COMPOSITION STUDIES ON TOBACCO. XXI*.—The Headspace Vapours of Leaf**

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A procedure for the collection of tobacco headspace vapours without the use of steam, heat, reduced pressure or elaborate apparatus is described. Typical results are presented showing the presence of at least 35 components in the concentrated vapours from Turkish tobacco examined by temperature programmed gas chromatography. The use of selective removal techniques to classify components by functional group reactions is described, and tentative identifications of some of these components by the use of retention indices are reported.

Introduction

Composition studies on tobacco have generally been concerned with large classes of substances grouped by solubility in various solvents[1] or by the possession of a common functional group.[2] Some time ago we initiated studies on the composition of the mixture of volatile compounds continually released to the air by cured tobacco, which accumulate in the headspace above tobacco stored in closed containers. Variations in the composition of this aroma-bearing mixture may influence the desirability of tobacco for manufacturing purposes and the character of the tobacco smoke.

Previous studies of the volatile compounds of tobacco have been concerned with the leaf substances obtained by solvent extraction[3] or steam distillation at ordinary pressure[4, 5] or under vacuum.[6] It was desired to avoid any procedure which might alter the natural composition of the headspace vapours during collection or which might include or generate additional components not normally present in the headspace itself. Also, since the moisture content of a tobacco sample has been shown to be related to the total amounts of certain volatile carbonyl compounds obtainable by steam distillation,[2] it was desirable that the procedure adopted would not alter the normal moisture content of the sample. Many alternative methods of collection were tried and discarded before a procedure was adopted.

The present paper describes details of a method for the collection of tobacco headspace vapours and the chemical composition of such vapours.

Experimental

Apparatus

A commercial cylinder of dry nitrogen equipped with a pressure-reducing regulator and needle valve was connected to the top of the tobacco container, a gas-washing bottle of 500 ml capacity with a standard-taper ground-joint and fittings for springs to hold the two parts together (Fig. 1). The gas passed downward through the tobacco to the bottom of the central tube, protected by a coarse fritted glass filter, then through the central tube to the exit at the top of the bottle. Entrance and exit tubes were further protected by loose plugs of glass wool. From the exit tube the mixture of vapours and nitrogen was led through glass tubing and

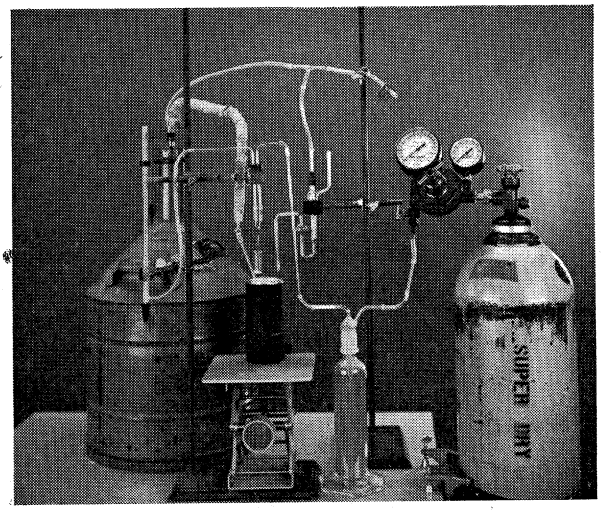

Fig. 1. *Photograph of apparatus for collection of headspace vapour*

adaptor to one of two 22-gauge hypodermic needles which extended through a rubber cap into the neck of a small serum bottle (nominal capacity 5 ml) arranged so that its lower portion could be immersed in a bath of liquid nitrogen maintained at constant level in a Dewar flask. During collection of the vapours, the serum bottle contained an amount of anhydrous sodium sulphate (0·1–0·3 g) sufficient to saturate the water expected to condense and to be added as aqueous classification test reagents. The exit needle was attached through glass tubing to a soap-film flowmeter. Gas chromatographic analysis of the collected headspace vapours was accomplished using a flame ionisation instrument (F. & M. Scientific Co. Model 1609*) equipped with a linear temperature programmer (Model 240) for the column oven.

Sample Collection

Initially, several collection methods were used similar to those employed for other natural products. A few comments on these attempts may be of interest.

Direct sampling of the unconcentrated headspace vapours by means of sampling valves or syringes (see references [7–9]) was not very successful because of the extremely small amounts present in tobacco headspace.

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The methods of Nawar *et al.*[10,11] involving mechanical circulation of vapours into various collectors presented many complications too numerous to detail. Modifications of the collecting traps including some similar to those of Farrington *et al.*[12] and West *et al.*[13] were relatively unsuccessful except for the use of a U-tube containing tobacco itself as an adsorbent, which proved to be an effective trap although the chromatographic results on the trapped volatiles were difficult to reproduce. The method of Hornstein & Crowe[14] involving collection of volatiles directly on a cooled chromatographic column and elution by warming the column was an effective method of trapping constituents. However, difficulties in obtaining leak-free connections prevented precise and consistent measurements of retention indices.

An adaptation of an apparatus for total collection of gas chromatographic eluates (F. & M. Scientific Co., Model TCS-3) was investigated. This apparatus permits collection in a large (300 ml) evacuated glass bulb of the total volume of carrier gas carrying an eluting gas chromatographic peak. After collection, a nipple in the bulb (5 ml volume) is cooled and the vapours within the bulb are condensed. This apparatus was employed to collect headspace vapours of tobacco by repeated filling of the bulb and condensation of the vapours. Chromatographic results similar to those reported above were obtained; however, several shortcomings were apparent, including impractically long waiting periods required for condensation of the vapours in successive samplings and leakage of laboratory air into the evacuated system.

Many other variations in technique were studied before the final method was adopted. Most of the variations failed to give satisfactory results and, in many cases, no chromatographic peaks were obtained. To reproduce the findings reported with the adopted method, strict adherence to the details of the method is required.

Final method adopted

The gas-washing bottle was filled loosely with cured tobacco leaves (250 g) ground to pass a 30-mesh screen and the top was attached by carefully working the central tube through the tobacco to the bottom of the bottle so that a tight fit at the ground joint was obtained and secured by attaching springs or rubber bands. Anhydrous sodium sulphate (0·1 g) was placed in a serum bottle (5 ml) which had first been flushed with dry nitrogen. The bottle was then closed with a rubber cap and was connected to the collection system by pushing the needles through the cap so that their tips just protruded into the wide part of the bottle. The entire system was then flushed with nitrogen at room temperature until all air had been removed. The bottom third of the serum bottle was gradually brought to liquid-nitrogen temperature, care being taken to maintain a positive flow of nitrogen through it at all times, as judged by the soap-film flowmeter. The upper third of the bottle was kept at a temperature above freezing by directing a stream of air across it in order to prevent plugging of the needles. When temperatures had reached equilibrium, the nitrogen flow was adjusted to 30–50 ml per minute and was continued at this rate during the 4-h collection period. At the end of this time the exit needle was withdrawn and the entrance needle was fitted with a gas-tight syringe (capacity 1·0 ml), preferably with the gas-flush modification. The bottle was then allowed to come to room temperature and was finally immersed in a bath at 60° for 3 minutes before removal of a 1·0-ml aliquot of the vapour for injection.

Gas–liquid chromatography

Stainless steel, packed, single columns (10 ft × ¼ in.) were used, with helium carrier gas flow rate of 50 ml/min. regulated by flow controller and needle valve; helium pressure at cylinder gauge, 30 psi; injection port temperature, 225°; detector block temperature, 250°; and flamehead temperature, 325°. With Carbowax 20M or Silicone SE-30 as the stationary phase (25% on Anakrom ABS), the oven temperature was programmed at 5° per minute from 75° to 220°. With tritolyl phosphate–glycerol (MacDonald & Brunet[15]), tritolyl phosphate alone or β,β'-oxydipropionitrile as the stationary phase, maximum temperatures were lower; isothermal operation was also used with these phases. Since the gas chromatograph used is a single-column instrument, temperature programmed runs show a rising baseline. With the flame ionisation detector, upper temperature limits for all phases were somewhat lower than would be the case with less sensitive detectors.

Classification tests

Component peaks were classified by use of the following selective removal reagents:[16,17]

(*a*) *Acidic hydroxylamine reagent:* 1·14 g of hydroxylamine hydrochloride was dissolved in 10 ml of N/1 sodium hydroxide solution and diluted to 50 ml with water.

(*b*) *Basic hydroxylamine reagent:* 1·14 g of hydroxylamine hydrochloride was dissolved in 50 ml of N/1 sodium hydroxide solution.

(*c*) *Mercuric chloride reagent:* a saturated aqueous solution was prepared at room temperature.

(*d*) *Potassium permanganate reagent:* 2·5 g of potassium permanganate were dissolved in 50 ml of water.

When a sample was to be used for classification tests, the amount of sodium sulphate in the serum bottle at the start of collection was increased to 0·3 g so as to saturate the water from the aqueous reagent to be added as well as that from the tobacco vapours. After the serum bottle had been allowed to come to room temperature with the 1·0 ml gas-tight syringe in place as described under 'Sample Collection', the syringe and needle were withdrawn and 0·5 ml of the classification reagent was added through the rubber cap from a liquid measuring syringe. The bottle was shaken for several minutes and was then set aside at room temperature for 1 h with occasional shaking. The gas-tight syringe and needle were then re-inserted, the bottle was immersed in the 60° bath for 3 minutes and a 1·0-ml aliquot of the vapour was withdrawn and injected into the gas chromatograph. Comparison of the resulting chromatogram with either a previously obtained chromatogram or a chromatogram on 1·0 ml of vapour removed before addition of the classification reagent, revealed which peaks were eliminated completely or reduced in size.

A mixture of nitrogen and the vapours of acetone, C_{3-5} n-aldehydes, methyl propionate, methanol, n-propanol and benzene was prepared in a serum bottle for use in checking the action of classification reagents. Both of the hydroxylamine reagents eliminated completely the peaks from aldehydes and ketones and diminished those from alcohols. Basic hydroxylamine removed methyl propionate as well. Potassium permanganate removed all but acetone, methyl propionate and benzene.

Identification

Kováts[18] retention indices as modified for use with linear

temperature programming by Van Den Dool & Kratz[19] were used to obtain the tentative identifications reported. Normal hydrocarbons and ethyl esters of straight-chain fatty acids were used as reference compounds. When ethyl esters were used the results were calculated to a n-hydrocarbon reference system as described by Van Den Dool & Kratz,[19] whose tables of retention indices were confirmed and supplemented as necessary by injections of pure compounds. The tables of isothermal retention indices of West *et al.*[20] who used tritolyl phosphate and β,β'-oxydipropionitrile stationary phases were also helpful.

Known compounds were injected as the vapour, using a technique similar to that used for the headspace samples. Small amounts of the pure liquids were placed in serum bottles from which all air had been removed by flushing with nitrogen. After the bottle had been closed with a rubber serum cap and time allowed for vapour-liquid equilibrium to be established, aliquots of the vapour varying in size from 1 μl to several hundred μl were measured at room temperature (or at elevated temperatures with less volatile compounds) in gas-tight syringes, the amount of each being adjusted until a peak similar in size to that of the corresponding unknown component was obtained. Retention temperatures were determined by the technique of Van Den Dool & Kratz,[19] the pyrometer temperature on the chart being marked at 5° intervals and the chart being as a graph. To correct for the rising baseline, an arbitrary line was drawn between inflexions at the start and end of well-separated peaks, and the point at which this line met a line tangent to the rising portion of the peak in question was used to locate the retention temperature. This is more desirable than use of the apex of the peak, the position of which depends to a larger extent on the amount injected. Reproducibility was as good as that reported by Van Den Dool & Kratz.[19]

Co-chromatography was accomplished by adding the required volume of vapour of the known substance to the serum bottle containing the unknown, agitating for a sufficient time to produce adequate mixing, and withdrawing an aliquot of the mixed vapours for injection into the gas chromatograph.

Frequent blank runs showed that insignificant amounts of impurities were contributed by the nitrogen gas used, by the short length of plastic or rubber tubing used in connecting together the various parts of the apparatus, and by the reagents used for selective removal studies. It was found, however, that unless laboratory air was rigidly excluded from the tobacco samples, the collection apparatus, and the gas-tight syringes by flushing with nitrogen before each use, artifacts could be introduced. Use of the gas-flush modification of the syringes was extremely helpful in this respect; ordinary gas-tight syringes without this modification tended to hold traces from previous injections tenaciously. Efficiency of the trapping system was proved by recovering trace amounts of known volatile compounds added to an empty gas-washing bottle instead of the tobacco and by the absence of detectable amounts of headspace vapour components in a second trap immersed in liquid nitrogen in series with the first.

Results and Discussion

Representative results

Table I and Fig. 2 show the results of classification tests and retention indices, and a typical chromatogram using the adopted method on cured Turkish (Smyrna) tobacco leaves. The standard retention indices reported in Table I are referred to the n-paraffinic hydrocarbon system in which the hydrocarbons with n carbon atoms are assigned the indices 100 n and other indices are calculated from retention temperatures using the following relationship,

$$I = 100i \frac{X—M_n}{M_{n+i}—M_n} + 100n$$

in which I is the standard retention index of the unknown component and X, M_n and M_{n+i} are the retention temperatures of the unknown component and two marker hydrocarbons of n and $n + i$ carbon atoms respectively. Over a limited range the index varies linearly with temperature. Under our conditions, in which programming was started simultaneously with sample injection at 75°, the change in index per degree was almost constant over the range 95–200°, but below 95° this ratio increased rapidly.

The chromatogram presented in Fig. 2 was made with a maximum sensitivity setting of attenuation 8 in range 1 since higher sensitivity gave an excessive noise level.

Components classified as inert in Table I were not removed by any reagent. Aldehydes were completely removed by both acidic and basic hydroxylamine and by potassium permanganate solution; the ketone was unaffected by permanganate. Evidence of carbonyl groups was confirmed by the formation of precipitates when the effluents, collected by means of a heated stream-splitting device inserted between the column exit and the detector, were reacted with 2,4-dinitrophenylhydrazine reagent. The ester was removed by basic hydroxylamine but not by acidic hydroxylamine or permanganate. Peaks 15, 16, 17, 20–23 and 25–27 were completely removed by permanganate but were not removed or were only partially reduced in size by the hydroxylamine reagents; these represent easily-oxidised, non-carbonyl compounds, which would include alcohols, aromatic compounds with oxidisable side-chains, unsaturated aliphatic hydrocarbons, etc. Saturated aqueous mercuric chloride did not remove completely any peaks in these tests on Turkish tobacco, although in some runs on this and other tobaccos evidence of removal of one or more peaks by this reagent was obtained. Although no peaks above number 27 were completely removed by any reagent, some of these may be unreactive only because of high molecular weight or poor solubility in the aqueous classification reagents under the test conditions, and others may be inert but reduced in size only because of low volatility after treatment with the reagent.

Peaks 1 and 2 of Table I appeared in all blank runs. They represent, at least partially, artifacts caused by pressure changes upon injection or possibly trace impurities. The possible presence of highly volatile headspace components in this region has not been entirely excluded.

These results show that the sample-collection method described permits the concentration and separation of the extremely small amounts of volatile compounds occurring in tobacco headspace vapours without elaborate trapping techniques under conditions closely similar to those obtaining in the natural state of the sample, which is not subjected to heat, steam, excess water vapour or the oxygen of the air. The vapours collected are not forcibly removed under the drastic conditions of a vacuum, but at a pressure close to that of the atmosphere. The moisture content of the sample is not changed appreciably during sampling (less than 0·25 ml of water is removed from 250 g of tobacco of normal moisture content during the specified collection period). Reproducibility is excellent and efficiency of collection is close to 100% even for very volatile substances at liquid nitrogen

TABLE I

Data on possible identity of vapour components in the headspace over Turkish tobacco separated on Carbowax

Peak no.	Chemical characteristics	Possible identity	Retention index of peak	Retention index of known substance
1	Inert	Artifact		
2	,,	,,		
3	,,	n-Pentane	500	500
4	,,	?		
5	,,	?		
6	,,	n-Hexane	600	600
7	,,	?		
8	,,	?		
9	,,	n-Heptane	707	700
10	Aldehyde	Acetaldehyde	743	?
11	,,	Propionaldehyde	805	796
12	Ketone	Acetone	823	823
13	Aldehyde	n-Butyraldehyde	873	866
14	Ester	Methyl propionate	883	893
15	Oxidisable	Methyl alcohol	912	911
16	,,	Ethyl alcohol	923	927
17	,,	?	948	
18	Inert	Benzene	958	953
19	Aldehyde	n-Valeraldehyde	1000	983
20	Oxidisable	?	1020	
21	,,	?	1032	
22	,,	?	1043	
23	,,	Toluene	1063	1058
24	Aldehyde	n-Caproaldehyde	1099	1089
25	Oxidisable	?	1121	
26	,,	*p*-Xylene	1149	1151
27	,,	*m*-Xylene	1169	1173
28	Uncertain	?	1203	
29	,,	?	1224	
30	,,	*o*-Xylene	1240	1242
31	,,	?	1256	
32	,,	?	1278	
33	,,	?	1299	
34	,,	?	1318	
35	,,	?	1338	
36	,,	?	1356	
37	,,	?	1371	

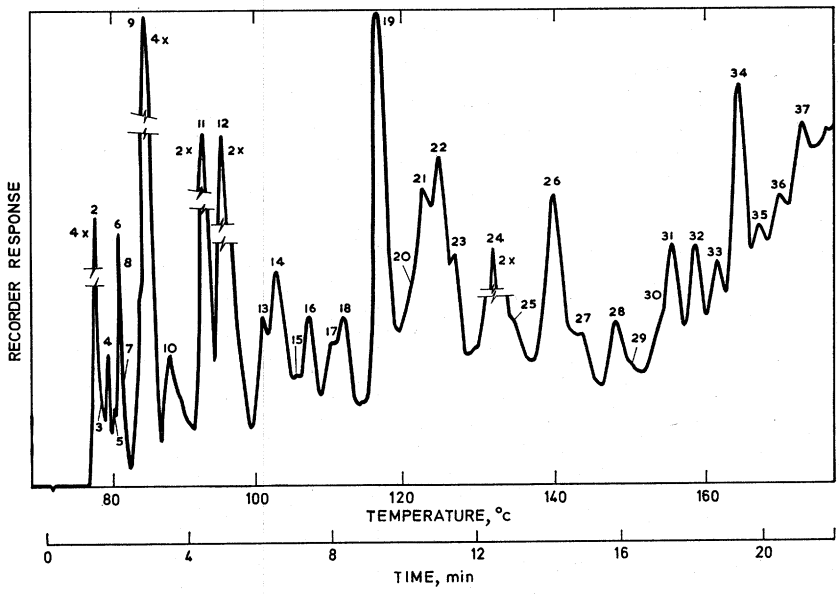

Fig. 2. *Gas–liquid chromatogram of headspace vapour from* 250 *g of Turkish* (*Smyrna*) *tobacco on Carbowax* 20 *M column*

IX=range 1, attenuation 8.

temperature. Cooling with Dry Ice was shown to be less efficient, giving chromatograms in which the early peaks are less prominent.

Tentative identifications

Table I also lists retention indices for known compounds calculated from the retention temperatures obtained with the pure substances injected under standard conditions on the Carbowax 20M column. Values for some, but not all, of these are contained in the tables published by Van Den Dool & Kratz.[19] Agreement with their values in most cases is good enough to confirm their conclusion that, although the retention temperature is not an absolute constant and will vary depending upon the heating rate, carrier gas flow-rate and other operating parameters, the difference between retention temperatures of members of a homologous series is relatively constant under a variety of conditions. The problem of determining with certainty to which homologous series within the same functional group class an unknown component belongs, still remains. Although we have supplemented the list of known compounds reported by Van Den Dool & Kratz[19] and confirmed the retention indices of many of the peaks on the SE-30 column, it is obvious that absolute identification cannot be achieved by these techniques alone. Nevertheless, the data in Table I indicate the probable occurrence of methanol, ethanol and higher alcohols; several aliphatic hydrocarbons, including n-pentane, n-hexane and possibly n-dodecane; simple aromatic hydrocarbons (benzene, toluene and xylenes); and a number of aliphatic aldehydes, ketones and esters. Co-chromatography with methanol, ethanol, propionaldehyde, methyl propionate, acetone and benzene tended to confirm these identifications, since the suspected peaks were enhanced without peak broadening. This of course does not exclude the possibility that the unknown is another compound of the same functional group class with closely similar retention. The presence of both methanol and ethanol was confirmed using the glycerol–tritolyl phosphate stationary phase recommended by MacDonald & Brunet[15] for better separations of low-boiling polar compounds; the methanol peak was much the smaller of the two. Polar compounds were not completely separated by the silicone SE-30 stationary phase, but produced broad, tailed peaks due to polar compounds which obscured the sharper peaks given by non-polar substances. This made interpretation of the results of functional group tests difficult. Acetone and a number of the other compounds listed were also detected in tobacco headspace vapours using the tables of retention times published by West *et al.*[20] for use with tritolyl phosphate and β,β'-oxydipropionitrile stationary phases.

The presence of the above hydrocarbons, n-caproaldehyde and methyl propionate in tobacco leaf has not been reported previously, although Onishi & Nagasawa[3] isolated an unidentified C_6-aldehyde from leaf.

Quantitative considerations

No attempt to determine absolute or relative amounts of the components of tobacco headspace vapours was made, but peaks of a size range similar to that observed for the main components in the headspace chromatograms were obtained when injections of 0·1–2·0 μg of benzene as vapour were made. Other tobaccos showed minor qualitative differences in headspace vapour chromatograms, but each exhibited a characteristic quantitative pattern.

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