

Preparation of painting material

Definitions

The composition and yield of cigarette smoke is dependant on many factors including the way in which the cigarette is smoked, the type of smoking machine used and the methods employed for condensation and concentration. The methods used for the production of smoke condensates for this work have been standardised as far as is practical and the type of material used for painting strictly defined.

Smoking Parameters All the smoke condensates used in this work have been prepared from smoke using the following smoking parameters:

Puff volume, 25 ml.; Puff duration, 2 seconds; Puff interval, 1 minute;
Butt length, 20 mm.

These parameters were chosen to simulate the manner used by the average habitual cigarette smoker in the United Kingdom (Bentley & Burgan, 1961) T.R.C. 1961
T.R.C. Research Paper No. 4.)

Whole Smoke Condensate is defined as the material remaining after condensing the whole smoke from cigarettes smoked with the standard smoking parameters on a Rotary Smoking Machine and using the particular trapping system described below.

Non-volatile Whole Smoke Condensate (NVWSC) is defined as the anhydrous residue remaining after an acetone solution of Whole Smoke Condensate has been evaporated to constant weight on a Rotary Evaporator using a boiling water bath and a water suction pump operating at 10 inches of mercury vacuum.

Stale Whole Smoke Condensate (S.W.S.) is defined as non-volatile whole smoke condensate which has been stored at -20°C for a period of four to eight weeks after production of the original smoke.

Freshly Applied Smoke Condensate (F.A.S.) is defined as the material remaining after the concentration of an acetone solution of whole smoke condensate (not more than 24 hours old) in a Rotary Evaporator on a water bath at 40°C , and at the full vacuum of a water pump until it reaches the concentration for painting at the highest dose rate. The material is painted on the mice 24 hours after the smoking of the cigarettes.

Neutral Fraction (N.F.) is defined as the material which is recovered under standard conditions after the ethereal solution of whole smoke

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condensate has been extracted with aqueous hydrochloric acid and aqueous potassium hydroxide solution.

Measurement of dose All doses of condensates used for paintings are expressed in terms of equivalent mg. of non-volatile whole smoke condensate. For example, in the case of the painting of freshly applied smoke condensate (F.A.S.) at 100 mg. equivalent dose the same aliquot is taken of the concentrated solution as would yield 100 mg. of non-volatile whole smoke condensate if taken to constant weight under the appropriate conditions. Similarly, the amount of neutral fraction (N.F.) painted at 100 mg. equivalent dose is that amount of neutral fraction produced from the appropriate quantity of whole smoke condensate which would give 100 mg. of non-volatile whole smoke condensate. Similarly, also the amount of stale whole smoke condensate (S.W.S.) painted at 100 mg. equivalent dose is that amount of stale whole smoke condensate produced from the appropriate quantity of whole smoke condensate which would give 100 mg. of non-volatile whole smoke condensate.

Smoking Procedures

Materials

Cigarettes Standard medium size plain cigarettes (length, 70 mm.; circumference, 25.3 mm.; weight, 25.2 cigarettes/ounce) were specially manufactured for this work from mixed blends of flue-cured tobacco commonly used in cigarettes smoked in the United Kingdom. The cigarettes were in batches of 50 in vacuum sealed tins and stored at 40° F. The seal was not broken until just prior to smoking.

Acetone Reagent grade (May & Baker)

Apparatus

Smoking Machine The smoke used in the preparation of all condensates was prepared using automatic rotary smoking machines designed by B.W. Harris of the Imperial Tobacco Co. Ltd. (obtainable from R.W. Mason (Engineers), Moor Lane, Clevedon, Somerset). These machines were designed so that each of 24 cigarettes secured in holders situated round a revolving disc is connected intermittently to a source of vacuum. The mechanism is such that individual cigarettes reach the smoking station at the top of the disc once per minute and are held in position for 2 seconds. The air flow is set at 12.5 ml. per sec by means of a bubblemeter (No. P107, Cigarette Components Ltd., 21-24 Chiswell Street, London, E.C. 1), and can be adjusted by the needle valve on the apparatus before smoking is carried out. Any changes in air flow occurring during smoking are corrected by resetting this valve to give the correct reading on the calibrated rotameter on the smoking machine.

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Lighting and Smoking Procedure The disc of the smoking machine is loaded with 24 cigarettes and each cigarette is lighted by holding an electrically heated coil against the open end during the 2 seconds that air is drawn through at the smoking station. When individual cigarettes have reached a butt length of 20 mm, the stubs are removed irrespective of positions on the disc and replaced with fresh cigarettes. These cigarettes are lighted as they reach the smoking post. The length of butt is read on a scale calibrated in mm. printed down the length of the cigarette.

Collection of Smoke Condensate The smoke is passed through a series of four glass traps cooled in a mixture of acetone and crushed solid carbon dioxide. Each trap consists of a vessel 25 cm. long x 7 cm. diameter with a B50 socket and carrying a two necked (B19) adaptor. The smoke enters the trap via a central tube (Q & Q) - MF 15/2, passing through the vertical neck of the adaptor and extending to 2 cm. from the bottom of the trap and leaves via the side neck of the adaptor. The traps are connected to each other and to the smoking machine by short lengths of PVC tubing. Traps 3 and 4 each contain 100g of glass helices (4mm. diam. single turn) to assist condensation. After smoking the requisite number of cigarettes to yield the appropriate quantity of smoke condensate for a given experiment, the glass traps are removed from the cooling mixture and allowed to attain room temperature. The condensed smoke is washed from the traps and connecting tubes with acetone (about 900 ml.), the washings filtered through glass wool to remove any helices and a suitable aliquot of the resulting solution removed for the checking of smoke yield by determination of nicotine. Over the two years of the experiments described in this report involving the smoking of ^{44,356,234} 1 cigarettes, the average yield of nicotine was 1.61 mg/cigarette with a range of 1.30 - 1.91 mg/cigarette.

Recovery of non-volatile whole smoke condensate

The solvent is removed from the final acetone solution obtained from the collection traps in a weighed flask on a Rotary Evaporator using a boiling water bath and a water suction pump operating at a vacuum of 10 inches of mercury. Evaporation is continued until the non-volatile residue attains constant weight. The average yield was 21.51 mg./cigarette with a range of 17.7 - 24.8 mg./cigarette.

Preparation of Mouse skin Painting Solution of Stale Whole Smoke Condensate (S.W.S.)

All non-volatile whole smoke condensate prepared in a period of four

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weeks is bulked to make a single sample and stored for a further four weeks before use. On basis of the yields of NVWSC determined at the time of smoking, painting solutions are prepared by dissolving the Stale Whole smoke condensate with constant stirring in 9:1 acetone/water (v/v) and made up to the appropriate volume (7 ml.) with the same solvent mixture. The standard dose volume of painting solution for the mouse skin test is 0.3 mls. per mouse. Thus, if the amount of equivalent NVWSC to be applied to each mouse is w mg. (100, 50 or 25 mg.) and the batch weight is W mg. then $V = \frac{W \times 0.3}{w}$ mls.

Preparation of Mouse Skin Painting solution of Freshly Applied condensate

(F.A.S.)

Using the average yield per cigarette of NVWSC obtained in the production of material for the preparation of old smoke condensate during the previous four weeks, the number of cigarettes is calculated which are required to be smoked to produce the correct amount of F.A.S. smoke condensate for the particular painting. This number of cigarettes is smoked and the smoke condensate collected and the traps washed out with acetone in the standard manner. All F.A.S. for one day's painting is bulked, mixed thoroughly and an aliquot of this solution is taken for nicotine determination to check that the correct smoke yield has been obtained. The bulk of the solution is then concentrated in a rotary evaporator using a water bath maintained at 40°C and the full vacuum of a water suction pump until it reaches the concentration for painting at the highest dose rate, an adjustment made for water content and divided into three parts for dilution for the three dose levels.

The necessary smoking is done on Mondays, Tuesdays and Thursdays and the condensate washed from the traps, concentrated and diluted on the following mornings.

Example of Freshly Applied Smoke Condensate preparation calculation

Total number of mice to be painted 1200

Dose levels 100 mg.; 50 mg.; 25 mg. equivalent NVWSC

Number of doses at each dose level 440

(400 + 10% for wastage)

Dose volume of painting solution = 0.3 ml.

Volume of painting solutions required (440 x 0.3 ml.)

= 132 ml. for each dose level.

Before making the 50 mg. & 25mg./dose painting solutions, the total bulk solution is diluted to the concentration of the highest dose level, i.e. 333.33 mg/ml. (100mg./0.3 ml.)

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Total volume of solution @ conc. of 33.3 mg./ml	= 231 ml.
Made up of solution for 100 mg. dose level	= 132 ml.
solution for 50 mg. dose level	= 66 ml.
solution for 25 mg. dose level	= 33 ml.

	231 ml.

231 ml. @ 33.3 mg./ml. = 77,000 mg. equivalent NWSC.

Average yield of NWSC obtained from cigarettes smoked over the previous four weeks = 20 mg./cigarette.

Cigarettes required to be smoked = $77,000/20 = 3,850$.

Thus the acetone solution of the smoke condensate collected from 3850 cigarettes is concentrated to 200 ml. and the appropriate amounts of acetone and water are added to give 231 ml. volume of solution in approximately 9:1 acetone/water (v/v). The actual volume of water to be added is arrived at by assuming that all the water in the original condensate has been removed during concentration and that the specific gravity of smoke condensate is 1.0. i.e. the final solution of 231 ml. @ 33.3 mg./ml. is made up of 77.0 ml. smoke condensate + 154.0 ml. acetone/water 9:1 (v/v). Thus 15.4 ml. of distilled water is added to the 200 ml. of smoke condensate concentrate and the solution made up to 231 ml. with anhydrous acetone.

The solution is divided in three portions of 132 ml. 66. & 33 ml. The first portion is used undiluted (100 mg./0.3 ml.), the other two portions are diluted to 132 ml. with 9 : 1 acetone/water (v/v) to give the painting solutions at 50 mg/0.3 ml. and 25 mg./0.3 ml. levels respectively.

Preparations of neutral fraction

Reagents

Diethyl ether Redistilled peroxide free Reagent grade. May and Baker.
(Treated with sodium to remove fluorescent material).

Hydrochloric Acid 2N aqueous solution. (Analar)

Potassium Hydroxide 3% W/V aqueous solution made from Reagent grade grade reagent (May & Baker).

Magnesium Sulphate Dried Magnesium Sulphate (British Drug Houses)

Procedure

The smoke condensate from a known number of cigarettes (about 2,000) is washed from the traps into a separating funnel with a minimum of 1 litre of ether and 900 ml. of 2N hydrochloric acid.

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The mixture is shaken, allowed to separate and the aqueous layer drawn off into a second separating funnel. Further extractions are carried out (2 x 200 ml., 5 x 100 ml. 2N HCl) and the combined acid extracts are washed with 2 x 100 ml. ether, the ether washings being added to the bulk ethereal solution left in the original separating funnel.

The combined ethereal solution is vigorously shaken with 200 ml. 3% (w/v) aqueous potash solution and the mixture allowed to stand for 1½ hours. The aqueous layer is drawn off and further extractions are carried out (1 x 200 ml., 5 x 100 ml., (w/v) KOH). Little emulsification occurs after the first extraction and the two layers separate in a few minutes.

The final ethereal solution is dried with anhydrous magnesium sulphate, filtered and the solvent removed in a Rotary evaporator using a water bath at 40°C and a water suction pump.

The average yield of neutral fraction was 6.68 mg./cigarette (36.6% NVWSC) with a range of 5.00 - 7.88 mg./cigarette (23.3 - 36.6% NVWSC).

A check to determine that the correct yield of smoke condensate had been obtained during the original smoking was made by carrying out a nicotine assay of a suitable aliquot of the acid extract.

Preparation of mouse painting solution of Neutral Fraction

Batches of neutral fraction produced over monthly periods are bulked and the amount of whole smoke in terms of equivalent NVWSC used in the preparation of the single sample is calculated from the total number of cigarettes smoked and the average yield of NVWSC obtained for old smoke condensate over the previous four weeks. The bulk sample of neutral fraction is dissolved in 9:1 acetone/water (v/v) in the same manner as for old smoke condensate and the solution diluted with the same solvent mixture to give three painting solutions containing the neutral fraction obtained from condensate equivalent to 100 mg./0.3 ml., 50 mg./0.3ml. and 25 mg./0.3 ml. NVWSC respectively.

Nicotine Assay The nicotine determinations done on the smoke condensate obtained from every individual smoking as a check on smoking performance and extraction efficiency have been carried out using the method of Willetts et al. ⁽¹⁹⁵⁹⁾ as modified by Lauren^s & Harrell⁽¹⁹⁵⁹⁾.

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Preparation of mouse painting solutions of Standard Carcinogens

3.4 Benzoyrene

9:10 Dibenzanthracene

Two weeks supply of 0.1% w/v solution in AR acetone of each carcinogen was prepared and the solution stored at -20°C in the dark when not in use.

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RESULTS

These must necessarily be sought in the appended computer results. However certain generalisations emerge. Firstly, that with all three treatments there is a dose response relationship; in other words the more condensate applied the more tumours result. But as regards the effect of the three treatments at any particular dose level, the neutral fraction appears to be the least powerful. Slight variations in response exist as between the other two treatments but substantial carcinogenicity exists in all three.

Although the final word rests with the computer, it may be said that these results were anticipated by preliminary estimations based upon the enumeration of papillomata as they first occurred.

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DISCUSSION

It was not the intention in planning this experiment to investigate the details of carcinogenesis - the comparative parts played by initiating and promoting agents, the pathways by which carcinogenic agencies reach susceptible cells, the influence of genetic factors, the nature of the susceptibility to cancer of individual animals, of tissues and of cells etc. It was thought sufficient for present purposes to consider the concept of carcinogenicity as being merely the obvious capacity of the material applied to give rise to tumours at the site of application. What is meant by 'tumour' and 'site of application' in this connection has been described above.

With regard to the statistical treatment it may be sufficient to say that the same techniques have been used as have been applied to human cancer epidemiology.

What has been described therefore is why this work was attempted, how it was done and what valid conclusions can be drawn from the results. To add anything further would seem to be contrary to Occam's principle, "Essentia non sunt multiplicanda praeter necessitatem".

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SUMMARY

A study has been made of the carcinogenicity of (1) freshly prepared cigarette smoke condensate when applied to the skins of I.C.I. mice. This carcinogenicity was compared with that following the application at equivalent dose rates of (2) a condensate which had been evaporated to constant weight on a water bath at 100°C and subsequent kept for over a month and (3) the neutral fraction. Reference to the computer tables will show that in spite of variations in relative carcinogenicity as between these treatments at different dilutions, substantial carcinogenicity remained in (2) and (3). From which it is to be concluded that the tar yielded by the average British cigarette contains material which is carcinogenic to mice and further that this material is not easily separated from the tar.

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Acknowledgements

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LEGENDS

Fig. 1. A smoking machine with traps.

Fig. 2. Penetration of the muscle of the
panniculus carnosus by groups of
epithelial cancer cells. Mag. x 400.

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