

DETERMINATION OF 3:4 BENZO-(a)-PYRENE IN

CIGARETTE SMOKE

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Experimental MethodMaterials and Apparatus

All chromatographic eluants should have extinction values less than 0.005 down to 360 m μ after purification.

<u>Benzene</u> -	May & Baker Ltd. (pure crystallisable) purified by redistillation (three times) through a fractionation column and rejection of the first 10% of distillate. The benzene is stored after drying with sodium wire.
<u>Petroleum ether</u> -	Analar - BP 40 ^o - 60 ^o C - purified by percolation through a 4.5 cm. diameter 15 cm. long fully activated alumina column and redistilled as for benzene. Dry with sodium wire.
<u>n-Hexane</u> -	Hopkin & Williams Ltd. (Hexane fraction).
<u>Diethyl ether</u> -	May & Baker Ltd. (Peroxide free). Dry with sodium wire.
<u>Cyclohexane</u> -	British Drug Houses Ltd. (Special for spectroscopy).
<u>Methyl alcohol</u> -	Hopkin & Williams Ltd.
<u>Hydrochloric acid</u> -	2N aqueous solution
<u>Sodium Hydroxide</u> -	2N aqueous solution
<u>Acetylated Chromotography Paper</u> -	Prepared as described by Spotswood. J. Chromat., 3, 1960, 101.
<u>Alumina A</u> -	Woelm Ltd. - neutral grade - activity 1. De-activated at 70% relative humidity for 3 days, re-activated by heating at 200 ^o C for 24 hours just before use.

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Alumina B - Woelm Ltd. - neutral grade - activity 1.
De-activated by addition of 3% water and
mixed in closed rotary mixer for 3 hours.

Silica gel - British Drug Houses - de-activated by
washing with methyl alcohol/water 1:1, dried
in air and activated by heating at 110°C
for 6 hours.

Nitrogen - British Oxygen Co. Ltd. - (oxygen free).

Solid Carbon dioxide - Imperial Chemical Industries Ltd.

7:10¹⁴-C benzpyrene - Specific activity 6 µ C/mg. purified by
paper chromatography as in procedure
(200 µg/6" strip). Log E₃₈₄ mµ = 4.41

Apparatus

All glass apparatus is cleaned with chromic acid and washed well with water
before use.

Spiral Glass traps - Drawing RR.100

Cambridge filter - Drawing RR.70/1, 70/2, 70/4a.

Glass separators - 500 ml.

Chromatography tubes - 4.5 cm. diameter - 50 cm. length.
3.0 cm. diameter - 45 cm. length.
Fitted with a sintered glass disc to
support adsorbent.

Agla Micrometer Syringe - Burroughs Wellcome Ltd.

Spectrophotometer - Unicam Ltd. SP.500.

Radiation counting - Labgear Ltd. - Scaler
equipment G-M tube and manual sample changer
Probe quenching unit.

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<u>Spectrograph</u> -	Hilger & Watts Ltd. - medium quartz E498
<u>Cells</u> -	Hilger & Watts Ltd. - Cat.No. H857
<u>Photographic plates</u> -	Ilford Ltd. - HPS (5" x 4")
<u>Photographic developer</u> -	Ilford Ltd. - Contrast FF
<u>Photographic fixer</u> -	Ilford Ltd. - Hypam fixer with Hypam hardener
<u>Spectrograph lens</u> -	Hilger & Watts - Cat.No. F100 ¹¹⁷²
<u>U.V. illumination</u> -	Mazda mercury vapour discharge lamp (125w,
<u>system</u>	MBW/μ) fitted into a photographic enlarger with objective lens removed. A Woods glass filter (2 mm) is fitted between the condensing lens and the lamp.

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Procedure

General Notes

All operations are carried out in subdued light to reduce the risk of photochemical decomposition of the benzpyrene, columns and flasks are covered with black paper.

All evaporations are carried out under reduced pressure in an atmosphere of nitrogen. When solvents are changed, the solutions are evaporated down with three successive 10 ml. fractions of the new solvent.

Smoke Collection

The cold traps are connected to the smoking engine via a Cambridge filter; the latter is a precaution in case of blocking or incomplete cold trap collection. The smoking engine is then calibrated to give a 35 ml puff, of 2 seconds duration, once a minute. The calibrator for puff volume is a soap film burette with a glass capillary attached, the resistance of which is approximately equivalent to that of the cigarettes to be smoked.

Smoke a sufficient number of cigarettes to produce 6 - 10 µg of benzpyrene (about 3 - 4g of condensate if dried at 100°C for 16 hours). Collect using a smoking cycle of 35 ml. puff volume, once per minute each puff of 2 seconds duration. Collect the condensate in the spiral cold traps surrounded by solid carbon dioxide (Note 1). Remove ~~the carbon dioxide from~~ the traps, and wash out the condensate with 6 x 10 ml. portions of a mixture of ether and hydrochloric acid (1:1). Transfer the washings into separators and each separator should contain approximately equal amounts of 1g of condensate. Wash out any condensate remaining in the traps with methyl alcohol, evaporate the alcohol and wash the residue into one of the separators with 10 ml. of warm ether/hydrochloric mixture. Extract the filter pads of the Cambridge filters with ether in a soxhlet apparatus for 1 hour and add the concentrated

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ether solution (20 ml.) to one of the separators. To each of the separators add equal volumes of ^{14}C -benzpyrene solution in cyclohexane such that a total of about 10 μg (accurately known) has been added in a total volume of 5.0 ml. cyclohexane.

Isolation of the neutral tar fraction

Separate the aqueous layer and discard. Shake the ether layer for 1 minute with 5 successive 100 ml. fractions of hydrochloric acid followed by 3 x 100 ml. fractions of water. Extract the ether solution with 6 x 100 ml. portions of sodium hydroxide and finally 100 ml. portions of water until the aqueous layer is neutral. Care should be taken to avoid emulsion formation especially with the sodium hydroxide solutions. Run the ether layer into a flask containing anhydrous sodium sulphate (5g) and allow to stand overnight

First Chromatography

Prepare a column (4.5 cm. diameter) containing 160g of alumina A and using n-hexane as the solvent (Note 2). Filter the ethereal neutral tar solution and wash the sodium sulphate with 6 x 20 ml. successive portions of dry ether, transferring the washings to the bulk of the solution. Change the solvent to n-hexane and apply the neutral fraction in 10 ml. of n-hexane to the column. Elute with n-hexane until all the green fluorescence has been removed from the column (Note 3). Continue the elution with 2 litres of benzene and collect the benzene eluate (Note 4). Evaporate the benzene, and change the solvent to petroleum ether and dissolve the residue in 10 ml. of petroleum ether.

Second Chromatography

Prepare the column (3.0 cm diameter) in petroleum ether using 120 g of alumina B. Apply the petroleum ether solution of the active fractions obtained from the first chromatographic separation to the column and elute with 500 ml. of petroleum ether. Collect 10 ml. fractions. Carry out a linear gradient elution commencing

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with 100% petroleum ether and finishing with 80% petroleum ether 20% benzene over 400 ml. of eluate (Note 6). Continue the elution with 80:20 petroleum ether/benzene taking 50 ml. fractions from the commencement of the gradient elution. Evaporate the solvent from each fraction and dissolve the residue in 5 ml. of petroleum ether. Determine the fluorescence spectra of the solutions using the spectrograph (Note 7 and Appendix 1), and combine these fractions showing the typical benzpyrene fluorescence spectral bands (404 m μ , 408 m μ , 427 m μ) together with one fraction either side. Evaporate the solution to near dryness and transfer quantitatively into a 10 ml. beaker using petroleum ether. Concentrate the solution to about 0.5 ml. on the steam bath whilst blowing a current of nitrogen over the surface.

Third Chromatography

Apply the solution containing the benzpyrene onto a base line drawn approximately 3 cm. from the edge of a 15 cm. wide strip of acetylated chromatography paper (Whatman 3MM) using an Agla micrometer syringe. Wash the beaker and syringe with small quantities of petroleum ether until all the fluorescent material has been applied to the paper. Develop the paper in methyl alcohol/ether/water (4:4:1) mixture for 6 hours. Withdraw the paper and allow to dry overnight, then replace in the chromatography tank for a further 6 hours. Examination of the paper under the ultra violet lamp shows the intensely fluorescent benzpyrene band 2 - 3 cm. from the base line.

Determination of the benzpyrene

Cut out the strip of paper showing the benzpyrene fluorescence and extract in a small soxhlet apparatus with benzene for one hour. Cool the benzene solution to room temperature and run the solution through a silica gel column [1 cm. diameter 2 cm. long (Note 8)] which is then washed with small portions of benzene (total volume 25 ml.). Change the solvent to cyclohexane and dissolve

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the benzpyrene in 5.0 ml. of cyclohexane. Measure the extinction of 1 cm. thickness of this solution using cyclohexane as a blank at 374 m μ (A), 384 m μ (B) 394 m μ (C).

Prepare 12 planchets for radioactive counting as described in Appendix 2; 6 containing benzpyrene solution from smoke and 6 planchets with the standard ¹⁴C-benzpyrene solution. Determine the count rate for each planchet over 10,000 counts as soon as possible after preparation and calculate the mean count rate for the standard and sample (Note 9).

Calculation of Result

$$\text{Benzpyrene found} = \frac{5 \times D \times F}{Y}$$

where $D = B - \frac{1}{2} (A + C)$ for the benzpyrene solution

$Y = B - \frac{1}{2} (A + C)$ for a standard benzpyrene solution of concentration F $\mu\text{g/ml}$.

$$\text{Benzpyrene recovery} = \frac{\text{count rate for the sample}}{\text{count rate for the standard}} = R$$

$$\text{Total benzpyrene} = \frac{5 \times D \times F}{Y \times R} \mu\text{g.}$$

$$\text{Benzpyrene in smoke} = \left[\frac{5 \times D \times F}{Y \times R} \right] - Z \mu\text{g.}$$

where Z = μg of benzpyrene added as radioactive tracer.

Notes on Experimental Procedure

1. Under these smoking conditions, (35 ml. puff, of 2 second duration, 1 per minute) each trap can be used to collect tar from 30 cigarettes. If the puff frequency is increased there is a tendency for the trap to block and the puff volume will decrease. The efficiency of the smoke collection was estimated as 98% as determined by measurement of the smoke deposited on the Cambridge filter placed at the suction end of the trap. The draw resistance of these traps was less than 0.2 cm. water gauge at a flow rate of 1050 ml./min.

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2. The activity of this alumina was found to be approximately No. 2 as determined by the Brockmann method²⁴ (Ber. Der. Deutschen, Chem. Gesell. 1941, 74, 73)

3. Fluorescence measurements showed that there is no loss of benzpyrene in the n-hexane eluate. About 1 - 1.4 litre of n-hexane is required to elute the green fluorescing substance.

4. Although most of the benzpyrene is eluted in the first litre of benzene, 2 litres are used to ensure the complete elution.

5. The activity of this alumina was approximately No. 3 as determined by the Brockmann method.

6. The gradient elution is conveniently carried out using the apparatus described by Bock and Nan-Sing Ling²⁵ (Anal. Chem. 1954, 16, 1545. fig 6.)

7. The spectra ~~are~~ recorded on Ilford HPS photographic plates developed for 3 minutes in Ilford Contrast FF developer.

8. This column removes paper fibres and suspended material which will interfere with the U.V. measurement.

9. 10,000 counts are taken because this is the minimum number of counts required to even out the effect of the natural random emission of the radiation. Provided the geometry of the counting system is reproducible the count rate is reproducible to within 1% with a theoretical confidence limit of 68%.

The uniformity of distribution of the sample on the dimple of the planchet was checked by screening of various areas, when the count rate was proportional to the exposed area of the dimple.

From 0 - 0.5 µg of ¹⁴C-benzpyrene the count rate was proportional to the amount of radioactive substance on the planchet showing that self absorption of radiation was negligible.

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The count rate fell by 1% per day when the prepared planchet was kept in the dark, this decrease doubled on exposure to daylight. Care must be taken to avoid counting in polluted atmospheres as certain substances cause a rapid decrease in count rate. Chloroform was found to cause a 20% reduction in 30 minutes when the planchet was exposed to the vapour.

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APPENDIX 1

Procedure for use of the Spectrograph

The spectrograph is set up using a slit width of 0.15 mm and slit height of 6mm. A half plate (5" x 4") is placed in the plate holder to photograph the 280 μ - 1,000 μ spectral range; the vacant space in the holder (5" x 4") is occupied by a piece of plain glass. A cell containing the solution is placed 48 cm. from the slit with one side facing the slit. A lens is placed 10 cm. from the slit to focus light from the cell onto the slit face. The cell is illuminated by a beam of light projected at 60° to the cell face. The spectrum is photographed using an exposure time of 3 minutes and a spectrum can be obtained on each plate. The plate is developed for exactly 3 minutes, washed in water for approximately 2 seconds and placed in the fixer for 20 minutes. The plate development is carried out in complete darkness and only after the plate has been placed in a covered fixing bath is light admitted in the darkroom.

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APPENDIX 2

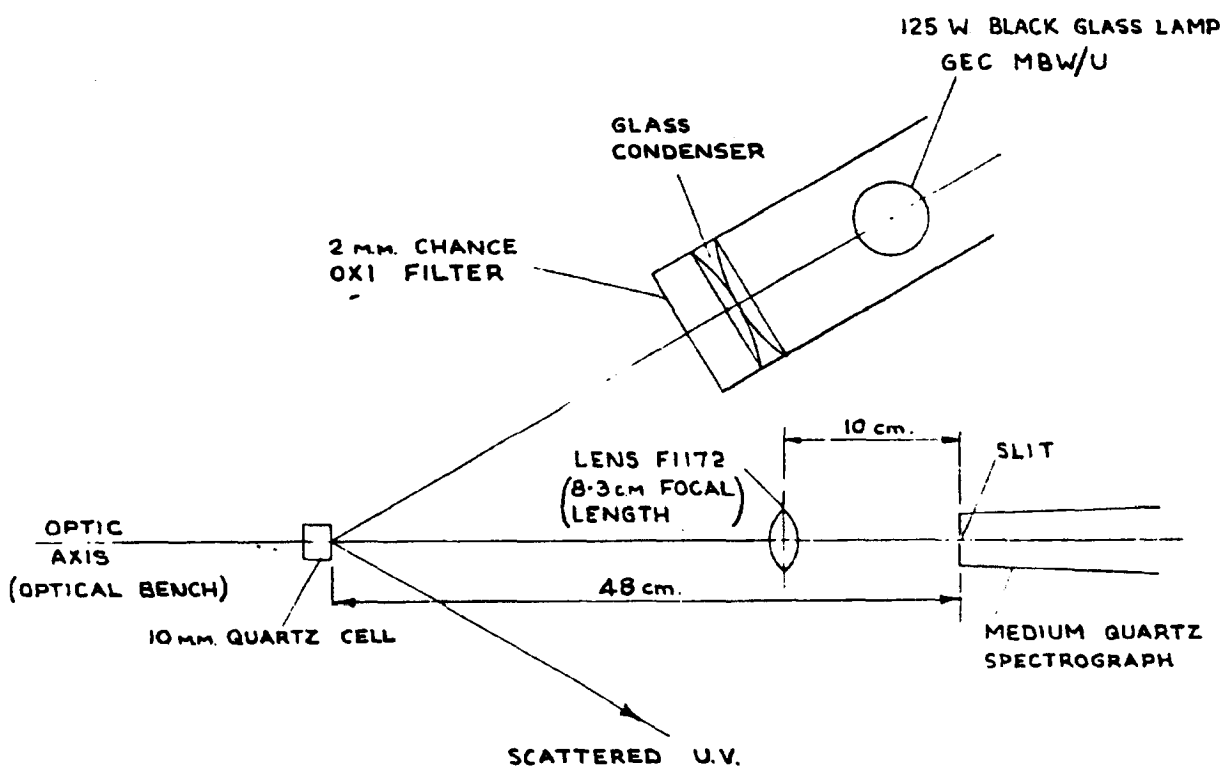
Preparation of samples for counting ^{14}C -benzpyrene

The planchets are cleaned by boiling with cyclohexane and wiped with a soft paper tissue to remove last traces of grease. The temperature of the heating apparatus is adjusted to 100°C and the planchet then placed in position. Using the agla syringe 0.04 ml. of solution is transferred into the dimple of the planchet and allowed to evaporate. This is followed by 2 x 0.08 ml. portions of sample. Immediately the last trace of solvent has evaporated, the planchet is removed and stored in a dark place before counting.

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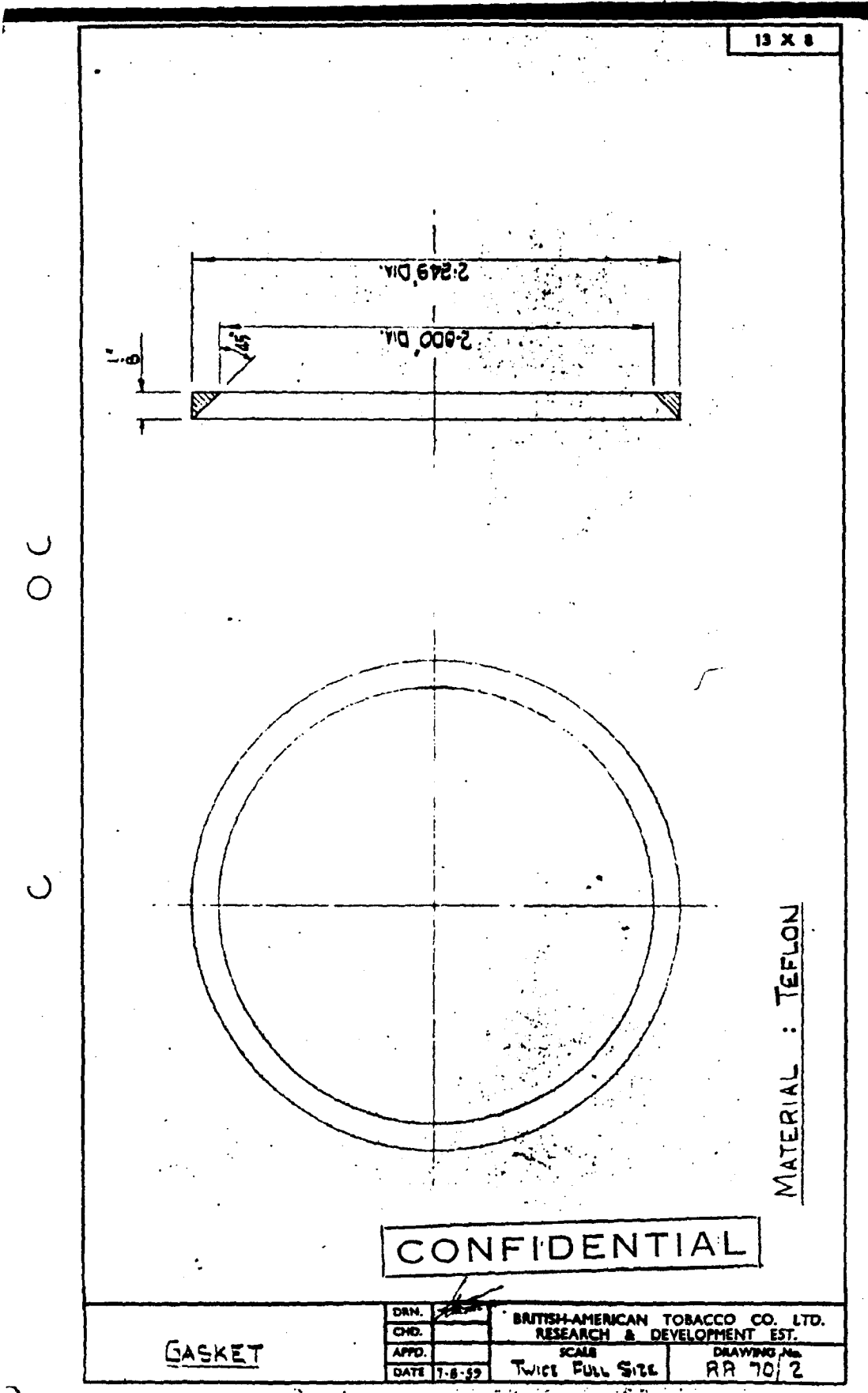
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OPTICAL ARRANGEMENT FOR
FLUORESCENCE SPECTROGRAPHY.



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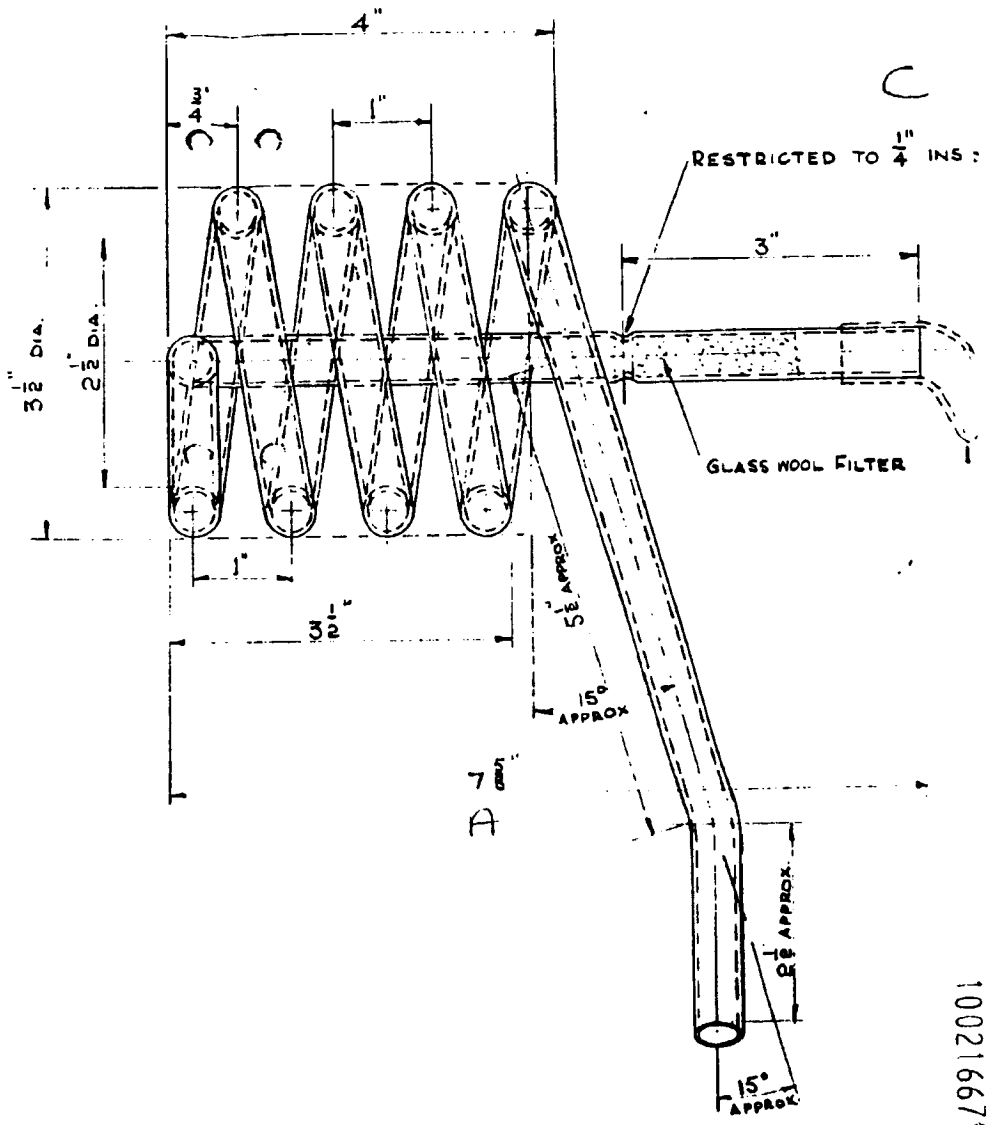
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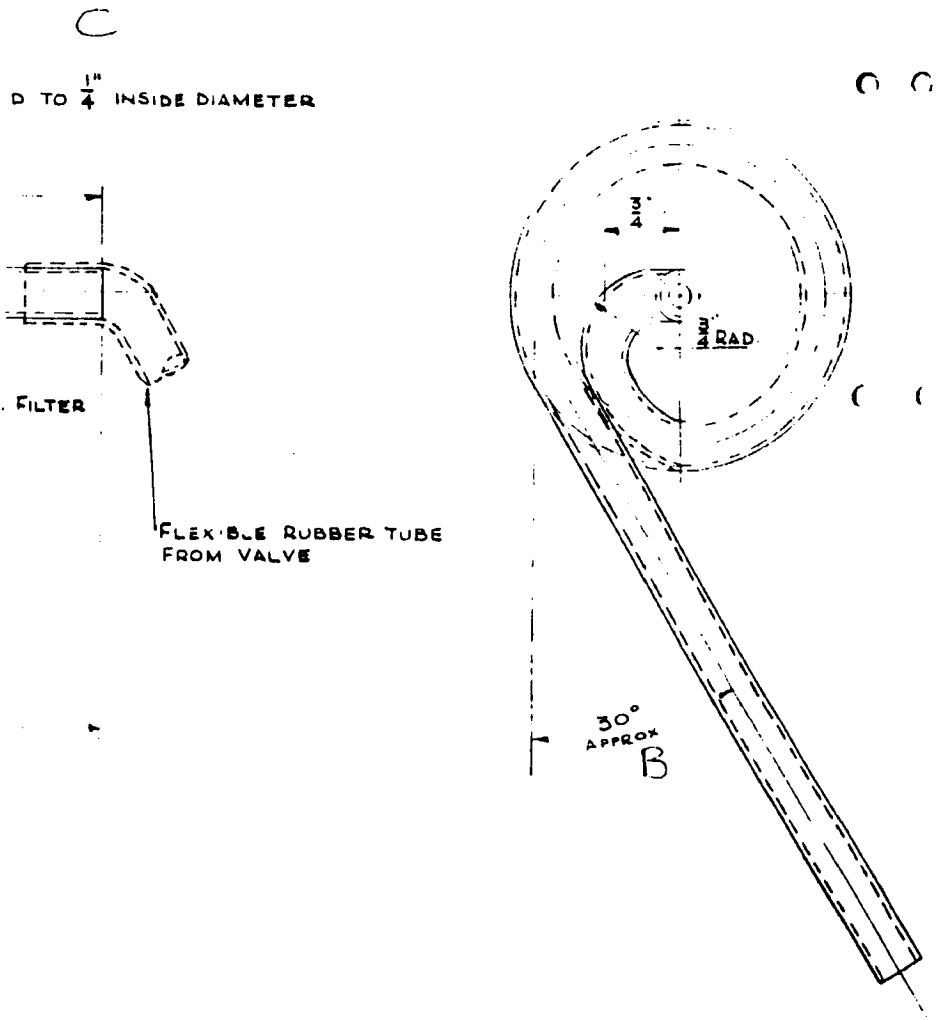
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DRG NO KR100.

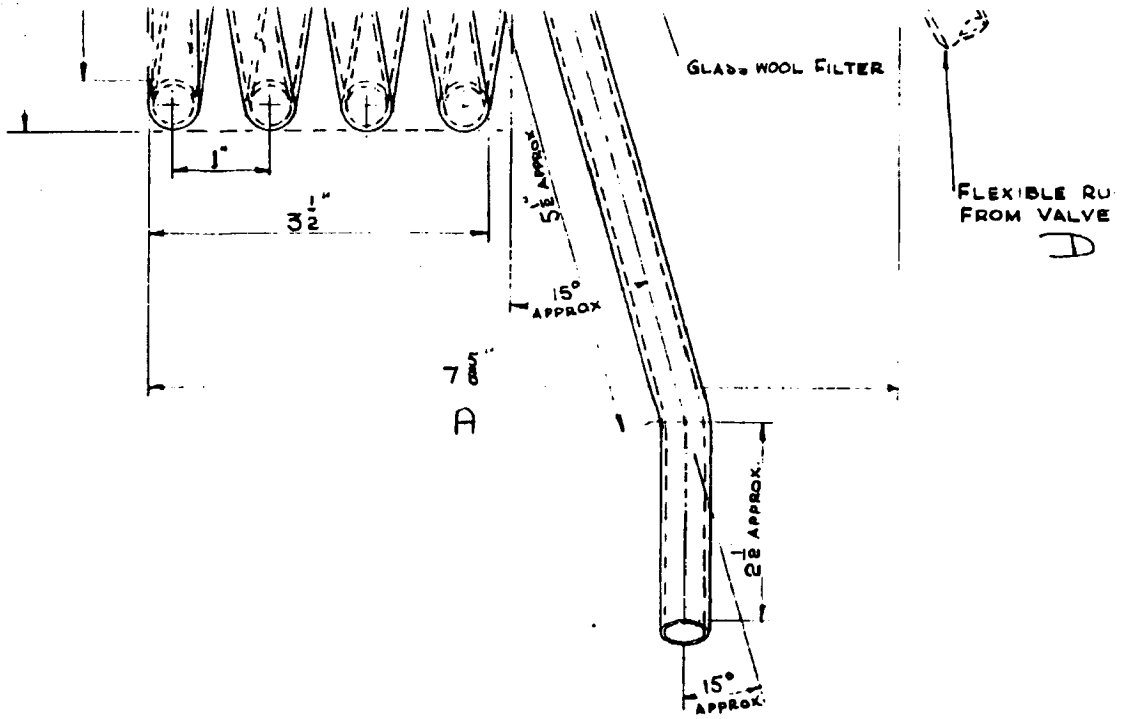


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30 X 20



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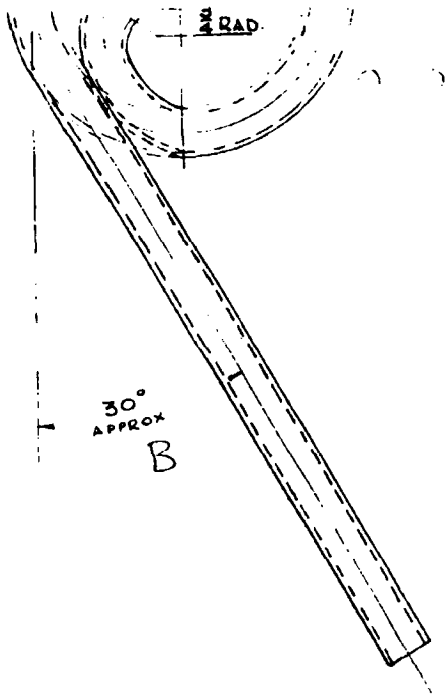


MATERIAL: PYREX. $\frac{1}{2}$ " O.D., $\frac{13}{32}$ " I.D. APPROX.

AMERICAN PROJECTION

HELICAL COLD TRAP

REF.	REVISION	DATE



FLEXIBLE RUBBER TUBE
COM VALVE

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30°
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CONFIDENTIAL

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DRN.	KHG	BRITISH-AMERICAN TOBACCO CO. LTD.	
CHD.		RESEARCH & DEVELOPMENT	
APPD.		SCALE	DRAWING No.
DATE	4/10/60	FULL SIZE.	RR. 100

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