19	1.80	13.47	11.3	6.46
C09	20APR93	16	17.08	1.26	2.06	13.76	11.3	6.88
C10	21APR93	2	18.72	1.30	3.12	14.30	12.0	6.34
C10	21APR93	7	18.30	1.32	2.19	14.79	13.4	7.00
C10	21APR93	12	19.38	1.41	2.52	15.45	13.2	7.02
C10	21APR93	17	16.70	1.26	1.52	13.92	12.3	6.50
C11	21APR93	3	16.40	1.27	1.72	13.40	14.4	6.20
C11	21APR93	12	17.20	1.34	1.83	14.02	11.9	6.26
	AVERAGE		16.97	1.27	1.84	13.86	12.1	6.60
	STD DEV		1.06	0.06	0.45	0.65	1.4	0.29
AVG/GM TOB BURNED 0.480			35.35	2.64	3.84	28.86	25.2	13.75

TABLE 5
Measurement of FTC Parameters
Cigarette K

80MM HPF
TIPPING LENGTH 24 MM
BUTT LENGTH 27 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	4	18.00	1.24	1.75	15.01	13.0	8.32
C06	19APR93	9	17.36	1.15	1.55	14.66	12.2	7.94
C06	19APR93	14	17.48	1.11	1.62	14.75	13.4	7.82
C06	19APR93	19	17.96	1.15	1.85	14.96	12.5	7.92
C07	19APR93	5	17.60	1.14	1.99	14.47	12.6	7.74
C07	19APR93	10	17.40	1.14	1.77	14.49	11.9	7.72
C07	19APR93	15	15.66	1.06	1.65	12.95	10.8	8.28
C07	19APR93	20	15.48	1.06	1.42	13.00	12.2	7.68
C08	20APR93	1	17.02	1.07	1.60	14.35	12.7	7.60
C08	20APR93	6	16.66	1.06	1.52	14.08	12.4	7.64
C08	20APR93	11	17.20	1.09	1.64	14.46	12.2	7.50
C08	20APR93	16	17.58	1.06	1.87	14.65	12.6	7.72
C09	20APR93	2	17.90	1.08	2.47	14.35	13.3	7.52
C09	20APR93	7	16.30	1.04	1.54	13.72	12.6	7.32
C09	20APR93	12	18.28	1.12	2.25	14.91	13.9	7.88
C09	20APR93	17	17.36	1.09	1.89	14.38	13.3	7.72
C10	21APR93	3	17.96	1.13	2.12	14.71	12.8	7.64
C10	21APR93	8	18.38	1.17	1.97	15.24	13.3	7.74
C10	21APR93	13	19.32	1.12	2.75	15.45	12.9	7.50
C10	21APR93	18	17.68	1.12	1.52	15.04	12.1	7.92
C11	21APR93	2	15.42	1.04	1.19	13.19	11.9	7.50
	AVERAGE		17.33	1.11	1.81	14.42	12.6	7.74
	STD DEV		0.98	0.04	0.37	0.70	0.7	0.21
AVG/GM	TOB BURNED 0.594		29.18	1.86	3.04	24.28	21.2	13.04

TABLE 6
Measurement of FTC Parameters
Cigarette L

85MM SPF
TIPPING LENGTH 25 MM
BUTT LENGTH 28 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	5	19.10	1.42	1.57	16.11	12.3	9.28
C06	19APR93	10	20.32	1.59	1.83	16.90	12.4	9.32
C06	19APR93	15	19.02	1.55	1.39	16.08	12.1	9.46
C06	19APR93	20	19.12	1.53	1.42	16.16	13.6	9.00
C07	19APR93	1	19.68	1.55	1.76	16.37	12.2	8.80
C07	19APR93	6	20.34	1.46	1.97	16.91	13.6	8.74
C07	19APR93	11	20.96	1.56	2.11	17.29	12.9	9.42
C07	19APR93	16	20.52	1.55	2.14	16.82	12.9	9.08
C08	20APR93	2	18.76	1.42	1.67	15.67	11.6	8.68
C08	20APR93	7	19.52	1.45	1.68	16.39	12.6	9.18
C08	20APR93	12	20.26	1.55	1.46	17.25	13.0	9.16
C08	20APR93	17	20.32	1.50	1.62	17.21	13.4	8.86
C09	20APR93	3	19.74	1.45	1.80	16.49	12.7	8.62
C09	20APR93	8	20.10	1.48	1.75	16.86	13.4	9.12
C09	20APR93	13	20.60	1.54	2.08	16.97	13.1	8.96
C09	20APR93	18	20.58	1.57	1.73	17.28	12.4	9.36
C10	21APR93	4	19.36	1.43	1.60	16.33	12.9	8.18
C10	21APR93	9	19.28	1.47	1.63	16.18	11.5	8.92
C10	21APR93	14	19.20	1.50	1.20	16.49	12.5	8.76
C10	21APR93	19	19.40	1.45	1.18	16.77	13.2	8.46
C11	21APR93	8	19.68	1.52	1.78	16.38	12.9	9.06
	AVERAGE		19.80	1.50	1.68	16.61	12.7	8.97
	STD DEV		0.63	0.05	0.27	0.45	0.6	0.33
AVG/GM	**TOB BURNED**		31.19	2.37	2.65	26.16	20.0	14.13
	0.635							

TABLE 7
Measurement of FTC Parameters
Cigarette #1R3F

CODE X 1R3F Reference Cigarette 85MM SPF
TIPPING LENGTH 25
BUTT LENGTH 28

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	1	21.26	1.27	3.51	16.48	19.5	7.86
C06	19APR93	6	22.02	1.31	3.30	17.41	19.1	8.16
C06	19APR93	11	22.86	1.41	3.77	17.68	19.9	8.58
C06	19APR93	16	22.70	1.47	3.50	17.73	19.2	8.04
C07	19APR93	2	20.46	1.40	3.15	15.92	16.7	8.08
C07	19APR93	7	20.72	1.34	3.08	16.29	18.7	7.82
C07	19APR93	12	21.34	1.35	3.51	16.48	18.3	8.32
C07	19APR93	17	21.68	1.39	3.71	16.58	18.7	8.56
C08	20APR93	3	20.14	1.25	2.75	16.13	18.2	8.10
C08	20APR93	8	20.00	1.25	3.12	15.63	17.2	7.62
C08	20APR93	13	21.38	1.34	3.25	16.79	19.8	8.34
C08	20APR93	18	19.78	1.17	2.78	15.83	17.7	8.20
C09	20APR93	4	21.78	1.33	3.81	16.64	19.2	7.88
C09	20APR93	9	20.70	1.25	3.35	16.10	18.8	7.86
C09	20APR93	14	21.20	1.22	3.35	16.63	20.2	8.20
C09	20APR93	19	22.02	1.29	3.47	17.26	20.5	7.98
C10	21APR93	5	22.74	1.32	4.43	16.99	19.8	8.18
C10	21APR93	10	22.14	1.32	3.46	17.36	19.6	7.74
C10	21APR93	15	21.06	1.20	3.45	16.42	19.5	8.56
C10	21APR93	20	20.84	1.33	2.73	16.78	20.0	8.10
C11	21APR93	1	21.04	1.30	3.27	16.47	19.3	7.92
C11	21APR93	6	20.00	1.30	2.53	16.18	18.6	8.28
C11	21APR93	11	19.68	1.41	2.89	15.38	18.7	7.64
	AVERAGE		21.20	1.31	3.31	16.57	19.0	8.09
	ST DEV		0.95	0.07	0.42	0.63	0.9	0.28
AVG/GM	TOB BURNED 0.695		30.50	1.88	4.76	23.98	27.34	11.64

TABLE 8
Tobacco-specific N-Nitrosamines in Test Cigarettes

brand	NNN	NAT	NAB	NNK	Total
			(ng/cig)		
KY 1R3F, 85 mm	193	210	29.3	222	654
L, 85 mm	287	216	26.5	194	724
K, 80 mm	272	250	25.9	173	721
#530, 100 mm	134	165	34.7	102	436
#531, 100 mm	86.7	101	20.5	70.9	279
			(ng/g tobacco)		
KY 1R3F	278	302	42.2	319	941
L	452	340	41.7	306	1140
K	458	421	43.6	184	1107
#530	279	343	72.1	212	841
#531	181	210	42.7	148	582
			(ng/mg tar)		
KY 1R3F	8.05	8.76	1.22	9.26	27.3
L	17.3	13.0	1.60	11.7	43.6
K	18.9	17.3	1.80	12.0	50.0
#530	5.98	7.36	1.55	4.55	19.4
#531	6.26	7.29	1.48	5.12	20.2

Ames Salmonella/Microsome Mutagenicity Test
Mean Summary Data

Sponsor: Brunnemann Date Initiated: 6/4/93
Study number: LRD57 Date Scored: 6/7/93
Test Articles: KY 1R3F, K, L, 92-30 (#530), 92-31 (#531)
Description: 5 tobacco smoke condensates; 4 doses each
Considerations: TA98, TA1535, TA1538; +/- Rat S9

TABLE 9
Controls

			Spontaneous Revertant Colonies/Plate (avg)		
Solvent Controls		S-9	TA98	TA1535	TA1538
DMSO		(-)	22	21	15
DMSO		(+)	34	14	23
Positive Controls (µg/pl)					
sodium azide	5	(-)	-	*1000*	-
2-Nitrofluorene	*5*	(-)	382	-	115
2-Anthramine	5	(+)	1964	114	1874

TABLE 10
Test Compound: XY 1R3F

	Total Revertant Colonies/Plate (avg)			
Dose Level (µg/pl)	S-9	TA98	TA1535	TA1538
50	(-)	25	19	18
100	(-)	41	15	15
200	(-)	52*	21	20
300	(-)	57*	18	22
100	(+)	169*	11	73*
200	(+)	195*	11	69*
300	(+)	157*	12	73*
400	(+)	172*	13	56*

* Positive response (threshold=2.000 x corresponding solvent).

TABLE 11

Test Compound: K

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	29	16	9
100	(-)	37	14	14
200	(-)	67*	20	26
300	(-)	ai*	15	26
100	(+)	194*	14	76*
200	(+)	162*	12	72*
300	(+)	172*	14	77*
400	(+)	167*	15	71*

Test Compound: L

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	36	19	17
100	(-)	40	18	18
200	(-)	47*	20	22
300	(-)	42	16	23
100	(+)	150*	16	71*
200	(+)	156*	14	61*
300	(+)	142*	18	5a*
400	(+)	143*	18	59*

* Positive response (threshold=2.000 X corresponding solvent).

TABLE 12

Test Compound: 92-30 (#530)

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	34	22	13
100	(-)	32	24	16
200	(-)	37	24	15
300	(-)	46*	17	16
100	(+)	83*	13	53*
200	(+)	102*	20	46
300	(+)	95*	14	54*
400	(+)	94*	11	53*

Test Compound: 92-31 (#531)

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	27	15	16
100	(-)	32	19	14
200	(-)	36	20	18
300	(-)	44	19	22
100	(+)	112*	13	43
200	(+)	129*	27	53*
300	(+)	104*	15	51*
400	(+)	85*	18	48*

* Positive response (threshold=2.000 X corresponding solvent).