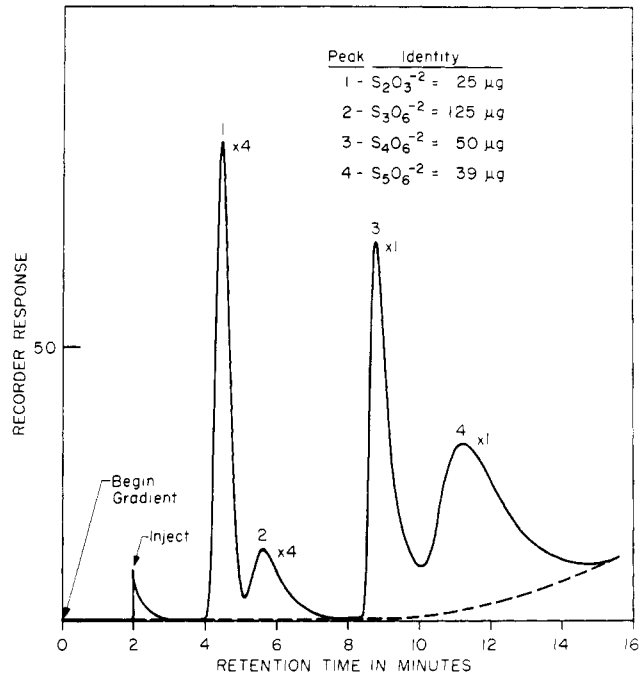

Figure 2. Separation of thionates in Wackenroder's solution

bon modifier to sharpen the $S_3O_6^{2-}$ peak in its separation from $S_2O_3^{2-}$ had a similar effect to increasing the pH with no modifier. However, above 1 volume-per cent THF in buffer, the separation became difficult to control. At pH 10.0 and 0.5 volume-per cent THF, an adequate $S_2O_3^{2-}$, $S_3O_6^{2-}$ separation occurred with a nearly Gaussian $S_3O_6^{2-}$ peak. The $S_4O_6^{2-}$ and $S_5O_6^{2-}$ peaks were then positioned on the chromatogram by the 2-minute injection-time lag. The finished chromatogram is shown in Figure 2. This solvent matrix continuously increasing in THF concentration over a 15-minute span produced a slight positive base-line drift as indicated in Figure 2 by the dotted line. The drift, however, has no effect if peak areas are measured by a digital peak analyzer.

Since increased concentrations of THF in buffer may raise the apparent pH of the buffer, it may be desirable to monitor the detector effluent with a pH meter and assume nearly complete removal of THF from the column during regeneration when the pH has returned to a value of 10.0. Our experience, however, has shown that 10 ml of solution A will return the pH to 10.0, yet 40 ml are required to restore the carbon to the condition of polarity required to effect a good separation of $S_2O_3^{2-}$ and $S_3O_6^{2-}$.

CONCLUSIONS

High-speed liquid chromatography offers a fast determination of the polythionates with no measurable decomposition during analysis. When samples of individual thionates were injected, one and only one peak resulted. The total sample is eluted from the column, and no column deterioration has been observed over a 2-month period. This method can be streamlined with a more sophisticated gradient generator and solvent turnover system.

Received for review May 21, 1973. Accepted June 28, 1973. Reference to specific equipment is made for identification only and does not imply endorsement by the Bureau of Mines.

Quantitative Chromatographic Determination of Maleic Hydrazide in Cigarette Smoke

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Maleic hydrazide (MH; 6-hydroxy-3(2*H*)-pyridazinone) is widely used to control tobacco sucker as demonstrated by the fact that at least 80% of all tobaccos harvested in the USA in 1968 and 1969 were treated with this agent (*1*). MH is commonly found in processed tobacco in concentrations of between 30 to 100 ppm (*1, 2*). G. J. Stone has assayed the fate of MH-^{14}C when cigarettes are smoked and found that 22% of the activity appeared in the mainstream smoke and that half of that β activity was located in the particulate phase. MH itself has not been identified in tobacco smoke prior to this investigation (*3-5*).

Maleic hydrazide is a proved mutagenic agent (*6, 7*), and is cytotoxic and antimitotic to mammalian cells *in vitro* (*8*), although it appears to be inactive as a carcinogen in adult rats and mice or as a tumor initiator on mouse skin (*9*). Epstein and Mantel reported that male mice treated in infancy with MH (0.4% hydrazine impurity) developed a high incidence of liver tumors (*10*).

This study reports a quantitative method for determining maleic hydrazide in tobacco and cigarette smoke. The determination employs a variety of techniques, including ion exchange chromatography, reaction with 4-chlorobenzyl chloride, absorption chromatography, and, finally, gas

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chromatography. Using a ^{63}Ni-electron capture detector, one can determine as little as 1 ng of MH. The transfer rate of unchanged MH from tobacco into smoke was determined for certain cigarettes.

EXPERIMENTAL

Apparatus. Cigarettes were smoked using a 20-channel automatic smoker RM 20/68 (*11*). A Hewlett-Packard gas chromatograph Model 7620A with FID and ^{63}Ni-ECD was used for the quantitative analysis. The β-radiation of the ^{14}C-labeled internal standards was counted with a Nuclear Chicago Isocap 300 Scintillation System. Ultraviolet absorption measurements were made with a Cary Model 118 and infrared spectra with a Perkin-Elmer Model 21. The mass spectra were determined with a Hitachi-Perkin-Elmer RMU-6D instrument by the Morgan Schaffer Corporation (Montreal, Canada) at 70 eV. The elemental analysis was carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich. Melting points were corrected. The laboratories were illuminated with yellow light (Sylvania Electric Tubes P-40 G.O.) which excludes radiation below 450 nm.

Reagents. All organic solvents were spectrograde. The other chemicals were analytical reagent grade. Absolute ethanol was dried over a type 3A molecular sieve, Fisher Scientific Company. 4-Chlorobenzyl chloride from Aldrich Chemical Company was purified by column chromatography on silica gel, which was washed before with *n*-hexane. Maleic hydrazide was a gift from Uniroyal Chemical. Its purity was checked by thin layer chromatography on silica gel in acetic acid/acetone/methanol/benzene (5:5:20:70). (No satisfactory gas chromatography system was found for MH.) Silica gel powder was purchased from J. T. Baker Chemical Company (mesh 80/100); analytical grade cation exchange resin (hydrogen form; mesh 200/400) from Bio-Rad Laboratories; precoated glass plates (aluminum oxide; 0.25-mm thickness; 5 × 20 cm) from Brinkmann Instruments; 3% XE 60 on Gas Chrom Q (mesh 100/200) was obtained from Applied Science Laboratories.

Cigarettes. Cigarettes without filter tips (85 mm) were purchased on the open market in July 1972 for use in establishing the analytical method. The experimental cigarettes (85 mm without filter) were obtained from T. C. Tso, Plant Genetics and Germplasm Institute, USDA, Beltsville, Md. The cigarettes were stored for at least 24 hours before smoking in a chamber with 60% rel. humidity (22 °C).

Internal Standards. 6-Hydroxy-3(2*H*)-pyridazinone-4,5-^{14}C (MH-2,3-^{14}C; spec. activity 2.8 mCi/mm) was purchased from Amersham/Searle, Arlington Heights, Ill.; its purity was checked by paper chromatography and TLC on silica gel. Bray's solution (60 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol, 20 ml glycol in 1000 ml dioxane) was used for counting; it gave efficiences of 83.5% for the unquenched MH-^{14}C.

2-(4-Chlorobenzyl)-6-(4-Chlorobenzyloxy)-3(2H)-Pyridazinone, [Bis(4-Chlorobenzyl)-MH; BB-MH]. For the analytical procedure, we explored the use of various synthesized derivatives of MH including the pentafluorosulfonate, the 3-chloropropyl carbonate, and the 2,2,2-trichloroethyl carbonate (*12*). Although the yields were rather high (>90%), we found that the derivatives are unsuitable for gas chromatography. 3-Chloropropyl carbonate, for example, decomposes during the gas chromatographic separation as shown by IR and TLC.

Yoneda and Nitta developed a method in which 56 mg of MH (0.5 mmol) was dissolved in 20 ml ethanol containing 60 mg of KOH and refluxed with 240 mg of 4-chlorobenzyl chloride (1.5 mmol) for 18 hours (*13*). The hot reaction mixture was then filtered and 60 mg of potassium in 20 ml ethanol and 240 mg of 4-chlorobenzyl chloride were added to the filtrate. The mixture was refluxed for 1 hour and filtered while hot. The filtrate was evaporated to dryness, dissolved in 10 ml water and extracted with ether. The ether extract was dried ($MgSO_4$) and the residue chromatographed on silica gel (70 g), eluting with *n*-hexane, *n*-hexane/benzene (1:1), benzene, and benzene/chloroform (4:1). The fraction eluted with benzene/chloroform contained BB-MH which was recrystallized from ethanol yielding 94 mg BB-MH (52%), mp 115–116 °C [lit. (*13*) mp 116 °C], UV max (98% ethanol) 312 nm (ϵ max 2345); IR (KBr) 1660 cm^{-1} (C=O); mass spectrum (70 eV) *m/e* (rel. intensity) 364 (0.65), 362 (3.2), 360 (4.8), 112 (34), 125 (100). *Anal.* Calcd. for $C_{18}H_{14}Cl_2N_2O_2$: C, 59.85; H, 3.91; Cl, 19.63; N, 7.76; O, 8.86. Found: C, 59.67; H, 3.98; Cl, 19.75; N, 7.83; O, 8.77

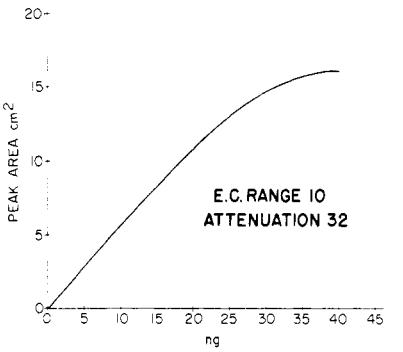

Figure 1. Linearity curve for 2-(4-chlorobenzyl)-6-(4-chlorobenzyloxy)-3(2*H*)-pyridazinone

Gas Chromatography. The most satisfactory separation of BB-MH from a concentrate from tobacco "tar" was obtained at 210 °C on a 3-mm × 0.6-m column packed with 3% XE 60 on Gas Chrom Q (mesh 100/200). Helium was used as carrier gas with a flow rate of 40 ml/min. The retention time for BB-MH was 24 minutes. Using a ^{63}NI-ECD, the detection limit was 1 ng (Figure 1). A 1:4 effluent splitter was installed to isolate BB-MH from a concentrate of tobacco smoke, so that 80% of the effluent could be collected in a glass capillary. The effluent was rechromatographed before spectral analysis.

PROCEDURE

Isolation and Quantitative Analysis of MH. *From Cigarette Smoke.* Two hundred cigarettes without filter tips were smoked under the standard conditions as reported in an earlier paper (*14*). The mainstream smoke was drawn through two gas wash bottles each filled with 200 ml of 2*N* sodium hydroxide. The smoke condensate suspensions were combined with the rinsing solvent (200 ml) and with 10 μg of MH-^{14}C. This suspension was filtered, washed, and extracted five times with 100 ml of ether. The back extractions of ether layers were combined with the water layer. This basic solution was acidified with concentrated hydrochloric acid and extracted five times with 100 ml of ether. The back washings of these ether layer extracts and the water layer were neutralized, evaporated to dryness, and continuously extracted with ethanol for 14 hours. The residue of the ethanol extract was dissolved in 5 ml of water and chromatographed on a cation exchange column (hydrogen form; 40 g) and eluted with water. We determined that after 40 ml of eluate were collected, the next 250 ml constituted the fraction with the β-activity. This fraction was neutralized and evaporated to dryness. The dry residue (P_2O_5) was dissolved in 50 ml of 2% ethanolic potassium hydroxide and refluxed with 4-chlorobenzyl chloride (2 g) for 18 hours. The hot filtrate was refluxed again with 0.35 g potassium in 20 ml absolute ethanol and 1 g 4-chlorobenzyl chloride for 1 hour, filtered while hot, evaporated to dryness, dissolved in water (10 ml) and extracted with ether (5 × 20 ml). The residue of the dry extract ($MgSO_4$) was chromatographed on about 60 g silica gel by eluting with *n*-hexane (100 ml), *n*-hexane/benzene (1:1; 200 ml), benzene (300 ml), and benzene/chloroform (4:1; 600 ml). The β-activity was found in the last 200 ml. The residue of this fraction was rechromatographed on five precoated aluminum oxide TLC plates with *n*-hexane/chloroform (2:3) as developing solvent (R_f 0.5). The BB-MH bands (visible at 254 nm) were extracted with ether and combined. The residue was dissolved in 0.3–0.5 ml of ethanol and analyzed by gas chromatography; the recovery rate was determined by counting of aliquots.

From Tobacco. Gas Chromatography. The tobacco from 5 to 10 cigarettes was extracted with water for 40 hours. After adding MH-^{14}C as internal standard, the extract was evaporated to dryness and passed through a cation exchange column (2 × 45 cm). The subsequent steps of the analytical procedure for MH were the same as for the analysis of MH in cigarette smoke.

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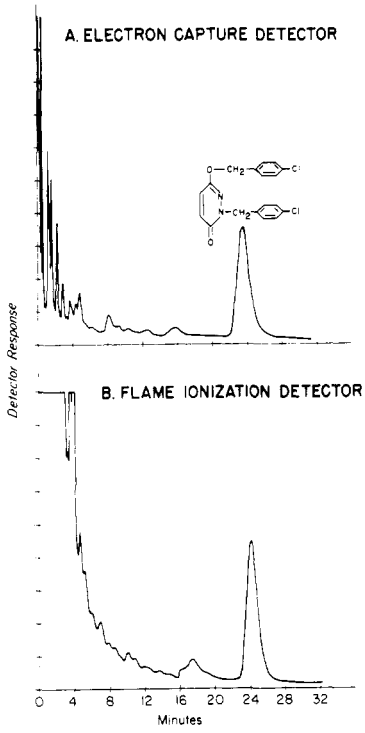

Figure 2. Gas chromatograms of a concentrate of 2-(4-chlorobenzyl)-6-(4-chlorobenzyloxy)-3(2*H*)-pyridzinone

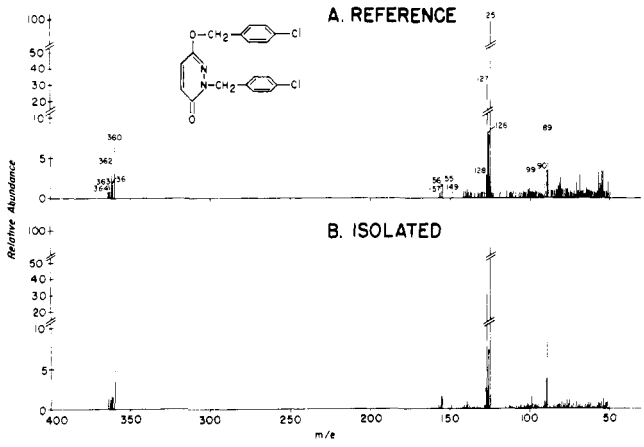

Figure 3. Mass spectra of 2-(4-chlorobenzyl)-6-(4-chlorobenzyloxy)-3(2*H*)-pyridazinone

Colorimetric Method. Wood (*15*) and Lane (*2, 16*) have determined MH by alkaline digestion of the tobacco sample, followed by rapid distillation of the sample together with zinc and ferrous chloride as reductants. In the distillate, hydrazine was identified by the yellow color which it forms with 4-dimethylaminobenzaldehyde.

RESULTS AND DISCUSSIONS

Gas chromatographs with FID and ^{63}Ni-ECD of a concentrate from cigarette smoke condensate with maleic hydrazide (MH) as bis(4-chlorobenzyl) derivative (BB-MH) are shown in Figure 2. The column effluent with the retention time of BB-MH was collected and identified by UV and IR spectra, and by its mass spectrum (Figure 3).

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Table I. Maleic Hydrazide (MH) in U.S. Blended Cigarette (Mainstream Smoke and Tobacco)

No. of Analysis	MH in smoke, μg/cig.[a]	Tobacco analyzed, dry wt; gram	MH in tobacco, μg[a] in 1 g dry tobacco	MH in tobacco, μg[a] MH/cig.
1	1.03	7.23	32.5	30.6
2	1.25	4.77	29.9	28.2
3	1.08			
4	1.24			
5	1.22			
Average	1.16			29.4
Standard deviation	0.10			1.6
Deviation coefficient	8.7%			5.4%
Transfer rate	3.95%			

[a] Calculated with the isotope dilution method.

Table II. Maleic Hydrazide (MH) in Experimental Cigarettes

Tobacco type	MH in smoke, μg/cig.[a]	MH in tobacco μg[a] Cigarette	1 g Dry Tobacco	Transfer rate, %
MH treated Burley cigarettes (1970 crop)	1.82	17.6	19.7	10.3
Hand suckered control Burley cigarettes (1970 crop)	<0.1	<0.1	...	...
MH treated flue-cured tobacco cigarettes (1970 crop)	1.76	25.2	24.7	7.0
Hand suckered control flue-cured tobacco cigarettes (1970 crop)	0.18	<0.1	...	...
Commercial cigarettes	1.16	29.4	31.2	3.95

[a] Calculated with the isotope dilution method.

Five times 200 standard cigarettes were smoked for the quantitative analysis. Maleic hydrazide-^{14}C was used as the internal standard. From the final gas chromatograms, it was calculated that the mainstream smoke of one 85-mm U.S. blended cigarette without filter tip contains 1.16 μg MH (Table I). The experimental deviation was ±8.7%. The recovery rate varied between 40–50%.

The transfer rate of MH from cigarette tobacco into the mainstream smoke was determined by analyzing the tobacco for MH according to Wood and Lane (*2, 15*) and further by the method reported above. Although we obtained reproducible data (±6.0%), the values obtained with the colorimetric method were 30–35% lower than those obtained with the method reported here. Lane *et al.* stated in an earlier paper that the distillate from tobacco contains material which causes "intensive red interference coloration" (*16*). Despite the suggested corrective measures for the optical measurements (*2*) we were unable to obtain data which were comparable with those of our method.

In addition to the standard nonfilter cigarettes, we also analyzed the tobacco and mainstream smoke of experimental cigarettes which were made from tobaccos high in maleic hydrazide or from hand suckered tobacco. The results of these analyses are summarized in Table II. They indicate that the transfer rate of MH from tobacco into mainstream smoke is partially dependent on the concen-

Table III. Analysis of Experimental Cigarettes and Some Selected Smoke Constituents[a]

Parameters	MH treated Burley tobacco	Hand suckered control Burley tobacco	MH treated flue-cured tobacco	Hand suckered flue-cured tobacco	Commercial cigarette
Dry weight, mg of tobacco	0.894	0.977	1.021	0.943	0.941
Draw resistance, cm[b]	6.5	7.5	5.5	5.5	8.5
Burning rate,[c] mg tobacco/min.	70.9	61.2	54.2	47.4	52.6
Average number of puffs	10.3	10.6	10.8	10.6	10.4
CO, vol. %[d]	3.98	3.99	3.64	3.83	3.85
CO_2, vol. %[d]	9.69	9.69	8.7	9.06	9.04
Dry TPM, mg[e]	28.7	27.5	40.9	39.7	28.6
Nicotine in tobacco, %[f]	7.5	8.2	4.8	5.9	2.75
Nicotine in smoke, mg	6.8	7.2	5.5	6.0	2.1
Nicotine transfer rate, %	13.9	12.3	15.3	14.8	11.1
MH in tobacco, μg/cig.	17.6	<0.1	25.2	<0.1	29.4
MH in smoke, μg/cig.	1.82	<0.1	1.76	0.18	1.16
MH transfer rate, %	10.3	...	7.0	...	3.95

[a] Smoke data derived from cigarettes smoked under standard conditions. [b] For an air flow of 17.5 ml/sec. (see ref. 4). [c] Method, see ref. 18. [d] Method, see ref. 17. [e] Federal trade Commission value for TPM (total particulate matter) = TPM wet minus water and minus nicotine (see ref. 19). [f] Based on dry weight of tobacco (see ref. 20).

tration of MH on the leaf. However, it appears likely that a number of other factors, such as burning rate and draw resistance, influence the transfer rate of MH as such factors are known to influence the transfer rate of nicotine (17) (Table III). However, more work is required in order to establish which factors are most influential on the transfer rate of MH.

This study established the presence of unchanged maleic hydrazide in the smoke of tobaccos treated with this sucker growth inhibitor. Although the data on the carcinogenicity of MH are ambiguous, MH is a proved cytotoxic and mutagenic agent. The analysis of maleic hydrazide in tobacco smoke is therefore essential.

It is also possible that the MH used in the cultivation of tobacco may serve as a precursor for carcinogenic hydrazines in tobacco smoke (21, 22) since the burning cone of a tobacco product is a reducing atmosphere. Recently, W. R. Johnson *et al.* found that in the burning cigarette, the reduction of nitrate nitrogen to ammonia is remarkably efficient. This and other observations support the hypothesis that part of the maleic hydrazide in tobacco not only appears unchanged in the smoke, but also that a portion may serve as a precursor for carcinogenic hydrazines.

Received for review April 12, 1973. Accepted May 15, 1973. No. XIX of "Chemical Studies on Tobacco Smoke."

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Analytical and Preparative Chromatography of Hemins on Polyamide Media

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Techniques for the purification and analysis of hemins are still rather primitive, despite the biochemical importance of these compounds. Chromatographic methods are available for separating hemin esters (1-3) and for separating hemin acids into classes according to the total number of carboxylic acid residues on the porphyrin ring (4). However, only two effective procedures have been described for the separation of the natural and synthetic hemin diacids. A reversed-phase paper chromatographic procedure that used silicone as the stationary phase allowed the separation of the dicarboxylic hemato-, deutero-, meso-, and protohemins (4). A second method, one more adaptable to preparative scale, is that of continuous elu-

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