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RAPID ELECTRON CAPTURE DETERMINATION OF CAFFEIC ACID AND QUERCETIN MOIETIES IN PLANTS*

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SUMMARY

A sensitive gas–liquid chromatographic method has been developed for the quantitative analysis of caffeic acid and quercetin moieties in plant leaf by means of electron capture detection. Caffeic acid, quercetin and their naturally occurring derivatives, chlorogenic acid and rutin, respectively, are directly extracted from plant materials with 1-propanol, and subjected to a transesterification–hydrolysis reaction to produce caffeic acid *n*-propyl ester and quercetin. After silylation of these compounds, the TMS derivatives are suitable for chromatography on 10% OV-101 in the case of caffeic acid and 1.5% OV-101 in the case of quercetin. The method requires about 5 h for their quantitative determination, and the approximate lower limits of detection in tobacco leaf samples are 20 ng caffeic acid and 300 ng quercetin.

INTRODUCTION

Caffeic acid and quercetin derivatives, for example chlorogenic acids and rutin, respectively, are among the most common soluble phenols in plant tissue[1]. Previous methods for analysis of these compounds were largely based upon absorption chromatography, paper chromatography, spectrophotometry and spectrophotofluorometry[2–4]. Gas chromatographic separation and detection of these and related phenolic moieties has recently been accomplished[5–9]. The use of volatile hydroxyl derivatives, especially trimethylsilyl (TMS) ethers, the development of stable silicone stationary column phases, and the sensitivity of flame ionization, argon ionization and thermal conductivity detectors facilitated this advance[5–9].

A quantitative gas chromatographic–flame detector method for caffeic acid moieties in tobacco was developed in our laboratories[10]. The major disadvantage of this method was the necessity for a preliminary separation of the plant phenol fraction. Compounds which have an affinity for electrons can often be detected at lower concentrations in an electron capture detector than in flame, argon ionization or thermal

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conductivity detectors[11–14]. In our present study the objectives were to: (1) extend the previous method to include an analysis for both caffeic acid and quercetin moieties in plant tissue, (2) increase the sensitivity of detection of these silylated phenolic derivatives by electron capture detection, and (3) shorten the analytical method by the elimination of a preliminary separation of the phenol fraction.

EXPERIMENTAL

Reagents

All chemicals were reagent grade unless otherwise specified. Acetonitrile and *n*-propyl alcohol were redistilled before use and the distillates were stored over anhydrous Na_2SO_4. Dry HCl gas was used. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Regis Chemical Co.*, Chicago, Ill. Chlorogenic acid hemihydrate, quercetin and rutin were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Kaempferol and myrcetin were obtained from K & K Laboratories, Inc., Plainview, N.J., and caffeic acid from Aldrich Chemical Co., Milwaukee, Wisc. Caffeic acid methyl ester and caffeic acid *n*-butyl ester were synthesized in our laboratories as previously described[10]. Caffeic acid *n*-propyl ester (new compound) was synthesized in the same manner as the methyl and *n*-butyl esters of caffeic acid[10]. The solid was recrystallized from methanol/water, m.p. 126° (uncorr.). C, H, found = 65.0, 6.3; C, H, calculated for $C_{12}H_{14}O_4$ = 64.9, 6.3.

Sample preparation

Some of the preliminary experimental work and most of the subsequent testing of the procedure for the preparation of samples for gas chromatography involved some or all of the steps given in the following analytical method for the determination of caffeic acid and quercetin moieties in plant tissues as esters, glycosides or in their free form.

Analytical method. Weigh 100 mg of freeze-dried tissue into an erlenmeyer flask and add 15 ml *n*-propanol. Insert a condenser and reflux 45 min. Filter the sample solution through Whatman No. 1 paper and collect the filtrate in a flask. Wash the residue several times with small portions of *n*-propanol and collect the washings in the same flask. Discard the insolubles. Pass dry HCl gas through the solution until it is saturated. Reflux this solution 1 h (cold-water condenser required), and then take to dryness on a rotary flash evaporator.

Add the following internal standards as weighed amounts sufficient to provide satisfactory peak heights during subsequent gas chromatography: Caffeic acid methyl ester, caffeic acid *n*-butyl ester, kaempferol and myrcetin. Add 1.00 ml acetonitrile and 1.00 ml BSTFA. Immediately cover the flask and swirl the mixture to effect complete solution. Transfer as much of the solution as possible (without washing) to a 5-ml sealable screw-type acylation tube, or a tube which can be sealed. Heat in an oil bath at 150° for 1 h. Cool to room temperature. Open the tube and inject 0.20–3.0 μl into the gas chromatograph.

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* Mention of proprietary materials in the text does not imply endorsement by the United States Department of Agriculture.

Gas chromatography

A Packard Model 7821 gas chromatograph was used in conjunction with a Packard Model 810 electron capture detector with tritium foil as the ionizing source.

In the analysis for caffeic acid moieties, the chromatograms were recorded on a Photovolt Microcord Model 44 recorder, using a chart speed of 6 in./h. A 6-ft. coiled glass column (4 mm I.D.) was used. The column was packed with 10% silicone stationary phase (OV-101) on 80–90 mesh Anakrom AS. The operating conditions were: inlet temperature, 220°; column temperature, 190°; detector temperature, 215°; and outlet temperature, 220°. Argon was used as a carrier gas at a flow rate of 100 cc/min. Detector voltage was 50 V.

In the analysis for quercetin moieties, the chromatograms were recorded on a Packard Model 561 recorder, using a chart speed of 10 in./h. The column size and packing were the same as that used for the caffeic acid analysis, except that 1.5% OV-101 was used. The operating conditions were: inlet temperature, 230°; column temperature, 220°; detector temperature, 220°; and outlet temperature, 230°. Carrier gas, flow rate and detector voltage were the same as those used for the caffeic acid analysis.

The peak areas were measured by planimetry at the retention times established with trimethylsilylated samples of caffeic acid *n*-propyl ester and quercetin. Retention times were calibrated by determining: (a) the relative peak positions of trimethylsilylated methyl, *n*-propyl and *n*-butyl esters of caffeic acid under the conditions specified for the 10% OV-101 column, and (b) the relative peak positions of trimethylsilylated kaempferol, quercetin and myrcetin under the conditions specified for the 1.5% OV-101 column. The peak areas of silylated caffeic acid *n*-propyl ester and silylated quercetin were compared with those from standard curves of peak areas from gas chromatographic analyses established with various amounts of chlorogenic acid hemihydrate and rutin added to a tobacco sample which had non-detectable amounts of caffeic acid and quercetin moieties. These samples were carried through the entire analytical method.

RESULTS AND DISCUSSION

Initial runs were made in which a high-phenol variety of tobacco leaf was extracted with either methanol, *n*-propanol or *n*-butanol, and, subsequently, prepared as described in the analytical method. GLC analyses of these samples were carried out by flame detection under the previously reported conditions[10]. Samples which were extracted and prepared with *n*-propanol and *n*-butanol yielded TMS-caffeic acid esters that were easily identifiable and there were few background peaks. However, methanol extractions and the preparation of TMS-caffeic acid methyl ester was not as suitable for quantitative GLC–flame detection analysis, because of greater background and overlapping peaks on chromatograms. *n*-Propanol was, therefore, selected for the extraction and preparation of samples for GLC–electron capture assay for caffeic acid and quercetin moieties in plant tissues. Tests showed that chlorogenic acid refluxed 1 h in *n*-propanol saturated with dry HCl was transesterified and yielded an equivalent amount of caffeic acid *n*-propyl ester; rutin treated in the same manner was hydrolyzed and yielded an equivalent amount of quercetin. Extraction of leaf samples with *n*-propanol and transesterification–hydrolysis with *n*-propanol saturated with dry HCl were, therefore, used in the analytical method.

TABLE I

RELATIVE PEAK POSITIONS[a] OF TMS DERIVATIVES OF CAFFEIC ACID ESTERS AND FLAVANOLS

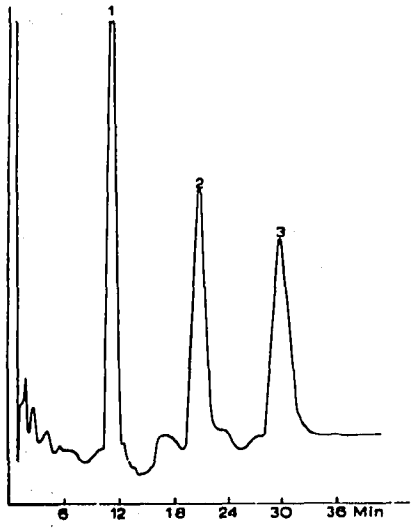
GLC column conditions	*t_{R_1} component*	*t_{R_2} component*	*Relative peak position*
10% OV-101, 190°	Caffeic acid methyl ester	Caffeic acid *n*-propyl ester	0.91
	Caffeic acid *n*-butyl ester	Caffeic acid *n*-propyl ester	(—) 0.31
1.5% OV-101, 220°	Kaempferol	Quercetin	0.53
	Myrcetin	Quercetin	(—) 0.22

[a] Relative peak position = $(t_{R_2} - t_{R_1})/t_{R_1}$, where t_R = retention time of a component measured from the start.

The synthetically prepared methyl, *n*-propyl and *n*-butyl esters of caffeic acid were silylated and chromatographed according to the conditions given in EXPERIMENTAL. The relative peak positions of the TMS-methyl, *n*-propyl and *n*-butyl esters are given in Table I.

Because the anthracene and pyrene internal standards previously employed in flame detection of TMS-caffeic acid esters[10] were unresponsive in the electron capture detector, the methyl and *n*-butyl esters were used as internal standards for the assay of caffeic acid moieties as TMS-caffeic acid esters.

TMS-caffeic acid *n*-propyl ester was separated from peaks of other compounds in tobacco, tomato, peach and buckwheat leaf extracts. For example, caffeic acid moieties were added by spiking a tobacco sample low in caffeic acid moieties with



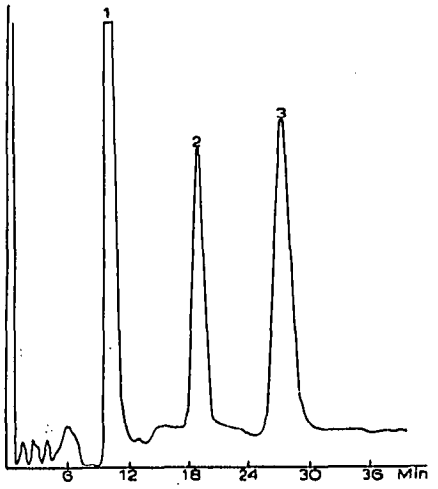
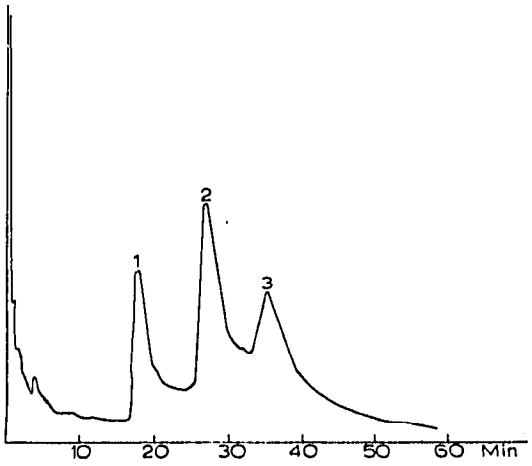


Fig. 1. Chromatogram of a 15-μg low-phenol tobacco sample spiked with 0.041 μg chlorogenic acid hemihydrate and subjected to the entire analytical method. Internal standards: 0.015 μg caffeic acid methyl ester (1) and 0.015 μg caffeic acid *n*-butyl ester (3). 2 = TMS-caffeic acid *n*-propyl ester assayed.

Fig. 2. Chromatogram of a 40-μg high-phenol tobacco subjected to the entire analytical method. Internal standards: 0.016 μg caffeic acid methyl ester (1) and 0.016 μg caffeic acid *n*-butyl ester (3). 2 = TMS-caffeic acid *n*-propyl ester assayed.



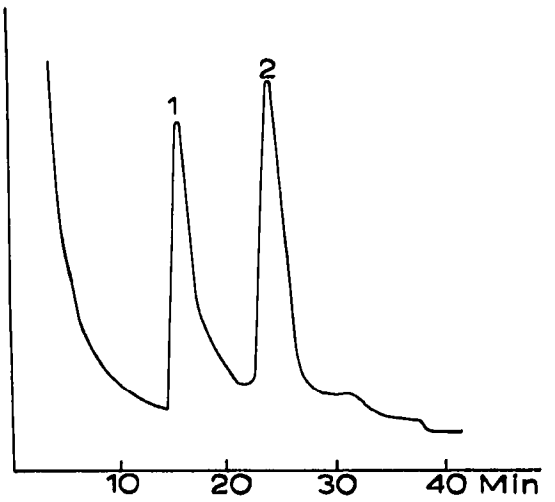


Fig. 3. Chromatogram of flavanols subjected to the silylation step of the analytical method. 1 = 0.45 μg kaempferol, 2 = 0.42 μg quercetin and 3 = 0.45 μg myrcetin.

Fig. 4. Chromatogram of a 75-μg low-phenol tobacco sample spiked with 1.50 μg rutin and carried through the analytical method. Internal standard: 0.75 μg kaempferol (1). 2 = TMS-quercetin assayed.

chlorogenic acid. The chromatogram obtained in Fig. 1 indicates the sensitivity of the analysis of caffeic acid moieties or derivatives, *e.g.* chlorogenic acid in tobacco leaf tissue. Similar results were obtained with other leaf samples. An advantage was the low level of background on chromatograms. Compared to the previous flame detector method for caffeic acid moieties in tobacco leaf[10], the electron capture method was more rapid, sensitive and had less background interference.

Leaves from tobacco (a high-phenol variety of *Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), peach (*Prunus persica*) and buckwheat (*Fagopyrum esculentum*) were analyzed for naturally occurring caffeic acid moieties. Chromatograms of 40 μg high-phenol tobacco (Fig. 2), 30 μg tomato and 30 μg peach all showed strong peaks corresponding to silylated caffeic acid *n*-propyl ester, but 50 μg buckwheat exhibited no detectable caffeic acid derivative.

Kaempferol, quercetin and myrcetin were silylated and chromatographed according to the conditions given in EXPERIMENTAL. The relative peak positions of the TMS-derivative of each of these compounds are given in Fig. 3 and Table I. The hydrocarbon 1,2:5,6-dibenz[*a*,*h*]anthracene was a suitable internal standard for the analysis of TMS-quercetin in preliminary runs in which a flame detector was used, but it was unresponsive to the electron capture detector. Kaempferol and myrcetin were, therefore, added prior to silylation to provide TMS-flavanol internal standards for the electron capture assay of quercetin moieties or derivatives (*e.g.* rutin) as TMS-quercetin.

A given quantity of silylated quercetin was two to three times more responsive with electron capture detection than it was with flame detection. There was also less background interference with electron capture than with flame detection when a high-phenol variety of tobacco leaf was prepared and assayed for quercetin. The increased sensitivity of TMS-quercetin detection with electron capture was substantial, but less than the parallel increased sensitivity noted with TMS-caffeic acid ester, *i.e.*,

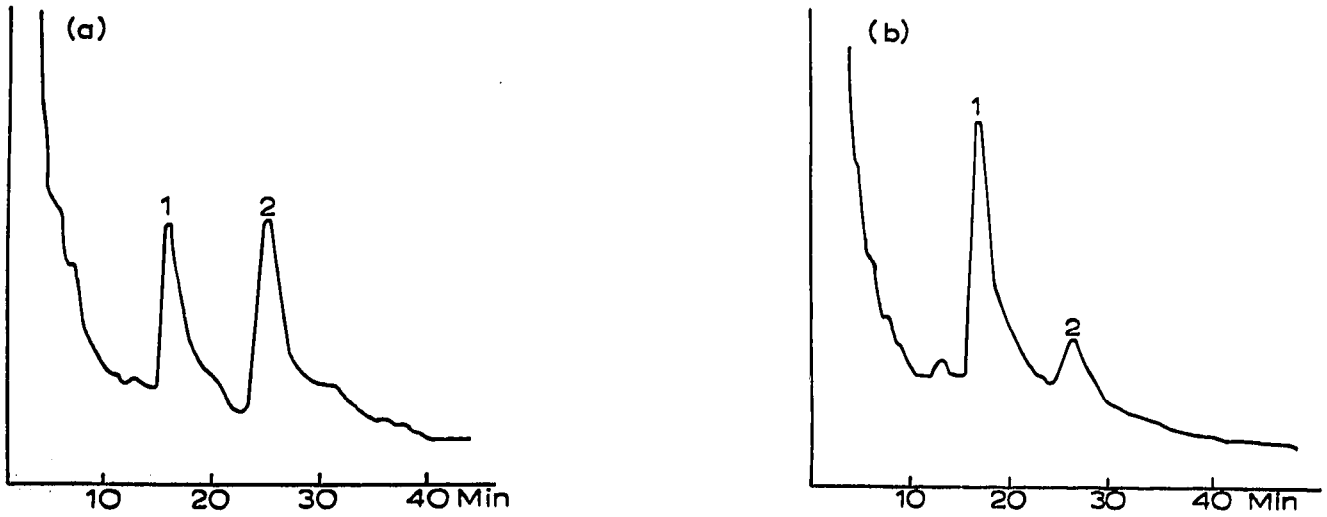


Fig. 5. (a) Chromatogram of 75 μg high-phenol tobacco carried through the entire analytical method. Internal standard: 0.45 μg kaempferol (1). 2 = TMS-quercetin assayed. (b) Chromatogram of 100 μg peach leaf carried through the entire analytical method. TMS derivatives assayed: kaempferol (1) and quercetin (2).

approximately twenty-fold. Apparently the TMS-caffeic acid ester contains a greater proportion of electrophilic sites than does TMS-quercetin.

TMS-quercetin was separated from peaks of other compounds in tobacco, tomato, peach and buckwheat leaf extracts. Quercetin moieties were added by spiking a tobacco leaf sample low in quercetin moieties with rutin. The chromatogram showed that the analytical method with electron capture provided a satisfactory method for analysis of quercetin moieties in tobacco leaf tissue with fairly low background on chromatograms (Fig. 4).

Leaves from a high-phenol variety of tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), peach (*Prunum persica*) and buckwheat (*Fagopyrum esculentum*) were analyzed for naturally occurring quercetin moieties. Chromatograms of 75 μg tobacco (Fig. 5a), 100 μg each of tomato, peach (Fig. 5b) and buckwheat all

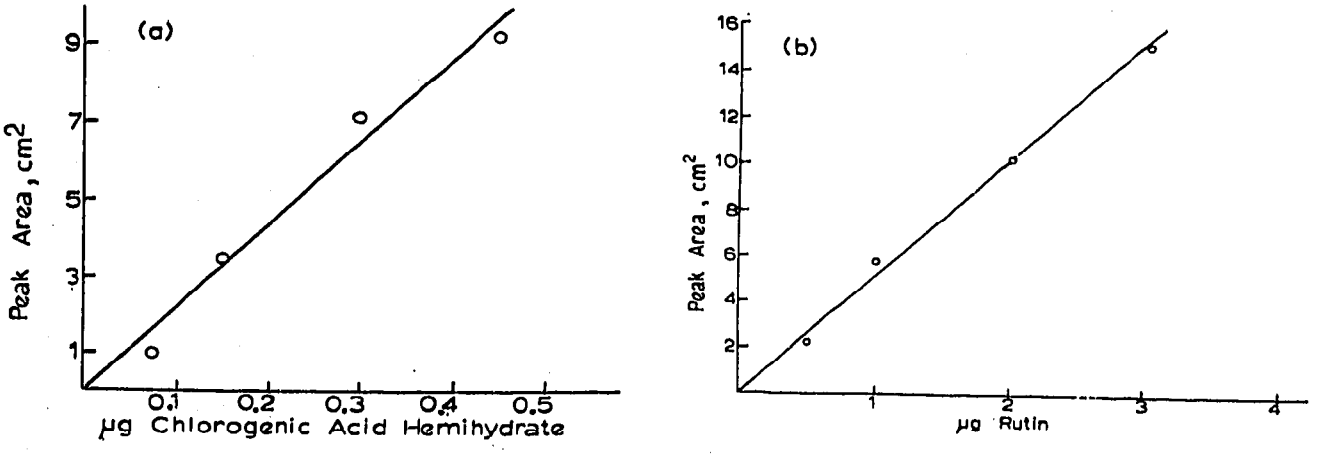


Fig. 6. (a) Recovery of chlorogenic acid hemihydrate (as TMS-caffeic acid *n*-propyl ester) spiked in 15 μg low-phenol tobacco. (b) Recovery of rutin (as TMS-quercetin) spiked in 100 μg low-phenol tobacco.

showed peaks corresponding to TMS-quercetin. In addition, the peach leaf (Fig. 5b) chromatogram showed the presence of naturally occurring kaempferol; this chromatogram was prepared without the use of a kaempferol internal standard.

Calibration curves for caffeic acid moieties (as chlorogenic acids) and quercetin moieties (as rutin) in leaf samples were plotted. In one case, for each analytical value 15 μg of a low-phenol tobacco variety sample was added to 0.10–0.50 μg standard chlorogenic acid hemihydrate (Fig. 6a). In the second calibration curve, each analytical value represented a 100-μg sample of the same tobacco variety which was spiked with 1.0–4.0 μg standard rutin (Fig. 6b). Similar calibrations were made with tomato, peach and buckwheat leaf samples.

A tobacco leaf sample high in plant phenols was analyzed five times for a determination of the precision of the analyses for caffeic acid and quercetin. The results and standard deviations obtained are as follows: caffeic acid = 1.32 $\pm$ 0.08; quercetin = 0.66 $\pm$ 0.08.

For comparison of GLC–electron capture analysis of caffeic acid and quercetin moieties in tobacco two other published methods were used for the analysis of tobacco leaf sample. In one case, a quantitative paper chromatographic–spectrophotometric analysis was performed for total chlorogenic acid isomers and rutin[15], and in the second case Arnow's nitrate–molybdate reagent[16], which yields a colored complex with *o*-dihydroxyphenols, was used. Assumptions[10] were made as follows: (1) caffeic acid and quercetin were not in the free state, and (2) chlorogenic acid isomers and rutin were the only caffeic acid and quercetin derivatives as well as the only *o*-dihydroxy plant phenols in the sample. Inspection of Table II shows the correlation

TABLE II

COMPARISON OF GLC ASSAY WITH OTHER METHODS FOR CHLOROGENIC ACID AND RUTIN IN A TOBACCO LEAF SAMPLE

Method	*Chlorogenic acid isomers (%)*	*Rutin (%)*	*Chlorogenic acid isomers plus rutin (%)*
GLC	0.30	0.23	0.53
Paper chromatography–spectrophotometry	0.38	0.10	0.48
Spectrophotometry (ARNOW's reagent)	0.58	0.16	0.74

among the results obtained by the three methods. In view of the dissimilarity of the analytical methodology and the necessary assumptions involved, the differences among the results do not seem great, and the use of the GLC–electron capture method for the estimation of the chlorogenic acid and rutin content of tobacco leaf seems valid.

The advantages of this method for the analysis of caffeic acid and quercetin moieties in plant leaf samples include: (1) a relatively short analytical period of approximately 5 h compared to a period about two times longer required for a quantitative conventional paper chromatographic analysis, (2) greater sensitivity than other GLC or paper chromatographic methods of specific plant phenol analysis[3,10] (*e.g.* approximately 20 ng caffeic acid and 300 ng quercetin are detectable), and (3) quantitation of results for these moieties in leaf samples.

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