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FLUORESCENT PHENOLS AS POSSIBLE INDICES OF TOBACCO QUALITY

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Abstract—A method employing fluorimetry and spectrophotometry on eluates from a single paper chromatogram was devised for the rapid analysis of chlorogenic plus neochlorogenic acids, rutin, 6-hydroxykynurenic acid, scopolin and scopoletin in flue-cured tobacco and was applied to fifty-six carefully graded samples from the 1964–65 North Queensland crop. Quality, as determined by the minimum price set for each grade, showed no overall correlation with the content of any of these compounds. Within a given leaf colour class there was a significant negative correlation of quality with scopoletin content. Within a given leaf position class there were no correlations. CGA, rutin and scopoletin content varied with leaf position, i.e. showed distribution gradients within the plant. Only CGA content varied with colour. The levels of CGA, rutin and 6-HKA were positively correlated with each other but not with those of scopolin or scopoletin.

INTRODUCTION

PHENOLIC compounds have been implicated for several decades as constituents having a positive influence on the quality of the cured tobacco leaf. By "quality" is meant the sum total of subjective impressions such as colour, aroma, etc. which determine even today the market value of the leaf. According to Shmuk and Semenova[1] and König and Dörr[2] these compounds exert their main effect on the flavour of the smoke. Better documented biochemically is their contribution, via enzymic oxidation, to pigments of high molecular weight[3] which are important in determining colour.

As a result of these and other studies a frequently used index of quality has been "total polyphenols", most commonly determined as the reducing capacity of the lead-precipitable material in the acid hydrolysate of a crude tobacco extract. Apart from the serious shortcomings of the analytical method,[4] this approach ignores the fact that the individual compounds, with their different biosynthetic relationships, cannot validly be assumed to behave as a group under changing physiological conditions. Thus, in the leaves of boron-deficient tobacco plants, there is a marked increase in scopolin content which is not accompanied by any increase in chlorogenic acid or rutin.[5] It is therefore not surprising that total phenols, either alone or in combination with other similar parameters, show no closer correlation with quality than, for example, total soluble carbohydrate.[6]

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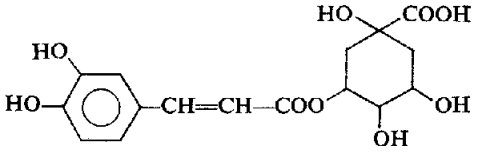
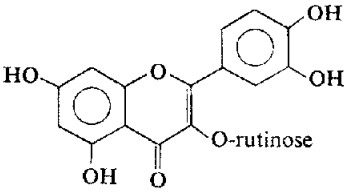
[1] A. A. SHMUK and V. SEMENOVA, *Publ. State Inst. Tobacco Makhorka Ind. Krasnodar, U.S.S.R.* Bull. No. **33** (1927).
[2] P. KÖNIG and W. DÖRR, *Biochem. Z.* **263**, 295 (1933).
[3] H. E. WRIGHT, W. W. BURTON and R. C. BERRY, *Arch. Biochem. Biophys.* **86**, 94 (1960).
[4] M. K. MIKHAILOV, *Tabak* **20**, 41 (1959).
[5] R. WATANABE, W. J. MCILRATH, J. SKOK, W. CHORNEY and S. H. WENDER, *Arch. Biochem. Biophys.* **94**, 241 (1961).
[6] C. PYRIKI and W. F. HOMANN, *Z. Lebensmittelunters. Forsch.* **97**, 281 (1953).

Since the detailed composition of the low molecular weight phenolic fraction of the tobacco leaf is comparatively well understood,[7] the time appears overdue for investigation of the trends with quality shown by individual phenols. Such an approach should yield information both susceptible to biochemical interpretation and of potential utility to the plant breeder.

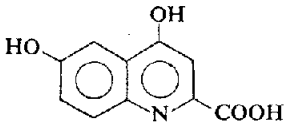
This paper examines the correlations between the quality of samples representing fifty-six grades of flue-cured tobacco from the North Queensland crop and their contents of chlorogenic acid (CGA) (I), rutin (II), 6-hydroxykynurenic acid (6-HKA) (III), scopolin (IV) and scopoletin (V). These compounds represent the three main classes of tobacco phenols and are formed by differing biosynthetic pathways.[7] 6-HKA, a new tobacco constituent,[8] is probably a metabolite of tryptophan.[9]



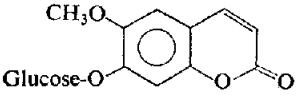
(I) Chlorogenic acid



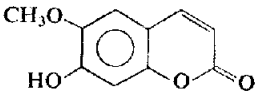
(II) Rutin



(III) 6-Hydroxykynurenic acid



(IV) Scopolin



(V) Scopoletin

The five phenols are the major components, on paper chromatograms of cured leaf extracts, that fluoresce in u.v. light. Johanson[10] had earlier reported that leaf of good quality fluoresces brightly whereas "trashy" leaf shows no fluorescence. It was of interest to ascertain *inter alia* whether the correlation implicit in this finding could be accounted for in terms of the phenolic compounds.

RESULTS AND DISCUSSION

The main difficulty in devising a rapid analytical procedure of adequate sensitivity arose from the wide disparity in levels of the phenols; it can be seen from Table 3 that the average CGA level was over 1000 times that of 6-HKA. Column chromatography appeared to offer the most flexible approach, but clean separation and adequate recovery of all compounds was not achieved on any of the adsorbents polyamide, polyvinylpyrrolidone, DEAE cellulose or activated carbon. Extensive screening of solvent systems and the use of a thick paper finally enabled adequate resolution of all five phenols on a single 10-in. two-dimensional paper chromatogram (Fig. 1). An advantage of the solvent pair employed, from the point of view of the present study, was that it did not resolve CGA from neochlorogenic acid, and owing

[7] See, e.g. K. HERRMANN, *Beitr. Tabakforsch.* **2**, 159 (1964).
[8] P. K. MACNICOL, *Biochem. J.* **107**, 473 (1968).
[9] M. SLAYTOR, L. COPELAND and P. K. MACNICOL, *Phytochem.* **7**, 1779 (1968).
[10] R. JOHANSON, *Nature* **171**, 753 (1953).

to the relatively short distance of travel did not separate *cis* and *trans* isomers. Although the major components CGA[11] and rutin[12] were readily estimated in the eluates by spectrophotometry, the resolvable amounts pf 6-HKA and scopoletin were too low for analysis by this technique even when chromogenic reagents such as diazotised *p*-nitroaniline[13] were used. Direct fluorimetry of these compounds on the chromatograms, using the apparatus of Bailey,[14] was found too imprecise owing to a pronounced influence of spot area on photocell response. Fluorimetry in eluates, however, showed good sensitivity and precision as long as the blank fluorescence of the paper and solvent were carefully allowed for.

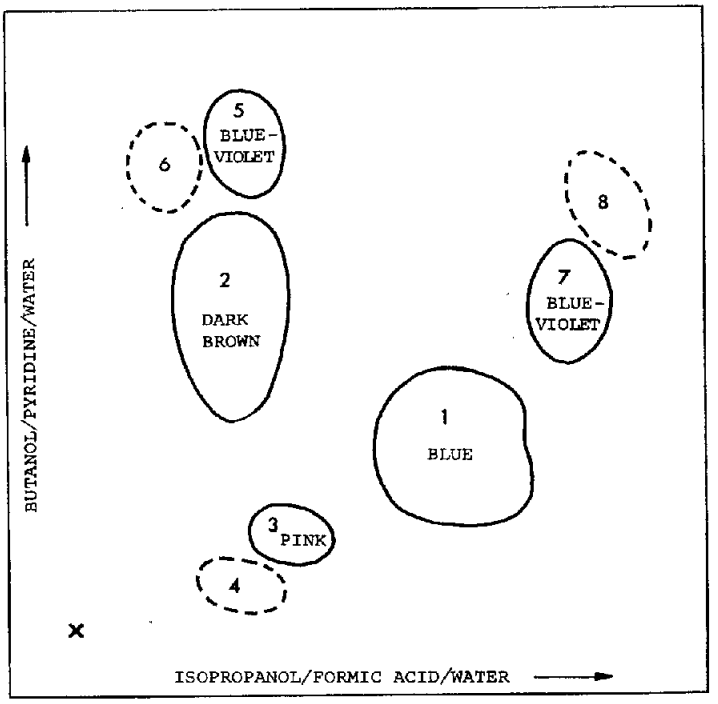


FIG. 1. DIAGRAM OF TYPICAL PAPER CHROMATOGRAM OF CURED LEAF EXTRACT.

Key to spots: 1, CGA; 2, rutin; 3, 6-HKA; 4, blank for 6-HKA; 5, scopoletin; 6, blank for scopoletin; 7, scopolin; 8, blank for scopolin. Colours refer to fluorescence under long-wave u.v. Minor fluorescent compounds omitted.

Table 1 gives the result of a representative recovery experiment. Taking into account the lability of the compounds to autoxidation and light, the recoveries ranging from 71 to 96 per cent were considered acceptable. A check on reproducibility, in which the same extract (grade T4K; see Experimental) was run on five chromatograms simultaneously, indicated an adequate degree of precision. The coefficients of variation were: CGA, 2 per cent; rutin, 9 per cent; 6-HKA, 6 per cent; scopolin, 9 per cent; and scopoletin, 10 per cent.

Table 2 shows that quality exhibited no overall correlation with the content of any of the

[11] R. F. DAWSON and E. WADA, *Tobacco Sci.* **1**, 47 (1957).
[12] T. B. GAGE and S. H. WENDER, *Anal. Chem.* **22**, 708 (1950).
[13] J. B. PRIDHAM, *Anal. Chem.* **29**, 1167 (1957).
[14] G. F. BAILEY, *Anal. Chem.* **32**, 1726 (1960).

five phenolic compounds or with "total cinnamic acid", a derived parameter (see Experimental) based on the common biosynthetic origin of scopoletin and large moieties of the CGA, rutin and scopolin molecules. Classification of the data according to colour and leaf position, however, revealed an interesting negative relationship between scopoletin content and quality within a given colour class. The correlation pooled over the three homogeneous

TABLE 1. RECOVERY OF STANDARD COMPOUNDS ADDED TO 1 g OF TOBACCO SAMPLE† PRIOR TO EXTRACTION

	CGA	Rutin	6-HKA	Scopolin	Scopoletin
Added to tobacco, mg	10	3	0·02	0·25	0·1
Endogenous, mg‡	11·9	5·38	0·0131	0·244	0·069
Recovered, mg§	8·4	2·31	0·0189	0·240	0·071
Recovery, % of theoretical	84	77	94	96	71

† Grade: X2M.
‡ Figures are means from duplicate chromatograms.
§ Total minus endogenous.

TABLE 2. CORRELATION COEFFICIENTS BETWEEN PRICE AND CONTENT OF FIVE PHENOLIC COMPOUNDS IN CURED TOBACCO SAMPLES CLASSIFIED ACCORDING TO COLOUR AND LEAF POSITION

Category	CGA	Rutin	6-HKA	Scopolin	Scopoletin	"Total cinnamic acid"
Classification according to colour						
Bright (B)	−0·14	−0·31	+0·11	−0·36	−0·52	−0·23
Orange (O)	+0·27	+0·22	+0·46	−0·18	−0·56*	+0·25
Mahogany (M)	−0·28	−0·28	−0·21	−0·36	−0·49	−0·32
Miscellaneous	−0·06	−0·11	+0·27	−0·22	+0·42	−0·07
Pooled correlation within colour classes O, B and M	−0·01	−0·11	+0·19	−0·28*	−0·52***	−0·06
Classification according to position						
Pooled correlation within all position classes	+0·09	−0·12	+0·04	−0·05	−0·20	+0·05
Correlation over all classifications	+0·06	−0·20	−0·02	0·00	−0·19	+0·01

*** Correlation significant at $P<0·001$, * at $P<0·05$.

colour classes bright, orange and mahogany was significant at the 0·1 per cent level. Omission of the "miscellaneous" class from the pooled correlation is justified in view of its heterogeneity.

Scopoletin is absent from the green tobacco leaf[15] but is formed in increasing amount during curing,[16] presumably from its β-glucoside scopolin, whose level decreases during

[15] L. J. DIETERMAN, C.-Y. LIN, L. ROHRBAUGH, D. V. THIESFELD and S. H. WENDER, *Anal. Biochem.* **9**, 139 (1964).
[16] P. T. PENN and J. A. WEYBREW, *Tobacco Sci.* **2**, 68 (1958).

TABLE 3. THE EFFECT OF LEAF COLOUR AND POSITION ON THE CONTENT OF PHENOLIC COMPOUNDS—ANALYSIS OF VARIANCE

	Mean content of phenolic compound (per cent dry wt.)†											
	CGA		Rutin		6-HKA		Scopolin		Scopoletin		"Total cinnamic acid"	
Category	Colour	Position	Colour	Position	Colour	Position	Colour	Position	Colour	Position	Colour	Position
1‡	2·45	2·35	0·71	0·78	0·0016	0·0016	0·076	0·043	0·0070	0·0067	1·24	1·20
2	2·03	2·27	0·65	0·77	0·0016	0·0017	0·066	0·059	0·0100	0·0088	1·04	1·17
3	1·82	1·94	0·68	0·58	0·0016	0·0015	0·064	0·072	0·0113	0·0092	0·96	0·99
4	2·00	1·73	0·69	0·60	0·0017	0·0016	0·058	0·089	0·0081	0·0118	1·04	0·92
Variance ratio	4·53*	5·31**	0·33	5·74**	0·51	0·49	0·67	4·48**	1·44	1·51	3·72*	5·01**

** Variance ratio significant at $P<0·01$, * at $P<0·05$.
† Adjusted for non-orthogonality.
‡ Colour: 1 = bright, 2 = orange, 3 = mahogany, 4 = miscellaneous; position: 1 = tips, 2 = leaf, 3 = lugs, 4 = sub-leaf.

curing.[17] Synthesis of scopolin may also occur during curing, utilizing the methyl group of nicotine;[18] whether it is quantitatively significant is not known. The wide variation (0·05–0·8) in scopoletin/scopolin ratio indicates that the scopoletin content of the cured leaf is influenced by factors additional to the scopolin content of the green leaf, further revealed by the correlation of scopolin but not scopoletin content with leaf position (Table 3). One factor might be greater activity of β-glucosidase during curing in leaf of lower quality, the activity being determined most probably by the physiological age of the leaf at harvest; "relative maturity" is in fact the major criterion of quality within a given position and colour class.[19]

The lack of correlation with quality shown by the major phenol CGA is not in conflict with the findings of Wilkinson *et al.*[20] The data of these workers show a positive correlation with leaf position rather than with quality, which the present work confirms (Table 3).

Significant trends of phenolic content with colour and leaf position were revealed when these components of the quality (grade) designation were separated by a two-way classification analysis of variance (Table 3). Only CGA and "total cinnamic acid", to which CGA was the overriding contributor, varied with colour, the highest content occurring in bright leaf,

TABLE 4. CORRELATION COEFFICIENTS BETWEEN COMPOUNDS OVER ALL CLASSIFICATIONS

Chlorogenic acid	Rutin	6-HKA	Scopolin	Scopoletin	
1·00	+0·75***	+0·30**	−0·09	−0·38**	Chlorogenic acid
	1·00	+0·24	−0·18	−0·18	Rutin
		1·00	+0·07	−0·06	6-HKA
			1·00	+0·36**	Scopolin
				1·00	Scopoletin

*** Correlation significant at $P > 0·001$, ** at $P > 0·01$.

followed by orange and mahogany in that order. Although CGA is certainly involved in the formation of brown pigments during curing,[3] the biochemical basis of variation in colour is only poorly understood. It has been suggested[21,22] that the nature of the nitrogenous bases (e.g. amino acids, pyrroles), with which the oxidized phenols couple, is important here.

CGA, rutin and "total cinnamic acid" increased from base to apex of the plant but scopolin showed the opposite gradient. These trends agree with those found by Wilkinson *et al.*[20] for CGA in tobacco, by Ahlgrimm[23] for rutin in buckwheat, and by Loche[24] for scopolin in tobacco.

The significant positive correlations (Table 4) between the levels of CGA, rutin and 6-HKA suggest that these compounds are more closely related biosynthetically to each other than to scopolin. Here it is assumed that the losses during the flue-curing are small in

[17] A. S. WEAVING, *Tobacco Sci.* **2**, 1 (1958).
[18] L. J. DEWEY and W. STEPKA, *Arch. Biochem. Biophys.* **100**, 91 (1963).
[19] V. BRINK (Rothmans Ltd.) personal communication.
[20] F. B. WILKINSON, M. PHILLIPS and A. M. BACOT, *J. Ass. Off. Agr. Chem.* **37**, 1004 (1954).
[21] W. W. REID, *J. Soc. Leather Trades' Chemists*, p. 75 (1956).
[22] S. BÄBLER, Dissertation, E.T.H., Zürich (1957).
[23] E.-D. AHLGRIMM, *Planta* **47**, 255 (1956).
[24] J. LOCHE, *Serv. Exploit. Ind. Tab. Allumettes Ann. Sect.* 2, **3**, 11 (1966).

relation to the initial levels. Other data,[9] however, do not support a phenylpropanoid origin of 6-HKA.

The samples analysed in this study were of the same tobacco cultivar grown in the same district in the same season. This ensured control over two important factors influencing leaf composition, namely genetic constitution and climate. Physiological age variation was circumscribed in that the leaves were picked when adjudged ripe, and effects of leaf position were extracted by the analysis of variance (Table 3). The major uncontrolled factor was therefore nutrition, particularly if this is used in a broad sense to include soil type.

"Quality" has been equated with the minimum price set for a particular grade (see Experimental) by the (Australian) Federal Tobacco Board; no other method of quantitation appears feasible at the present time. Although the allocation of quality grades was based on leaf position and the subjective properties of colour, texture and aroma, it was carried out by highly trained personnel. This approach has two drawbacks: firstly, leaf of very low quality has to be omitted from consideration because no price is given to it; secondly, the prevailing conception of quality changes with time. Thus the present tendency is to a preference for tobacco of a thinner texture, producing a smoke of lower alkaloid and tar content. This has reduced the importance of colour and increased that of leaf position, since nicotine content increases from the base towards the apex of the plant.[25]

From the agronomic point of view the main finding of this work is the negative relationship of scopoletin content to quality within a given colour class. Although the pooled correlation coefficient of $-0{\cdot}52$ (Table 2) does not give adequate basis for quality prediction within such a class, the use of other denominators than dry weight might eliminate sufficient of the variation for it to do so.

The use of scopoletin as a parameter of quality is supported by biochemical considerations. Scopoletin and scopolin seem to be metabolically rather mobile, as shown by the accumulation of scopoletin around virus lesions[26] and under high sodium chloride nutrition,[27] by the accumulation of scopolin in boron deficiency,[5] and particularly by the incorporation of nicotine methyl groups into scopolin.[18] Skoog and Montaldi[28] found the scopolin/scopoletin equilibrium in tobacco pith cultures to be sensitive to the balance between auxin and cytokinin in the medium. These facts suggest that in leaf of lower quality the equilibrium is shifted further towards scopoletin as a result of greater physiological stress during curing.

EXPERIMENTAL

CGA was obtained from the California Corporation for Biochemical Research, rutin from L. Light & Co. and scopoletin from Mann Research Laboratories Inc. 6-HKA was synthesized by the method of Makino and Takahashi.[29] Scopolin was isolated from the roots of tobacco plants that had been sprayed with 2,4-dichlorophenoxyacetic acid[15] and was crystallized from methanol. All of these reference compounds were homogeneous by paper chromatography. They were dried over P_2O_5 *in vacuo* before use.

The cured samples had been graded according to the system used in the U.S.A., Canada, Rhodesia and Australia, whereby each grade is characterized by three symbols, the first denoting leaf position, the third colour and the second, quality within this position-colour group. Thus "X2M" means "mahogany-coloured lugs of second quality". The samples were in four leaf position categories: "tips" (highest; 7 samples), "leaf" (upper middle; 22 samples), "lugs" (bottom; 12 samples) and "sub-leaf" (pieces resulting from physical damage, mainly from lower stem positions; 15 samples). Owing to fungal attack (blue mould, *Peronospora tabacina*) during the growing season, "cutters" (lower middle) were not available. The colour categories

[25] F. R. Darkis, L. F. Dixon, F. A. Wolf and P. M. Gross, *Ind. Engng Chem.* **28**, 1214 (1936).
[26] R. J. Best, *Australian J. Exp. Biol. Med. Sci.* **14**, 199 (1936).
[27] H. D. Fowler, *Nature* **188**, 1044 (1960).
[28] F. Skoog and E. Montaldi, *Proc. Nat. Acad. Sci. U.S.* **47**, 36 (1961).
[29] K. Makino and H. Takahashi, *J. Am. Chem. Soc.* **76**, 6193 (1954).

represented were "bright" (12 samples), "orange" (15), "mahogany" (15), "mahogany-red" (4), "off-type" (3), "green-bright" (2), "green-orange" (2), "green-mahogany" (2) and "dark" (1).

After collection from the sales floor the samples (each consisting of a representative "hand") were stored briefly in the dark in sealed polythene bags, then the hands were sub-sampled by taking the same number of 10-cm² discs from the centre of each leaf so that a total weight of *ca.* 3 g was obtained. In taking these discs, blue mould lesions were carefully avoided. The discs were dried overnight at 95°, ground to a fine powder in a Glen Creston mill and stored over silica gel at −20°.

Ground sample (1 g) was weighed accurately into an Erlenmeyer flask and 15 ml of extractant (70% ethanol containing 7 mg/100 ml potassium metabisulphite[30]) added. The suspension was gently refluxed for 5 min and filtered hot through a Whatman No. 42 paper. The residue was washed with a little hot 70% ethanol, returned to the flask and again refluxed with 15 ml of extractant. The suspension was filtered and washed as before until the washings were colourless. The combined extract was shaken with an equal volume of petroleum ether (b.p. 60–80°) and the aqueous phase concentrated to approx. 1 ml in a rotary film evaporator at 35–40°. This extract was made up to exactly 5 ml with 50% ethanol and stored at −20°.

For separation of the phenols, 40 μl of extract was subjected as a spot to ascending two-dimensional chromatography on a 10-in. square sheet of Whatman No. 3 paper, with *n*-butanol–pyridine–water (14:3:3, v/v/v; equilibration overnight) followed by isopropanol–formic acid–water (5:1:94, v/v/v; 3 hr equilibration). Spotting was done in the cold, and equilibration and chromatography performed in the dark, owing to the sensitivity of CGA and rutin to autoxidation and of scopolin and scopoletin to light.

After drying the chromatogram in a current of cold air the phenols were detected by their characteristic fluorescence in long-wave u.v. light (Fig. 1). The spots were cut out, weighed, cut into small squares and placed in stoppered tubes containing the eluent. In the case of 6-HKA, scopolin and scopoletin neighbouring blank spots of approximately the same area were cut out and treated in the same way. For CGA (and neochlorogenic acid, which was not resolved from it) the eluent was 50% methanol (10 ml), for rutin it was 0·01 M aqueous $AlCl_3$ (5 ml) and for the remaining compounds and blank spots it was 50% methanol (5 ml). The tubes were shaken 2 hr in a water-bath at 50°, allowed to cool and the supernatants decanted.

CGA and rutin were determined by spectrophotometry at 326 nm and 412 nm respectively; standard solutions of the reference compounds gave the following relations for a 1 cm light path: $\mu g\ CGA/ml = 19{\cdot}8 \times A_{326\ nm}$ and $\mu g\ rutin/ml = 30{\cdot}8 \times A_{412\ nm}$. 6-HKA, scopolin and scopoletin were determined by fluorimetry using an Eppendorf photometer with fluorescence attachment, 366 nm primary filter and 420–3000 nm secondary filter. The readings were first corrected for fluorescence due to the solvent and then for that due to the paper, taking into account the ratio of weights of sample spot and blank spot in each case. Standard curves prepared with reference compounds were linear for all three phenols in the required range (0·008–0·1 μg 6-HKA/ml, 0·1–2·0 μg scopolin/ml, 0·01–0·4 μg scopoletin/ml). "Total cinnamic acid", a derived parameter based on common biosynthetic origin, was calculated as $(0{\cdot}42 \times CGA) + (0{\cdot}24 \times rutin) + (0{\cdot}42 \times scopolin) + (0{\cdot}77 \times scopoletin)$, i.e. as the sum of the separate cinnamic acid contributions.

All analyses were performed at least in triplicate.

Acknowledgements—We are indebted to Mr. M. L. Dudzinski for statistical advice and analysis and to Messrs. V. Brink, G. Landy, P. Avis and B. Dare of Rothmans Ltd. for their friendly co-operation in procuring and grading the leaf samples for us, as well as for several valuable discussions. We also wish to acknowledge the capable technical assistance of Miss H. Davies and Mrs. L. Weir.

[30] L. VUATAZ, H. BRANDENBERGER and R. H. EGLI, *J. Chromatog.* **2**, 173 (1959).