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HIGH-PERFORMANCE REVERSED-PHASE LIQUID CHROMATOGRAPHY OF NATURALLY OCCURRING PHENOLIC COMPOUNDS*

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SUMMARY

A method is described for the analysis of phenolic compounds in plant tissue. Extraction of the phenols with aqueous alcohol is followed by analysis by high-performance liquid chromatography. Separation by reversed-phase chromatography on μBondapak C_{18} was achieved by gradient elution. The method is applied to the major phenolic compounds in tobacco (*Nicotiana tabacum* L.).

INTRODUCTION

Many methods have been published for determining phenols in tobacco and other natural products. Most of the procedures involve separation of the phenolic compounds and other extracted materials by paper chromatography[1–3], thin-layer chromatography[4,5], column chromatography[6], or gas chromatography[7–9]. Except for the gas chromatographic procedures, quantitation is usually based upon their absorption maxima in the UV region or by the formation of colored complexes[10–12]. However, none of the procedures are ideally suited to both the routine processing of large numbers of samples and the determination of individual phenolic compounds. The objective of the present work was to develop a fast, dependable, and accurate method using high-performance liquid chromatography (HPLC).

Flue-cured tobacco contains considerable amounts of caffetannins[13,14]. These substances have been identified as chlorogenic acid (3-O-caffeoylquinic acid), its isomers, and their hydrolytic products caffeic and quinic acids. Several flavanoids have been found in tobacco[13,14]. Rutin (quercetin 3-rhamnosidoglucoside), a major flavanol, occurs along with much smaller amounts of isoquercitrin (quercetin 3-glucoside), quercetin (3,3′,4′,5,7-pentahydroxyflavone), kaempferol-3-rhamnoglucoside, and kaempferol (3,4,5,7-tetrahydroxyflavone). Scopoletin (6-methoxy-7-hydroxycoumarin) and its glycoside, scopolin (scopoletin-7-glucoside), are the most frequently found coumarins. A number of other minor phenolic substances have been reported in tobacco[13,14].

* Contribution No. 111.

The phenolic constituents of tobacco have traditionally been regarded as important indicators of tobacco leaf quality[13–15]. The relationship of these substances to the colour of the leaf has long been recognized[15]. They have also been regarded as important contributors to smoke flavour and aroma[15]. The latter relationship remains somewhat speculative but has been responsible for much of the work on these substances. In recent years, more attention has been directed towards their role in the formation of undesirable phenols in smoke. Studies using individual phenolic compounds and tobacco extracts have shown that they contribute significantly to the formation of smoke phenols[16,17].

EXPERIMENTAL

Apparatus

Liquid chromatographic separations were performed on a Waters Assoc. Model ALC201 liquid chromatograph with Model 6000 pumps. The detector was a Schoeffel SF770 variable-wavelength UV detector at 350 nm. Samples were injected via a Model U6K injection system. A Spectra-Physics System I computing integrator was used for all computations.

Column

A 30 cm × 4 mm I.D. μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) column was used. μBondapak C_{18} has a monomolecular layer of octadecyltrichlorosilane chemically bonded to Porasil beads having an average particle size of 10 μm. A short precolumn of C_{18}/Corasil Bondapak (37–50 μm) was placed immediately before the μBondapak C_{18} column.

Mobile phase

The gradient elution employed methanol and 0.1 *N* potassium dihydrogen phosphate. The gradient was from 16% methanol to 40% methanol in 15 min at a flow-rate of 1.0 ml/min (Fig. 1).

Reagents

Phenols commercially available were obtained from Sigma (St. Louis, Mo., U.S.A.), Supelco (Bellefonte, Pa., U.S.A.), and K and K Labs. (Plainview, N.Y., U.S.A.).

Scopolin was synthesized from scopoletin and O-tetracetyl-α-glucosidyl bromide according to the proccdure used by Chaudhury *et al.*[18].

The isomers of chlorogenic acid were purified by column chromatography on silicic acid following the procedure reported by Sondheimer *et al.*[19].

Procedure

Tobacco leaf tissue was ground to pass through a 40-mesh screen and dried prior to extraction. Samples were defatted with petroleum ether (b.p. 40–60°), and extracted twice in a Soxhlet apparatus (for 4 and 15 h) with 150-ml portions of 80% aqueous methanol. The combined extracts were concentrated under vacuum, transferred to a 50-ml volumetric flask, and filtered through Celite prior to analysis.

RESULTS AND DISCUSSION

A typical liquid chromatogram of the phenolic constituents of flue-cured tobacco is shown in Fig. 1. A list of the compounds identified is given in Table I. Included in the table are several reference compounds not detected in this particular sample.

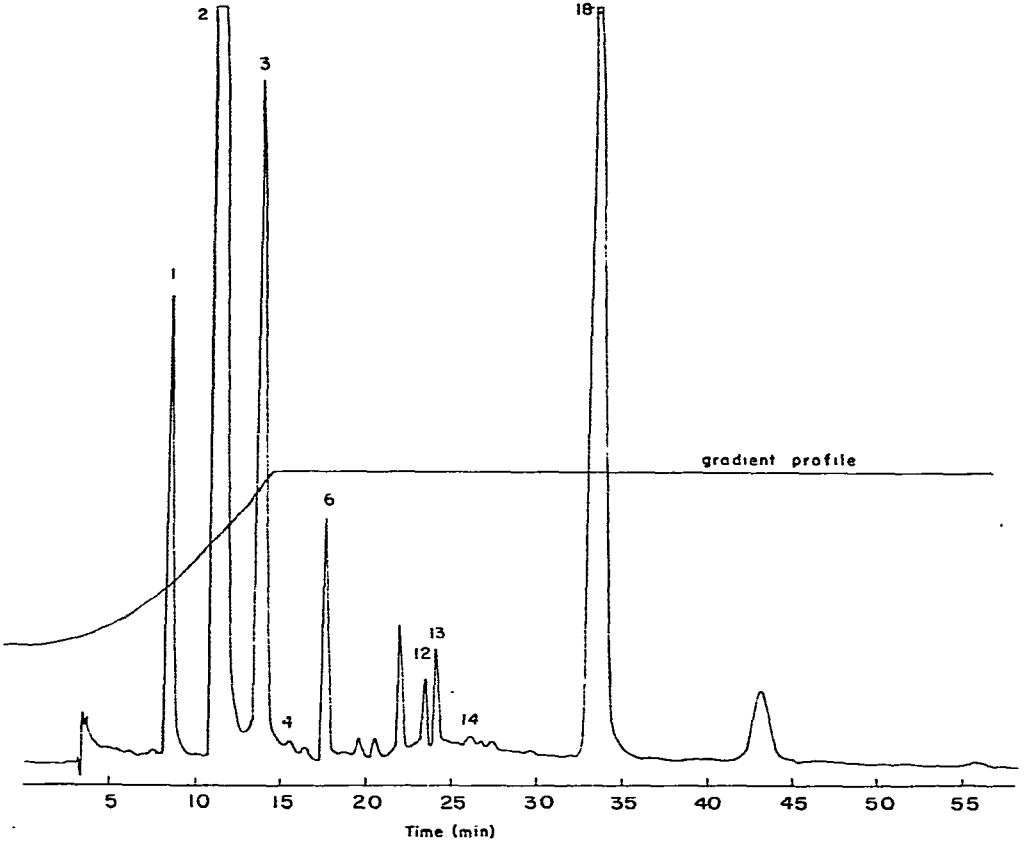

Fig. 1. Separation of phenolic constituents of flue-cured tobacco on a μBondapak C_{18} column (30 cm × 4 mm I.D.). Eluents, methanol and 0.1 *N* KH_2PO_4; flow-rate, 1.0 ml/min; pressure, 1000 p.s.i.; detector, UV photometer; wavelength, 350 nm; sensitivity, 0.2 a.u.f.s.; injection volume, 5 μl.

Chlorogenic acid and rutin are by far the major phenolic constituents of flue-cured tobacco. On the basis of their retention times and UV spectra, they were readily assigned as peak 2 and peak 18, respectively. The UV absorption spectra of peaks 1 and 3 corresponded closely to that of chlorogenic acid. In addition to chlorogenic acid, several other derivatives of caffeic and quinic acid have been identified in tobacco. They include 5-O-caffeoylquinic acid (neochlorogenic acid), 4-O-caffeoylquinic acid ("Band 510"), and isochlorogenic acid[14,20]. The latter material has been shown to be primarily a mixture of a number of dicaffeoylquinic acids[21]. A reference sample of isochlorogenic acid gave two major, one somewhat smaller, and several

TABLE I

PHENOLIC CONSTITUENTS OF FLUE-CURED TOBACCO

Peak No.	*Retention time (min)*	*Compound*
1	8.6	neochlorogenic acid
2	11.4	chlorogenic acid
3	13.9	4-O-caffeoylquinic acid
	14.9	esculin
4	15.5	caffeic acid
6	17.7	scopolin
	23.5	isochlorogenic acid
	25.5	
	26.0	
13	24.2	scopoletin
18	33.6	rutin
	42.2	quercitrin
	67.1	quercetin

minor peaks under the chromatographic conditions. The retention times of the major constituents corresponded to peak 12 and peak 14 in Fig. 1. The smaller peak, which is not discernible in Fig. 1, eluted immediately prior to peak 14. No attempt has been made to determine if peak 14 represents one of the components of isochlorogenic acid. However, by comparing the reference sample with the tobacco samples, it is apparent that peak 12 is primarily some as yet unidentified tobacco constituent. Attempts to obtain pure 4-O-caffeoylquinic acid and 5-O-caffeoylquinic acid by paper chromatography, using several different solvent systems, proved unsatisfactory. However, they were readily separated by column chromatography on silicic acid. In this manner peak 1 was identified as neochlorogenic acid and peak 3 as the remaining positional isomer. Caffeic acid was shown to have a retention time corresponding to peak 4. Since this acid was usually present in only very small amounts, no attempt was made to confirm this assignment.

The major coumarins in flue-cured tobacco are scopoletin and its glycoside scopolin. They were identified as peaks 13 and 6, respectively. Esculin (6,7-dihydrocoumarin-6-glucoside) and esculetin, the aglycone of esculin, have been reported in tobacco in very small amounts[8]. A standard sample of esculin eluted from the column shortly after the much larger constituent 4-O-caffeoylquinic acid. Esculin was not detected in any of the tobacco samples. Quercetin, the aglycone of rutin, was also not present in sufficient quantities to be detected routinely.

After the extraction of the plant tissue, the time required for one analysis by this method is less than 1 h. Quantitation was readily achieved since the detector response was linear over the observed concentrations. The column was found to perform satisfactorily when the precolumn was changed periodically, and the column was cleaned occasionally following the manufacturer's recommendations. However, individual columns may require slightly different conditions for optimum performance. In addition, a certain amount of peak tailing was observed with one of the columns which was tested. This phenomenon improved considerably after several injections and did not interfere with the analysis of the major constituents.

In addition to the components reported in Table I, it is evident from Fig. 1

that a number of minor constituents have as yet not been identified. Several of these have been tentatively identified but will not be reported until they are fully characterized. However, this does not detract from the feasibility of the method for the determination of the major phenolic constituents in plants and in particular in flue-cured tobacco.

CONCLUSIONS

High-performance reversed-phase liquid chromatography is an effective, dependable method for the separation of plant phenolic constituents. The much shorter analysis time compared with current methods allows the processing of many more samples with improved accuracy.

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