

National Cancer Institute
Smoking and Health Program

Report No. 5

Toward Less
Hazardous
Cigarettes

Summary:
Four Skin
Painting Bioassays
Using Condensate
from Experimental
Cigarettes

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September 1980

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SUMMARY REPORT ON THE FOUR SKIN
PAINTING BIOASSAYS USING CONDENSATE
FROM EXPERIMENTAL CIGARETTES

Smoking and Health Program
Division of Cancer Cause and Prevention
National Cancer Institute
Bethesda, Maryland

SUMMARY: TOWARDS LESS HAZARDOUS CIGARETTES

Four Skin Painting Bioassays Using Condensate from Experimental Cigarettes

1. Introduction

Since the 1964 Report to the Surgeon General on the hazards of smoking, the National Clearinghouse for Smoking and Health, the American Cancer Society, and other public health oriented organizations have expanded their efforts to reduce the degree of cigarette smoking nationwide. However, these attempts have been only partially successful. Today, between 50 and 60 million Americans smoke cigarettes.

The National Cancer Institute (NCI), in coordination with the National Heart, Lung and Blood Institute (NHLBI) and the Department of Agriculture, established the Smoking and Health Program to provide guidelines for the reduction of the risks of cigarette smoking. The program is advised by consultants representing a wide spectrum of disciplines.

A systematic approach has been taken toward the development of less hazardous cigarettes, one of the principal objectives of the Smoking and Health Program. The first phase of this Program involved the design of a variety of experimental cigarettes and the chemical and biological analyses of their condensate and smoke.

The primary objective of these cigarette experiments is to determine the tumorigenic activity of condensate from each experimental cigarette when equal weights of dry smoke condensate (as contrasted to equal numbers of cigarettes or equal numbers of puffs) are applied to mouse skin. The components of the tobaccos, the cigarette smoke condensates and whole smokes, and the physical characteristics of the cigarettes provide extensive laboratory data. Analyses of these data include correlations with the mouse-skin tumor bioassay data for insights into which smoke components are associated with adverse health effects. The analyses provide an evaluation of the relative toxicity of the experimental cigarettes and serve as the basis for the design of advanced cigarette experiments. The ultimate objective for these experiments is to identify the characteristics of less hazardous cigarettes that will serve as guidelines for future commercial cigarettes. Success is hindered by the uncertain relationship between tumors resulting from mouse skin painted with condensate and human lung cancer and by the virtual absence of information on the cardiovascular and respiratory effects of these cigarettes (beyond the permissible inferences from their physical and chemical characteristics). Therefore, the skin painting bioassays are viewed as screening experiments. It is assumed that reduction of mouse dermal tumorigenic response from smoke condensate is a valid indicator of viable lines of investigation that are worth pursuing through more sophisticated (and more costly) tests, such as direct inhalation of whole smoke in suitable animal models. Thus, the experiments are considered initial steps in the process of improving cigarette characteristics.

Separate reports on each of the four skin painting experiments were published by the U.S. Department of Health, Education and Welfare in March, 1974, June, 1975, June, 1977, and March, 1980. This present report is a summary of the major findings from the experiments. The reader is referred to the individual comprehensive reports on each experiment for detailed presentations of the protocol, chemical analyses, statistical analyses and interpretation of results.

2. Materials and Methods

This section summarizes the materials and methods used in the skin painting experiments. Only slight variations occurred among experiments, these being primarily differences in which condensate and smoke chemicals were measured. Specific details for each experiment are contained in their respective reports.

2.1 The Cigarettes. During each experiment, the cigarettes were distributed by code to the participating laboratories using a random blind code. Unless noted otherwise, all cigarettes were made according to the following specifications:

Filter: None
Length: 85 mm
Circumference: 25 mm
Weight: Varied with firmness characteristics of the tobaccos
Draw Resistance: Varied with each tobacco
Paper: Schweitzer 556 except variable No. 40 with Schweitzer "special porous" paper
Print: Ring 23 mm from end of cigarette and cigarette code number
Packaging: 3800 cigarettes in each cardboard "filter tray," sealed in polyethylene bags and stored at -20° C

There was a separate Standard Experimental Blend (SEB) cigarette prepared for each experiment, all having identical compositions. They differed only in the manufacturer and crop year. The blend of the SEB cigarettes was (by weight):

Glycerol	2.8%
Invert sugar	5.3%
Flue-cured	32.5%
Burley	20.0%
Maryland	1.1%
Turkish	11.1%
Reconstituted sheet ¹	27.2%
	100.0%

¹ Stems and fines in a slurry process.

2.2 Tobacco Analyses. These analyses were conducted at several laboratories, coordinated by the Tobacco Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture. The analyses include:

Sand	Chlorogenic acid
Moisture	Rutin
Water	Total phenols
pH	Total polyphenols
Ash	Total alkaloids
Sodium Hydroxide (NaOH)	Total volatile bases (TVB)
Hydrochloric acid (HCl)	TVB-nicotine
Potassium (K)	Ratio nicotine/TVB
Sodium (Na)	Ammonia (NH ₃)
Calcium (Ca)	Nitrate-Nitrogen (NO ₃ -N)
Magnesium (Mg)	Amino-nitrogen
Manganese (Mn)	α Amino-nitrogen
Chlorine (Cl)	Nitrate (NO ₃)
Reducing sugars	Total nitrogen
Total sugar	Glycerine
Starch	Over volatiles
Cellulose	Wax
Malic acid	Phyto-sterols
Citric acid	Petroleum ether extract (PEE)
Oxalic acid	Nicotine

2.3 Condensate Preparation. The cigarettes were stored in a freezer. When needed, they were removed and conditioned at 24.0 ± 1.0 C and $60\% \pm 5\%$ relative humidity for not less than 48 hours prior to smoking.

The condensates were prepared by Meloy Laboratories. The cigarettes were smoked on machines built by Process and Instruments Corporation, according to the following specifications:

Operation	Direct smoking (negative pressure puffing)
Capacity	Approximately 2,000 cigarettes per hour
Puffs	1/min, 35 ml, 2-sec. duration; no more than 10 puffs per cigarette; ejected earlier if smoked to butt
Ambient Air Conditions	Room air 24.0 ± 1.0 C, $60\% \pm 5\%$ relative humidity; exhaust designed to avoid drafts that could influence the burning rates of cigarettes

Condensate collection. The condensate was collected in four traps at 7.00 C; the first two traps used 4-mm Pyrex beads, and the second two used Teflon filaments.

Extraction. The condensate was extracted with freshly distilled acetone. The condensate was concentrated under reduced pressure at 40° C until less than 6% water remained. Weighed amounts of acetone and water were added, aliquots taken, and the water and nicotine contents were analyzed by gas chromatography. The mixture was adjusted so that the final product contained 250 or 500 mg of dry condensate per ml.

Storage. The condensate was stored at -20° C until sent to the bioassay laboratories and was packed in dry ice for transfer to users.

Production Schedule. Condensate preparation schedules were arranged so that all condensate samples were less than two months old when used for mouse skin painting.

Quality Control. As a part of quality control, samples from each batch of experimental cigarettes were used to determine: average cigarette weight and pressure drop; static burn rate in draft-free air; combustion zone temperature at 2 butt lengths; and amount of potassium (K), sodium (Na), magnesium (Mg), ash, hexane solubles, nitrate, calcium (Ca), nicotine, total reducing sugar, neophytadiene; and the pH of material collected in the filter.

Process Monitoring. Measurements were made of the mean butt length after smoking, total dry condensate yield and dry condensate yield per cigarette, pH of the condensate, and percent nicotine in the condensate and per cigarette.

2.4. Smoke and Condensate Analyses. The smoke and smoke condensate from the various cigarettes were tested by Oak Ridge National Laboratory. During the course of the skin painting experiment, condensate was sent to Oak Ridge National Laboratory three times at approximately 6-month intervals. Each shipment was analyzed once within that 6-month period, with quadruplicate determinations for each sample analyzed. The following analyses were conducted on whole smoke, gas phase, and particulate matter:

Cigarette Characteristics

Weight
Resistance to draw
Puff number

Condensate

Nicotine
Weak acids
Very weak acids
Total weak acids
Alkalinity (pH)
Oleic + linoleic + linoleic acid
Palmitic acid
Stearic acid
Neophytadiene
Catechol
Indole
Skatole
Benz [a] anthracene
Benz [a] pyrene
Total free fatty acids

Cigarette Smoke

Total particulate matter
Tar
Water
Nicotine
Acetaldehyde
Acrolein
Isoprene
Hydrogen cyanide (HCN)
Formaldehyde
Nitrogen oxides (NO_x as NO₂)
Carbon monoxide (CO)
Carbon dioxide (CO₂)
TPM phenolics

Values for smoke components analyses were expressed in five ways: per cigarette, per puff, per liter of smoke, per gram of tobacco, and relative to total particulate matter. Values for condensate components were recorded on a weight-to-weight basis.

In addition to the above determinations, several special analyses were performed on selected condensate batches. These include glycerol, colorimetric phenolics, nicotine alkaloids, phenol + cresols, o-cresol, m- + p-cresols, and phenol.

2.5 Skin Painting Bioassays. The skin painting bioassay was conducted at Hazleton Laboratories. Each condensate was tested at two or three dose levels on groups of 100 mice each, the daily application being 0.10 ml of a condensate suspension containing 12.5 mg, 25 mg, or 50 mg of dry smoke condensate.

Three control groups were used: mice with dorsal hair clipped but no skin painting (sham); mice painted with acetone only to test the effect of vehicle without condensate; and mice painted with benzo[a]pyrene in acetone at three dose levels (positive control), to test the response of the system to a known carcinogen.

Mice. ICR Swiss female mice were randomized five to a cage; cage occupancy was maintained but cage positions were changed weekly.

Painting. Dorsal hair was clipped weekly, while the condensate was applied daily (Monday through Saturday). The volume was measured by syringe and spread uniformly over the test site with a glass rod. Condensate solutions were thoroughly shaken (by machine) prior to each application. Painting was continued for the duration of the experiment (18 months).

Observations. Routine observations of the mice were made daily by laboratory technicians. If a suspected tumor was observed on any animal for 3 consecutive weeks, it was recorded as a "visually observed tumor." Data entered once a month into computer storage included (where applicable): date of the first visually observed tumor, description of tumor (wart-like or gross carcinoma), number of tumors, weight of the animal, and date of death.

Necropsy. All mice dying during the experiments, sacrificed if moribund, or sacrificed on termination of the experiment at 18 months were necropsied and their tissues were fixed in formalin. The target tissue of those mice visually observed to have tumors or suspected of having tumors at necropsy was histopathologically examined. The statistical analysis was based on histopathologically verified tumors.

Survival probabilities. Actuarial methods were used to estimate the probability (Pf) that an animal within a given group would not develop a tumor if the animal were to survive the 18 months of the experiment. Adjustments were made for those animals that died during the experiment without developing a tumor. In addition, estimates were calculated of the latent periods (number of days since the initiation of the experiment) to 75%, 50%, and 25% survival (T75, T50, T25).

3. Histopathological Confirmation

As stated previously, statistical analyses of tumor incidence among the experimental animals were based on histopathologically confirmed tumors. At the conclusion of the first skin painting experiment, a set of 84 slides from the mice painted with SEB condensate was sent to three independent pathologists for separate readings. The purpose was to estimate the extent of consistency among independent reviews of the slides, with reference to the findings of the Hazleton pathologist.

Although the four reports of histopathology were expressed in different format and in somewhat different terminology, it was possible to test the degree of concurrence among them concerning the presence or absence of malignancy. For one slide, Hazleton reported negative and the other three pathologists reported positive. For another slide, Hazleton reported positive and the other three pathologists reported negative. There were four slides on which the consensus was evenly split (i.e., two positives and two negatives). Discounting these latter four slides, Hazleton was in agreement with the consensus of other pathologists in approximately 98% (78/80) of the slides examined. A summary follows.

All four positive	39
All four negative	33
<hr/>	
Subtotal	72 = 86%
3 positive, 1 negative	4
3 negative, 1 positive	4
2 positive, 2 negative	4
<hr/>	
Subtotal	12 = 14%
TOTAL	84

This high level of consistency between the Hazleton results and those of the three independent pathologists suggested that the histopathological data were sufficiently reliable for analytical purposes.

4. Factors Analyzed in the Experiments

A complete list of all experimental cigarettes tested during these experiments is given in Table 1. The purpose in choosing specific cigarettes was to test selected factors for relative levels of tumorigenicity. The major factors of interest, by experiments, are listed below.

<u>Skin Painting Experiment</u>	<u>Major Factors for Analysis</u>	<u>Types of Cigarettes Tested</u>
I	Laminae/Stems	<ul style="list-style-type: none"> • SEB I, laminae only • flue-cured, laminae only • Burley, laminae only • SEB I, stems only, rolled and cut
	RTS Paper Process	<ul style="list-style-type: none"> • stems only • medium density • additives, low density • additives, medium density • additives, high density
	RTS Slurry Process	<ul style="list-style-type: none"> • stems and fines only • medium density • additives, low density • additives, medium density • additives, high density

<u>Skin Painting Experiment</u>	<u>Major Factors for Analysis</u>	<u>Types of Cigarettes Tested</u>
I (Cont.)	Other	<ul style="list-style-type: none"> • citrate paper • phosphate paper • potassium nitrate (2 x SEB I) • coarse cut tobacco (20 cuts/inch) • fine cut tobacco (60 cuts/inch) • combinations of the above
II	Nicotine/Fertilizer Application	<ul style="list-style-type: none"> • Burley, normal/normal • Burley, low/normal • Burley, low/high • Flue-cured, normal/normal • Flue-cured, low/normal • Flue-cured, low/high • Flue-cured/Burley (3:1) normal/normal low/normal low/high
	Suckering	<ul style="list-style-type: none"> • Fatty alcohol, normal • Fatty alcohol, X100 • Hand suckered
	Tobacco Processing	<ul style="list-style-type: none"> • Reynolds puffed • Philip Morris expanded • Freeze-dried
	Artificial Tobacco Substitutes (ATS)	<ul style="list-style-type: none"> • ATS-A • ATS-A/SEB II, 50/50 • ATS-B • ATS-B/SEB II, 50/50
III	Paper Porosity	<ul style="list-style-type: none"> • Low (5 cm/min) • Standard (30 cm/min) • High (60 cm/min) • Very High (100 cm/min)
	Filters	<ul style="list-style-type: none"> • Cellulose Acetate • Dilution • Permanganate
	Additives	<ul style="list-style-type: none"> • Sugar • Humectant • Cocoa (powdered) • Other (magnesium nitrate, zinc oxide)

<u>Skin Painting Experiment</u>	<u>Major Factors for Analysis</u>	<u>Types of Cigarettes Tested</u>
III (Cont.)	Artificial Tobacco Substitutes	<ul style="list-style-type: none"> • ATS-A/SES III, 30/70 • ATS-B • ATS-B/SES III, 50/50
IV	Reconstituted Tobacco Sheet (RTS)	<ul style="list-style-type: none"> • Paper vs. slurry process • Steam vs. water extraction • Hexane/ethanol vs. water/mechanical extraction • Inorganic fillers • Additives • Burley vs. Bright leaf
	Nicotine	<ul style="list-style-type: none"> • 0.0, 0.5, 1.0, 1.5 mg/cig
	Expanded stems	<ul style="list-style-type: none"> • Expanded stems, 100% • Expanded stems/SES IV, 50/50
	Artificial Tobacco Substitutes	<ul style="list-style-type: none"> • ATS-B • Ecusta/SES IV, 30/70
	Pesticide-Treated Plants	<ul style="list-style-type: none"> • Pesticide-free • Pesticide-treated
	PMC	<ul style="list-style-type: none"> • 3-phenyl, 5-methyl, 1-2-3-oxdiazole (chemical analyses only; no skin painting)

5. Consistency of Experimental Results

As mentioned previously, the University of Kentucky (IR1) and the Standard Experimental Blend (SEB) were used as common reference cigarettes throughout the four skin painting experiments. Chemical analyses and biological responses from these cigarettes were used to measure the consistency of protocol from one experiment to the other.

Tables 2 and 3 summarize the chemical analyses for the IR1 cigarettes, for the smoke and the smoke condensate, respectively. The results indicate a high level of consistency in the deliveries of all constituents. For the condensate analysis, stearic acid and indole produced the greatest variations from the Series I-IV averages. The BaA, BAP, and skatole levels were, however, much closer to the running averages than those of the Series III condensates. The cresols were not protocol constituents, but were measured in one batch of condensates. Hence, batch-to-batch contributions to the variability of

this constituent were not averaged, and the reported data differ from previous measurements. Further analyses would be required to determine if these differences are real.

Tables 4 and 5 summarize similar results of the chemical analyses for the SEB cigarettes. Re-analysis of the SEB-I cigarette throughout the experiments suggested little effect from aging in cold storage, with the exception of the oxides of nitrogen (NO_x) content of the gas phase. Over the course of the four experiments, NO_x delivery of the SEB-I appeared to increase by about 20%. To determine if this is a definite trend, or if this is normal $\pm 10\%$ variation about a mean value, requires additional analysis. No regeneration of the Series I condensate was performed in Series IV.

There are relatively small differences in the average smoke and condensate constituent concentrations of the SEB's manufactured for Series II, III, and IV, suggesting the difficulty of exactly reproducing the SEB blend and generating the condensate from year to year. However, the variations are minor and probably represent the best results achievable with current technology.

Biological response data for the reference cigarettes are summarized in Table 6 in terms of the final probability of survival (P_f) and associated standard errors, by experiment and dose level.

As a test of consistency among experiments, the P_f values were compared pairwise within common dose levels for the IRI cigarettes, and separately for the SEB cigarettes. The P_f values themselves are maximum likelihood estimates and, as such, are asymptotically normally distributed. On the basis of this latter property, in conjunction with the large animal group sample sizes used in these bioassays, the normal deviate test was used for the comparisons. A list of the pairwise comparisons performed is given in Table 7, with statistically significant differences noted if such differences are significant at the 5% (or higher) level.

As seen from this table, no statistically significant differences were found in comparisons between the IRI cigarettes. However, six statistically significant differences were found when comparing the SEB cigarettes--three at the 5% and three at the 1% level of statistical significance. It is to be noted that the three tests resulting in differences at the 1% level all involved the SEB I cigarette from the Series II bioassay. This same cigarette was also involved in one of the tests that resulted in a difference at the 5% level of significance. The SEB I cigarette from the Series I bioassay was involved in all three tests that resulted in differences significant at the 5% level.

SEB I from Series I had an unusually high P_f value and SEB I from Series II had an unusually low P_f value. In comparing all SEB P_f values simultaneously for each given dose level, it is the SEB I cigarette from Series II that emerges as the potential outlier.

Of the 19 SEB tests not involving SEB I from Series II, only two result in statistically significant differences, and both of these are at the 5% level. This is not incompatible with tests of significance (there is a 25% chance that two or more of the 19 tests would be significant, solely by chance). If all 27 tests among the common reference cigarettes are considered (exclusive of SEB I from Series II), the chances of two or more tests being significant increase to 40%.

The tests considered here imply that one should be cautious in making use of SEB I to draw inferences from the skin painting experiments; however, the overall test results are compatible with consistency among the four experiments.

6. General Summary of Results

Detailed analyses of the data and presentations of the results are included in the separate comprehensive reports of each experiment. Table 8 summarizes the results for the major factors of interest in terms of tumorigenicity relative to SEB.

Several significant findings were obtained from the first experiment. Cigarettes made with high-porosity paper, those made of tobacco stems only, and those made with reconstituted sheets all provided condensates found to be less tumorigenic to mouse skin than SEB I. Neither the width of tobacco cuts nor the doubling of nitrates content to SEB I appeared to affect the condensate tumorigenicity, but cigarettes made of tobacco laminae only were so toxic to the mouse that the skin painting with their condensate had to be discontinued.

For Series II, the low nicotine/normal fertilizer and low nicotine/high fertilizer blends showed significantly lower tumorigenicity than the normal nicotine/normal fertilizer blends. There were no significant differences, however, between the low nicotine/normal fertilizer and low nicotine/high fertilizer blends.

Condensates of the Reynolds puffed, Philip Morris expanded, and freeze-dried SEB II blends showed no statistically significant differences among themselves, but the expanded and freeze-dried SEB II blends showed significantly lower condensate tumorigenicity than SEB II.

One of the two artificial tobacco substitutes (ATS) had the lowest condensate tumorigenicity of all blends tested; the other ATS had the highest. Blends of these non-tobacco materials combined 50/50 with SEB II, however, were not significantly different from SEB II itself. Condensates from the ATS materials were not as homogenous as tobacco condensates and appeared to differ in physical properties. Further testing of the ATS materials was done as part of the fourth cigarette experiment.

The fatty alcohol, fatty alcohol x 100, and hand-suckered blends showed no significant differences among themselves or from the SEB II blend.

The results of the correlation analyses of several constituents of the tobacco, leaf, and condensates of the second cigarette experiment complement those of the first experiment. The concentrations of both nicotine and tar, as constituents of the condensate, were highly correlated with the incidence of tumorigenic activity on mouse skin painted with the condensates. Static burn rate was negatively correlated with tumorigenic activity. Since static burn rate can affect the chemical composition of the smoke, a fast burning rate may be a factor in developing less hazardous cigarettes. Other compounds that were negatively correlated with tumorigenicity in both experiments were acetaldehyde, formaldehyde, NO_x , CO , and acrolein. Total phenolics in the leaf, moisture content of the cigarette filter, and benz[a]anthracene in the condensate were positively correlated with tumorigenicity.

Comparisons among the tobacco additive variables for Series III indicate that magnesium nitrate reduces the tumorigenicity of cigarette condensate.

When the additives sugar, humectant, and cocoa are compared, neither sugar nor humectant seems to affect the tumorigenicity of the tobacco smoke at lower (12.5 mg) dose levels but may contribute to tumorigenicity at higher dose levels (25 mg). Powdered cocoa appears to increase the tumorigenicity of the smoke at both dose levels.

The air dilution filter proved to be effective in reducing the tumorigenicity of the cigarette condensate applied on an equivalent weight basis. Neither the permanganate filter nor the cellulose acetate filter reduced the tumorigenicity of the condensate.

There were no statistically significant differences in tumorigenicity among the paper porosity variables or between these variables and SEB III.

Of the two artificial tobacco substitutes (denoted by ATS-A and ATS-B) included in this experiment, the ATS-A cigarette fared well with respect to lower tumorigenicity compared to SEB III, whereas the ATS-B cigarette fared poorly. Experimental difficulties arose with ATS-B regarding the choice of solvent used in the second and third experiments. This cigarette was re-tested during the fourth experiment using a different solvent.

Series IV was the last of the skin painting experiments. Overall, the Bright tobacco produced condensates slightly less tumorigenic than the Burley tobacco, based on results from the mouse skin painting. However, the Bright and Burley variates were comparable with respect to selected chemical yields.

One of the paper process cigarettes (10% cellulose fiber additive, nicotine added back in the form of nicotine citrate) had the lowest measure of survival for all variates tested at two dose levels. Otherwise, both the

paper and slurry processed tobacco had survival measures either comparable to or significantly higher than SEB.

No significant differences were observed between cigarettes made from pesticide-treated tobacco leaves and pesticide-free tobacco leaves.

Based on both skin painting bioassays and yields of selected chemicals measured in the condensates, cigarettes made from expanded stems were significantly less tumorigenic than SEB.

A positive correlation was observed between nicotine content of condensate and biological response. Because of variations in condensate yields, it is not clear from these experiments if the correlation suggests a causal relationship.

Results from this fourth experiment support the finding from the third experiment that, in the design of less tumorigenic cigarettes, it may not be necessary to go beyond a paper porosity of approximately 60 cm/min. However, as pointed out in the report on the third experiment, toxic gas phase constituents can be reduced further through the use of more porous paper.

There was a slight improvement in the measure of survival for the ATS-B cigarettes when a modified solvent was used. However, there is still no evidence that the ATS-B cigarettes produce condensate that is less tumorigenic than SEB.

The reader is referred to the separate reports on each of the four skin painting experiments for detailed presentations of the protocol, chemical analyses, statistical analyses, and interpretations of results. These reports are available through the Smoking and Health Program, National Cancer Institute, and entitled:

- Report No. 1, Towards Less Hazardous Cigarettes. The First Set of Experimental Cigarettes. DHEW Publication No. (NIH) 76-903.
- Report No. 2, Towards Less Hazardous Cigarettes. The Second Set of Experimental Cigarettes. DHEW Publication No. (NIH) 76-1111.
- Report No. 3, Towards Less Hazardous Cigarettes. The Third Set of Experimental Cigarettes. DHEW Publication No. (NIH) 77-1280.
- Report No. 4, Towards Less Hazardous Cigarettes. The Fourth Set of Experimental Cigarettes. March, 1980.

Table 1. Experimental Cigarettes Tested in
Skin Painting Experiments

Var. No.	Cig. Code	Experiment I Cigarette Description
1	1	University of Kentucky Reference (1R1)
2	2	SEB I
3	3	SEB I, High porosity citrate paper (Schweitzer #505) (48 cm/min)
4	4	SEB I, Low porosity phosphate paper (Ecusta Reference A of Schweitzer Regular Verge) (11 cm/min)
5	5	SEB I, Nitrates added as KNO ₃ to 2X natural level of SEB I.
6	6	SEB I, cut coarse (20 cuts/in)
7	7	SEB I, cut fine (60 cuts/in)
8	8	SEB I, with low porosity phosphate paper and coarse cut (combination of codes 4&6)
9	9	SEB I, with high porosity citrate paper and high nitrate content (combination of codes 3&5)
10	10	SEB I, Laminæ only (only leaves of the formula used)
11	11	SEB I, Flue-cured Laminæ only (only flue-cured leaves of formula used)
12	12	SEB I, Burley laminæ only (only Burley leaves used)
13	13	SEB I, Stems only (only flue-cured and Burley stems used, rolled and cut)
14	14	SEB I, Stems only, made into RTS by Schweitzer paper process
15	15	SEB I, Stems and fines only, made into RTS by AMF slurry process
16	16	RTS of whole SEB I by Schweitzer paper process, no additives, medium density
17	17	RTS of whole SEB I by Schweitzer paper process, 7.5% wood pulp added, low density
18	18	RTS of whole SEB I by Schweitzer paper process, 7.5% wood pulp added, medium density
19	19	RTS of whole SEB I by Schweitzer paper process, 7.5% wood pulp added, high density
20	20	RTS of whole SEB I by AMF slurry process, no additives, medium density
21	21	RTS of whole SEB I by AMF slurry process, additives*, low density
22	22	RTS of whole SEB I by AMF slurry process, additives*, medium density
23	23	RTS of whole SEB I by AMF slurry process, additives*, high density

*In addition to glycerine 2.81% and invert sugar 5.31%, additives were:

Methocel	7.35%
Refined unbleached sulfite pulp	4.59%
Ethylhydroxyethyl cellulose	1.34%

Table 1. (Cont.)

Var. No.	Cig. Code	Experiment II	
		Cigarette Description	(May 23, 1973)
1	40	University of Kentucky Reference (1R1)	
2	41	SEB I	
3	42	SEB II	
4	43	SEB II	
5	44	SEB II	
6	45	SEB II	
7	46	ATS-A, 100%	
8	47	ATS-A, 50% & SEB II, 50%	
9	48	RJ Reynolds puffed SEB II	
10	49	Phillip Morris expanded SEB II	
11	50	Freeze-dried SEB II	
12	51	Straight Burley, normal nicotine, normal nitrogen fertilization *(NN)	
13	52	Straight Burley, low nicotine, normal nitrogen fertilization (LN)	
14	53	Straight Burley, low nicotine, high nitrogen fertilization *(LH)	
15	54	Straight flue-cured, normal nicotine, normal nitrogen fertilization (NN)	
16	55	Straight flue-cured, low nicotine, normal nitrogen fertilization (LN)	
17	56	Straight flue-cured, low nicotine, high nitrogen fertilization (LH)	
18	57	Blend 3 parts flue-cured, 1 part Burley (NN)	
19	58	Blend 3 parts flue-cured, 1 part Burley (LN)	
20	59	Blend 3 parts flue-cured, 1 part Burley (LH)	
21	60	Hand suckered (no suckering chemicals used)	
22	61	Fatty-alcohol - normal	
23	62	Fatty-alcohol-x100	
24	63	ATS-B straight (100%)	
25	64	ATS-B, 50% & SEB II, 50%	
Special Use Cigarettes			
	65	Remake of SEB II	
	66	Remake of Code 48 Series II	
	70	Remake of SEB I	
	71	Remake of Code 19 Series I	
	LN	Low Nicotine	
	NN	High Nicotine (spiked)	

*Normal Nicotine variety, NC95.

Low Nicotine variety, LN90.

Normal Nitrogen rate, 61lb/acre

High Nitrogen rate, 100lb/acre

Note: ATS = Artificial Tobacco Substitute

Table 1. (Cont.)

Var. Cig.		Experiment III	(May 21, 1978)
No.	Code	Cigarette Description	
1	40	University of Kentucky Reference (IRI)	
1A	41A	SEB I	
3	41B	SEB I	
4	75A	SEB III	
5	75B	SEB III	
6	72	SEB III	
7	73	SEB III	
8	74	SEB III	
9	76	SEB III (Schweitzer paper 489-14; Low porosity) (5cm/min)	
10	77	SEB III (Vergé 55 paper-high porosity) (60 cm/min)	
11	78	SEB III (Schweitzer perforated paper-very high porosity) (100 cm/min)	
12	80	SEB III No sugar, with humectant	
13	81	SEB III With sugar, no humectant	
14	82	SEB III With 1% cocoa, no sugar, no humectant	
15	83	SEB III No sugar, no humectant (no casing)	
16	84	SEB III With L&M additive #1 (Magnesium Nitrate, 5.72%)	
17	85	SEB III With L&M additive #2 (Zinc Oxide, 7.09%)	
18	86	SEB III With L&M additive #3 (Magnesium Nitrate, 5.61%, Zinc Oxide, 6.95%)	
19	87A	SEB III Burley Blend, cased with sugar, no humectant	
20	87B	SEB III Burley Blend, cased with sugar, no humectant	
21	88	SEB III Burley Blend, no casing (no sugar)	
22	89	SEB III With dilution filter	
23	90	SEB III With dilution filter and Schweitzer perforated paper (100 cm/min)	
24	91	SEB III With cellulose acetate filter	
25	92	SEB III With Permanganate filter	
26	93	ATS-A, 30% & SEB III, 70%, plus flavor	
27	95	ATS-A, 30% & SEB III, 70%, plus flavor	
28	97	ATS-B, 100% (old material, old dyes)	
29	99	ATS-B, 100% (new material, no dyes)	
30	0	ATS-B, 100% (old material, no dyes)	
31	01	ATS-B (old material, no dyes) 50% & SEB III, 50% (casing applied to tobacco portion only)	
32	79	SEB III	
33	94	ATS-A, 30% & SEB III, 70% plus flavor with Vergé 60cm/sec paper	
34	96	ATS-A, 50% & SEB III, 50% (casing applied to tobacco portion only)	
35	98	ATS-B, 100% (old material, new dyes)	
36	00	ATS-B, 100% (new material, new dyes)	

Note: Ecusta 556 paper used on cigarette codes 72 thru 75, 75 thru 89, 91 thru 93, 96 thru 99, and on 00, 0, and 01.

Note: ATS-A = artificial tobacco substitute A
ATS-B = artificial tobacco substitute B

Table 1. (Cont.)

Var. No.	Cig. Code	Experiment IV Cigarette Description (May 4, 1973)
1	30	University of Kentucky Reference (1R1)
1A	38	SEB III (Same as Code 75, Series III, L&M)
2	32	SEB IV
3	14	SEB IV
4	29	SEB IV
5	04	SEB IV
6	33	SEB IV, RTS, PJS paper process, no additives
7	09	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber)
8	06	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), no return of water soluble substances
9	02	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), nicotine reduced by proprietary process
10	28	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), nicotine reduced and added back in form of nicotine citrate to level of Code 32, SEB IV
11	10	SEB IV, RTS, AMF slurry process, no additives except 2.8% glycerine, 5.31% invert sugar
12	17	SEB IV, RTS, AMF slurry process, 13% additives (see Note 1)
13	22	SEB IV, RTS, AMF slurry process, 13% additives plus 6% pH adjustment (see Note 2)
14	25	SEB IV, RTS, AMF slurry process, extracted with hexane-isopropyl alcohol azeotrope, 13% additives (see Note 1)
15	18	SEB IV, RTS, AMF slurry process, extracted with isopropyl alcohol- water azeotrope, 13% additives (see Note 1)
16	12	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), waxy substances reduced
17	03	SEB IV, RTS, AMF slurry process 57%; plus 30% calcium carbonate plus Note 1
18	35	SEB IV, RTS, AMF slurry process 27%; plus 60% calcium carbonate plus Note 1
19	16	SEB IV, RTS, AMF slurry process 27%, extracted with isopropyl alcohol-water azeotrope, plus 60% calcium carbonate plus Note 1
20	27	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber) plus 25% inorganic fillers (calcium carbonate 18%, clay 7%)
21	19	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber) H ₂ O ₂ treated
22	15	SEB IV expanded stems, 100%
23	08	SEB IV expanded stems 50%, SEB IV 50%
25	24	Ecusta material 30%, SEB IV 70%
27	13	SEB IV nicotine removed
28	11	SEB IV nicotine at 0.5 mg/cig.
29	31	SEB IV nicotine at 1.0 mg/cig.
30	23	SEB IV nicotine at 1.5 mg/cig.
31	26	Burley leaf with full return of stem
32	37	Burley leaf RTS, AMF slurry process, 15% additives (see Note 3)
33	21	Burley HLC RTS, AMF slurry process, 15% additives (see Note 3)
34	20	Bright leaf with full return of stems
35	36	Bright leaf RTS, AMF slurry process, 15% additives (see Note 3)
36	05	Bright HLC RTS, AMF slurry process, 15% additives (see Note 3)

Table 1 (Cont.)

Var. No.	Cig. Code	Experiment IV Cigarette Description
37	L8	Pesticide free tobacco
38	M6	Pesticide treated tobacco (See Note 4)
39	67	SEB IV, treated with PMO (1.5% by weight)
40	68	SEB IV, with special 100cm/min paper
		<u>Repeat ATS Experiment</u>
	A	Code 75, Series III
	C	Code 97, Series III (ATS-B 100%, old material, old dyes)
	D	Code 0, Series III (ATS-B 100%, old material, no dyes)

Note 1. Additives: Refined unbleached sulfite pulp 6.05%
Galacto-Mannan Gums 5.85%
Cellulose Ether Gums 0.52%
Dialdehyde Crosslinker 0.58%
Total 13.00%

Note 2. Additives: Same as Note 1, plus
Sodium Hydroxide 3.20%
Citric Acid 2.30%
Total 6.00%

Note 3. Additives: Refined Unbleached Sulfite Pulp 6.25%
Triethylene Glycol 2.25%
Galacto-Mannan Gums 4.48%
Cellulose Ether Gums 1.42%
Dialdehyde Crosslinker 0.60%
Total 15.00%

Note 4. Additives: The soil fertility, pesticide residues and pesticides used on Codes L8 and M6 are listed in a separate document.

RTS = Reconstituted Tobacco Sheet
PJS = Peter J. Schweitzer
AMF = AMF, Inc.
HLC = Monogenized Leaf Cured.

Table 2. Smoke Analyses of Kentucky Reference Cigarette (1R1) Across the Four Skin Painting Experiments

<u>Delivery Per Cig.</u>	<u>Series I</u>	<u>Series II</u>	<u>Series III</u>	<u>Series IV</u>	<u>Average</u>
TPM, mg	43.2	44.4	43.1	43.8	43.6
Water, mg	5.55	5.07	4.16	5.58	5.09
TA, mg	2.65	2.61	2.61	2.60	2.52
Tar, mg	35.3	36.6	36.6	35.6	36.0
Acetaldehyde, μ g	957	985	1084	1058	1021
Acrolein, μ g	126	128	123	110	122
Isoprene, μ g	585	585	565	576	572
HCN, μ g	422	413	417	416	417
Formaldehyde, μ g	32	32	31	30	31
NO _x (as NO ₂), μ g	282	269	278	285	278
CO, (ml)	17.6	18.0	16.2	16.8	17.2
CO ₂ , (ml)	32.8	33.9	34.2	34.7	33.9
Phenolics, μ g	---	209	236	214	220
TPM Palmitic Acid, μ g	171	178	---	---	175
Ol-Lin-Lin Acids, μ g	256	293	---	---	275
Steric Acid, μ g	65	57	---	---	61
Total Free Fatty Acids, μ g	492	528	---	---	510

Table 3. Condensate Analyses of Kentucky Reference Cigarette (1R1) Across the Four Skin Painting Experiments

Per Gram Dry Weight	Series I	Series II	Series III	Series IV	Average
Nicotine (mg)	97.1	92.5	93.1	87.6	92.5
Phenol (mg)	4.29	4.18	4.19	4.07	4.18
o-Cresol (mg)	0.86	0.77	0.62	0.47	0.68
m + p Cresol (mg)	2.45	2.10	1.87	1.33	1.94
Phenol + Cresol (mg)	7.60	7.05	6.68	5.86	6.80
Weak Acids (meq)	1.31	1.35	1.42	1.32	1.35
Very Weak Acids (meq)	0.91	0.83	0.95	0.77	0.87
Total Weak Acids (meq)	2.22	2.20	2.37	2.09	2.22
BaP (ug) ^d	0.76	0.78	1.08	0.97	0.90
BaA (ug) ^e	1.30	1.01	1.26	1.17	1.19
Palmitic (mg)	---	6.7	6.2	7.2	6.7
Ol-Lin-Lin (mg)	---	11.8	10.6	9.2	10.5
Stearic (mg)	---	2.6	1.9	3.0	2.5
Total Fatty Free Acids (mg)	---	21.1	18.7	19.4	19.7
Indole (ug)	---	633	577	438	549
Skatole (ug)	---	387	308	350	348
pH	4.94	5.36	5.23	5.20	5.18
Neophytadiene(s)	---	---	9.1	8.5	8.8
Catechol	---	---	5.5	5.5	5.5

Table 4. Smoke Analyses of Standard Experimental Blend (SEB) Cigarettes Across the Inner Skin Painting Experiments

Smoke Component Per Cigarette	SEB I				SEB II				SEB III				SEB IV			
	Series I	Series II	Series III	Series IV	Series I	Series II	Series III	Series IV	Series I	Series II	Series III	Series IV	Series I	Series II	Series III	Series IV
TM (mg)	30.7	33.3	31.6	33.3	32.8	32.8	31.7	35.5	31.3	31.7	35.5	31.3	31.3	31.3	31.3	31.3
Water (mg)	2.87	3.48	2.76	3.29	3.80	3.79	2.68	4.72	3.64	3.80	4.72	3.64	3.64	3.64	3.64	3.64
Total Alkaloids (mg)	1.68	1.80	1.92	1.80	1.79	1.79	1.79	1.92	1.92	1.79	1.79	1.92	1.92	1.92	1.92	1.92
Tar (mg)	25.9	28.0	26.7	28.0	27.1	27.1	27.2	28.6	28.7	27.1	27.2	28.6	28.7	28.7	28.7	28.7
Acetaldehyde (μ g)	1065	1049	1090	1036	986	986	1122	985	985	986	1122	985	985	985	985	985
Acetone (μ g)	109	109	106	110	101	101	109	109	109	101	109	109	109	109	109	109
Isoprene (μ g)	540	515	549	527	500	500	462	510	510	500	462	510	510	510	510	510
MEK (μ g)	399	394	372	—	394	394	338	394	394	394	338	394	394	394	394	394
Formaldehyde (μ g)	36	36	34	—	31	31	34	31	31	31	34	31	31	31	31	31
NO_x (as NO_2) (μ g)	367	393	416	444	395	395	439	455	455	395	439	455	455	455	455	455
CO (ml)	16.1	18.2	16.8	16.4	15.8	15.8	16.2	16.1	16.1	15.8	16.2	16.1	16.1	16.1	16.1	16.1
CO_2 (ml)	31.0	33.6	32.9	32.7	30.5	30.5	32.5	31.0	31.0	30.5	32.5	31.0	31.0	31.0	31.0	31.0
100 Phenolics (μ g)	—	164	172	164	112	112	164	164	164	112	164	164	164	164	164	164
Phenolic Acid (μ g)	140	135	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Di-Lin-Lin Acids (μ g)	223	235	—	—	192	192	—	—	—	192	—	—	—	—	—	—
Stearic Acid (μ g)	64	48	—	—	35	35	—	—	—	35	—	—	—	—	—	—
Total Free Fatty Acids (μ g)	477	419	—	—	239	239	—	—	—	239	—	—	—	—	—	—

Table 5. Condensate Analyses of Standard Experimental Blend (SCB)
Cigarettes Across the four Skin Painting Experiments

Condensate Components Per Gram Dry Condensate	SIB I							
	Series I		Series II		Series III		Series IV	
	98.4	98.5	99.1	99.1	98.4	97.1	97.3	91.5
Nicotine (mg)	4.35	3.46	3.60	NA	3.00	3.84	4.25	3.48
Phenol (mg)	0.79	0.57	0.60	NA	0.60	0.70	0.51	0.56
o-Cresol (mg)	1.83	1.23	2.11	NA	1.83	1.92	1.53	1.58
m,p-Cresol (mg)	6.96	5.76	6.31	NA	6.23	4.46	6.27	5.61
Phenol+Cresol (mg)	1.33	1.52	1.41	NA	1.34	1.38	—	1.58
Weak Acids (mg)	0.83	0.87	0.90	NA	0.80	0.85	—	0.91
Very Weak Acids (mg)	2.16	2.39	2.31	NA	2.14	2.23	—	2.49
Total Weak Acids (mg)	0.72	0.72	0.85	NA	0.71	1.02	0.87	0.69
BAP (ug)	1.20	0.95	1.76	NA	0.93	1.81	1.15	1.18
BAF (ug)	—	6.2	6.7	NA	6.4	6.6	—	6.4
Palmitic Acid (mg)	—	12.2	12.6	NA	11.4	12.9	—	10.0
Ol-Lin-Lin Acids (mg)	—	2.3	2.6	NA	2.2	2.1	—	2.0
Steric Acid (mg)	—	20.8	21.7	NA	20.0	21.6	—	19.4
Total Fatty Free Acids (mg)	—	417	542	NA	521	599	375	511
Indole (ug)	—	376	297	NA	348	315	293	359
Shatoke (ug)	4.72	5.25	5.17	NA	5.34	5.30	—	5.05
pH								

Table 6. \overline{Pr} and Associated Standard Errors for Common Reference Cigarettes, by Series and Dose Level

Common Reference Cigarette	Series I		Series II		Series III		Series IV	
	Dose Level		Dose Level		Dose Level		Dose Level	
	25.0 mg	50.0 mg	25.0 mg	50.0 mg	12.5 mg	25.0 mg	12.5 mg	25.0 mg
101	.4541, .059	.5971, .059	.3431, .057	.4506, .059	.7971, .044	.8341, .055	.8671, .060	.8806, .057
510 I	.5171, .075	.4806, .027	.3781, .052	.2211, .051	.7711, .047	.4141, .056	—	—
510 II	—	—	.4471, .029	.4141, .029	—	—	—	—
510 III	—	—	—	—	.7711, .026	.4841, .028	.7541, .044	.7151, .057
510 IV	—	—	—	—	—	—	.7751, .031	.4881, .030
								.5111, .060

Table 7. Pairwise Comparisons Among
Common References Cigarettes

DOSE LEVEL (mg)	COMPARISON CIGARETTE	SERIES	COMPARISON CIGARETTE	SERIES	SIGNIFICANCE*
12.5	1R1	III	1R1	IV	
25.0	1R1	I	1R1	II	
	1R1	I	1R1	III	
	1R1	I	1R1	IV	
	1R1	II	1R1	III	
	1R1	II	1R1	IV	
	1R1	III	1R1	IV	
50.0	1R1	I	1R1	II	
12.5	SEB I	III	SEB III	III	
	SEB I	III	SEB III	IV	
	SEB I	III	SEB IV	IV	
	SEB III	III	SEB III	IV	
	SEB III	III	SEB IV	IV	
	SEB III	IV	SEB IV	IV	
25.0	SEB I	I	SEB I	II	5%
	SEB I	I	SEB I	III	
	SEB I	I	SEB II	II	5%
	SEB I	I	SEB III	III	
	SEB I	I	SEB III	IV	5%
	SEB I	I	SEB IV	IV	
	SEB II	II	SEB III	III	
	SEB II	II	SEB III	IV	
	SEB II	II	SEB IV	IV	
	SEB III	III	SEB III	IV	
	SEB III	III	SEB IV	IV	
50.0	SEB I	I	SEB I	II	1%
	SEB I	I	SEB II	II	
	SEB I	I	SEB IV	IV	
	SEB I	II	SEB II	II	1%
	SEB I	II	SEB IV	IV	1%
	SEB II	II	SEB IV	IV	

* Not statistically significant unless noted otherwise.

Table B. Summary of Results--Major Factors of Interest.
Tumorigenicity of Experimental Cigarettes
Compared to Tumorigenicity of the Standard
Experimental Blends (SEB)

• LESS TUMORIGENIC THAN SEB

<u>Factor</u>	<u>Exp.</u>	<u>Code Number(s)</u>
RTS paper process	I	14-23
Citrate paper	I	3, 9
Stems only	I	13-15
Potassium nitrate additive	I	5
Philip Morris expanded	II	49
Freeze-dried	II	50
Low nicotine (plants)	II	51-59
ATS-A	II	46, 47
ATS-A (30%), SEB (70%)	III	93, 95
Dilution filter	III	89, 90, 95
Magnesium nitrate additive	III	84, 86
Paper process, water extracted (10% additives)	IV	6
Slurry process, IPA, water azeotrope (13% additives)	IV	18
Expanded stems (freon)	IV	15
Bright leaf, slurry process (15% additives)	IV	36

• MORE TUMORIGENIC THAN SEB

<u>Factor</u>	<u>Exp.</u>	<u>Code Number(s)</u>
ATS-B	II	63
Cellulose acetate filter	III	91
Potassium permanganate filter	III	92
Slurry process (no additives)	IV	10
Paper process, extracted water (65%), inorganic (25%), additives (10%)	IV	27
Paper process (nicotine same as SEB IV)	IV	28

Table 8. (cont.)

• TUMORIGENICITY COMPARABLE TO SEB

<u>Factor</u>	<u>Exp.</u>	<u>Code number(s)</u>
Tobacco cut (fine, coarse)	I	6-9
Phosphate paper	I	4, 8
RTS slurry process	I	14-23
Hand suckered	II	60
Fatty alcohol suckered	II	61, 62
Reynolds puffed	II	48
ATS/SEB mixes	II III	46, 47, 63, 64 0, Q1, 93, 95, 97, 99
Paper porosity	III	76-78, 90
Sugar	III	80, 83
Humectant	III	81, 83
Zinc oxide additive	III	85, 86
Powdered cocoa	III	82
Paper process, no additives	IV	33
Paper process, 10% additives	IV	09
Slurry process 13% additives	IV	17
Expanded stems 50%, SEB IV 50%	IV	08
Ecusta material 30%, SEB IV 70%	IV	24
Nicotine at 1.0 and 1.5 mg/cig	IV	23, 31
Burley leaf with full return of stem	IV	26
Burley leaf, slurry process, 15% additive	IV	27
Burley HLC, slurry process, 15% additive	IV	21
Pesticide-free, pesticide-treated tobacco	IV	18, M6

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