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QUANTITATIVE DETERMINATION OF CHLOROGENIC ACID IN PLANT TISSUE BY COMBINED POLYVINYLPYRROLIDONE COLUMN AND GAS CHROMATOGRAPHY*

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SUMMARY

A new, specific, and sensitive chromatographic method for the quantitative determination of chlorogenic acid in plant tissue has been developed. Polyvinylpyrrolidone column chromatography is utilized for preliminary quantitative isolation of chlorogenic acid from whole extracts of plant tissue. The partially purified chlorogenic acid, after conversion to the trimethylsilyl derivative, is then further purified and quantitatively determined by gas chromatography. The essential features of the method appear readily applicable to the analysis of other plant phenolic compounds.

INTRODUCTION

Chlorogenic acid (3-O-caffeoylquinic acid) occurs almost universally in higher plants[1]. The function and metabolism of this compound and related depsides remain, however, largely undefined, even though chlorogenic acid has received much attention for years. As is the case with many other plant phenolic compounds, a major problem in the study of function and metabolism of chlorogenic acid in plants is the quantitative determination of this compound when its isomers and other closely related compounds are present with it in small samples of plant tissue. Methods involving silicic acid column chromatography or paper chromatography and spectrophotometry have previously been most used for this purpose[1-3]. This paper describes a new, specific, and sensitive method which we are now using in our laboratory for the quantitative analysis of chlorogenic acid in plants. This procedure involves preliminary quantitative isolation of chlorogenic acid from whole extracts of plant tissue by polyvinylpyrrolidone column chromatography, followed by further purification and quantitative determination of the chlorogenic acid as the trimethylsilyl derivative by gas chromatography. We have employed this method mainly for analysis of tissue

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of tobacco, tomato, and bean plants, but it should be generally useful for determination of chlorogenic acid in plant tissue. The method also appears to have potential, with minor modification, for application to determination of other plant phenolic compounds.

EXPERIMENTAL

Description of the analytical method

Preparation of plant tissue extracts

Freshly harvested plant tissue is weighed, immediately plunged into boiling methanol for approximately 15 min to inactivate enzymes, and stored under refrigeration until further processing is convenient. The plant tissue, except for root samples, is then ground thoroughly with the suspending methanol in a Waring Blendor. Root samples are hand chopped, since grinding usually produces a gelatinous mass which cannot be efficiently extracted. The macerated tissue–methanol suspension is quantitatively transferred to a Soxhlet extraction thimble supported in a short piece of glass tubing of slightly larger dimensions equipped with a drip tip; the filtrate is collected, and the solid residue is washed successively with the following solvents: 2-propanol–water (1:1 v/v); 2-propanol–benzene–methanol–water (2:1:1:1, v/v/v/v, hereafter called IBMW); and 2-propanol–water azeotrope (87.9:12.1, w/w). The volume of each wash solvent used is three to four times the fresh weight of the sample. The residue is then extracted in a Soxhlet extractor for 24 h with 2-propanol–water azeotrope and finally for 24 h with pure 2-propanol. The combined filtrate, washings, and extracts are evaporated to dryness *in vacuo* on a rotary evaporator, and the resulting residue is removed from the walls of the flask by an emulsion of benzene–water (1:1, v/v). The emulsion is brought to one phase by addition of a volume of 2-propanol equal to the combined volumes of benzene–water, and one-half this volume of methanol is next added. The sample solution is adjusted to a final volume of two to three times the original fresh weight of the tissue in a volumetric flask with IBMW solvent.

Preliminary separation of chlorogenic acid from plant tissue extracts by column chromatography

Preliminary quantitative separation of chlorogenic acid is achieved by chromatography of plant tissue extract on a column of Polyclar AT polyvinylpyrrolidone (General Aniline and Film Corporation, Grasselli, N.J.). The Polyclar AT is thoroughly washed in bulk with N,N-dimethylformamide–acetic acid–water–methanol solution (1:2:6:4, v/v/v/v) followed by distilled water and is stored as an aqueous slurry. A column 6.5 cm in depth is prepared by packing a suitable quantity of this slurry, under 5 lb. pressure, into a 14.5 mm I.D. column equipped with stopcock and 250 ml reservoir. The column is washed successively with 50 ml of methanol and 50 ml of IBMW solvent, and then is charged with 5 ml of IBMW solution of plant tissue extract. For tobacco, 5 ml of the plant tissue extract in its original concentration are used. For tomato and bean plants, which usually contain much less chlorogenic acid and other phenolic compounds than tobacco, the original extract is concentrated just before application to the column by evaporating 20–25 ml to dryness on a rotary evaporator and redissolving the residue in 5 ml of IBMW solvent by first suspending it in 2 ml of benzene–water (1:1, v/v) and then adding 2 ml of 2-propanol and 1 ml

methanol. This concentration is necessary to insure that the polyvinylpyrrolidone step yields sufficient chlorogenic acid to permit subsequent quantitation by gas chromatography. The extract is applied with a 5 ml transfer pipette, with care being exercised not to disturb the surface of the column. The walls of the column are carefully washed with 2–3 ml of IBMW. When the rinse solution has moved into the column the solvent is changed to benzene–methanol (9:1, v/v), and elution of the column under 5 lb. pressure is begun. The solvent sequence is: 50 ml benzene–methanol (9:1, v/v); 150 ml benzene–methanol (3:1, v/v); and 300 ml pure methanol. Each solvent is added just as the last of the preceding solvent moves into the top of the column packing. The column is operated at 22 to 26°, and fractions collected correspond in volume to the eluting solvents. Under these conditions, all the chlorogenic acid is contained in fraction 3 (300 ml methanol). Two isomers of chlorogenic acid which usually accompany it in plant tissue, namely 4-O- and 5-O-caffeoylquinic acid, are also eluted in fraction 3. This fraction is collected directly into a round bottom flask and evaporated to dryness on a rotary evaporator. The dried residue is then transferred to a 4 dram vial with four 10 ml washings of methanol, and the methanol is evaporated under a stream of nitrogen at low heat (50–60°).

Further purification and quantitative determination of chlorogenic acid by gas chromatography

The trimethylsilyl derivative, suitable for gas chromatography, of the chlorogenic acid in the Polyclar column fraction 3 is prepared by reacting this fraction with hexamethyldisilizane in N,N-dimethylformamide. Hexamethyldisilizane (hereafter referred to as HMDS, obtained from Applied Sciences Laboratories, State College, Pa., or synthesized by the method of LANGER, CONNELL, AND WENDER[4]) is redistilled in small quantities, maintained in sealed containers, and kept for not more than 3 weeks after redistillation to insure dryness and purity. N,N-Dimethylformamide (called DMF) is redistilled in quantities needed for 2 to 4 weeks supply. The dry fraction 3 residue is dissolved in 0.7 ml of DMF and then 0.3 ml of HMDS is added to yield a final volume of essentially 1 ml. The vial containing this mixture is stoppered with a polyethylene cap and vigorously shaken for approximately 30 sec, then left to stand loosely stoppered for 30 min to 1 h to allow escape of ammonia produced in the silylation reaction. Finally, the vial is resealed and the sample allowed to stand at least 2 h more. Suitable duplicate aliquots of this silylated fraction 3 are then subjected to gas chromatographic analysis, and the quantity of chlorogenic acid present is determined from the average peak area produced by its trimethylsilyl derivative.

For gas chromatography, we employ an F&M Model 810 research chromatograph equipped with flame ionization detector and a Honeywell Model 153 recorder with Disc integrator for determination of peak areas. Helium is carrier gas, and oxygen is used as purge gas to increase the sensitivity of the detector[5]. The column is 6 ft. × 1/4 in. O.D. stainless steel packed with 3% UCW-98 coated on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.), by the method of PARCHER AND URONE[6]. Essential operating parameters are as follows: column temperature, 240°; injection port temperature, 300°; detector temperature, 280°; carrier gas flow, 80 ml/min. Fig. 1 shows a typical chromatogram obtained when Polyclar column fraction 3 from tobacco leaf extract is silylated and subjected to gas chromatography under these conditions. As this figure shows, the TMS derivative of

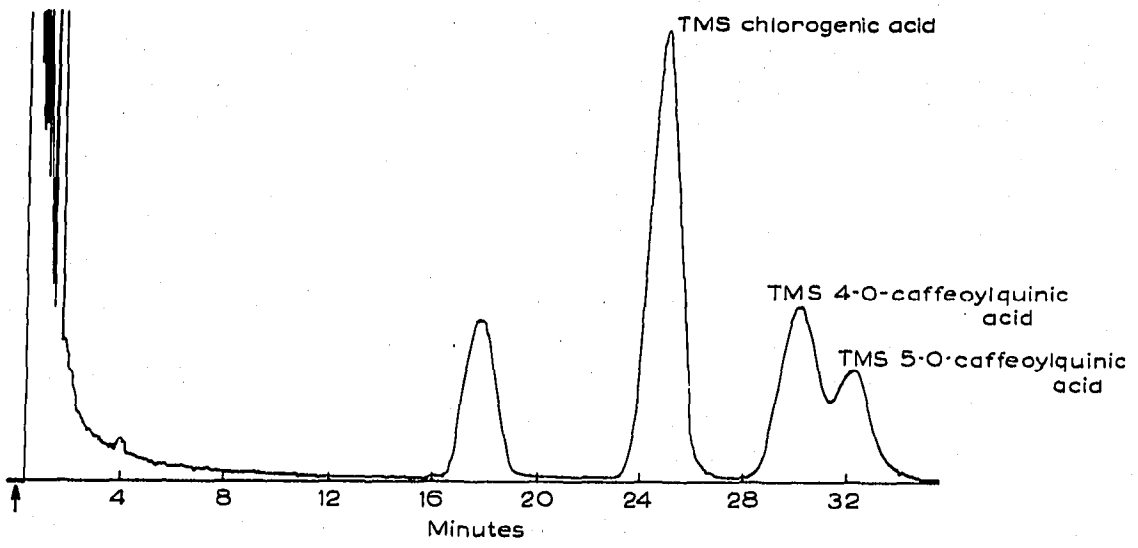

Fig. 1. Gas chromatogram of TMS derivatives of Polyclar column fraction 3 from tobacco leaf extract. Column: 6 ft. × 1/4 in. stainless steel, UCW-98 (3 %) on Gas Chrom Q (100–120 mesh); column temp. = 240°; injection temp. = 300°; detector temp. = 280°; helium flow rate = 80 ml/min.

chlorogenic acid is cleanly separated from other components of fraction 3 and can be easily quantitated. TMS derivatives of 4-O- and 5-O-caffeoylquinic acids are not completely resolved, but relatively accurate quantitative estimation of these compounds can be made, if desired.

Under the conditions employed here, the relationship between peak area and quantity of chlorogenic acid becomes non-linear for quantities of this compound below 0.8 μg. Therefore, the volume of silylated fraction 3 which is chromatographed must be sufficient to contain this minimum quantity. For analysis of tobacco, tomato, or bean tissue as described herein, 0.8–3.0 μl aliquots of fraction 3, injected with 1 μl or 5 μl syringes (Hamilton 7101N or 75), are used. Standards consisting of 1 and 10 mg of pure chlorogenic acid (Fluka AG, Buchs, Switzerland, "puriss" grade) in methanol solution are carried through the evaporation, silylation, and gas chromatography steps with each set of samples in order to establish the standard curve to be used for quantitation of chlorogenic acid in these samples.

Studies relating to the precision and accuracy of the method

The precision of the method was studied by performing the analysis for chlorogenic acid successively on six samples of a single tobacco leaf extract. An average of 1.13 mg chlorogenic acid per gram of fresh tissue was obtained, with a standard deviation of 0.04 mg and a coefficient of variance of 3.5 %.

Another study was made to determine if chlorogenic acid, when added to plant tissue extract, could be quantitatively detected by the analytical method. Accordingly, 0.6 mg of pure chlorogenic acid was added to an aliquot of tobacco leaf extract which, from the precision studies, had been found to contain 1.3 mg chlorogenic acid, and the enriched solution was then subjected to analysis. The chlorogenic acid found was 105 % of the calculated value.

RESULTS

Application of the method

To illustrate the use of the method, Tables I and II present results obtained when the chlorogenic acid in various plant tissues was determined by this new procedure. Table I shows the wide variation in chlorogenic acid concentration in leaves of bean, tomato, and tobacco plants. The change in chlorogenic acid content with age of bean plants and the difference in concentration of this compound between young leaves and old leaves on the same tobacco plant are also revealed. In Table II, the changes are shown in chlorogenic acid content of leaves, stems, and roots of normal and 2,4-dichlorophenoxyacetic acid (2,4-D) treated tobacco plants observed over a period of 35 days after spraying of the treated plants with herbicide.

TABLE I

CHLOROGENIC ACID IN VARIOUS PLANT TISSUE

Sample	*μg/g fresh wt.*
Bean leaf, 13 days old[a]	30
Bean leaf, 16 days old[a]	77
Tomato leaf, 49 days old[b]	70
Tobacco, young leaves[c]	500
Tobacco, old leaves[d]	300

[a] Red kidney bean; extract contained all leaves from single plant.
[b] Extract contained all leaves from single plant.
[c] Extract contained all leaves less than 3 in. long from a single plant, 91 days old.
[d] Extract contained all leaves greater than 3 in. in length, from the same plant as c.

TABLE II

CHLOROGENIC ACID CONTENT OF LEAVES, STEMS, AND ROOTS OF NORMAL AND 2,4-D TREATED TOBACCO PLANTS

Days after spraying[a]	*Control leaves*	*Treated leaves*	*Control stems*	*Treated stems*	*Control roots*	*Treated roots*
4	670	730	1210	850	600	430
8	1480	1400	730	1700	470	310
20	2200	1300	740	1000	700	[b]
35	1130	3700	480	1250	1150	[b]

[a] Plants sprayed 60 days after planting.
[b] Below measurable levels.

DISCUSSION

The quantitative method described here is capable of relatively precise and accurate determination of chlorogenic acid in plant tissue. This precision and accuracy result from the very effective preliminary quantitative separation procedure, including both extraction and column chromatography steps, and from the specificity obtained through the coordinated column and gas chromatography steps. The method usually

requires only a few grams of fresh plant tissue for analysis, and its essential features appear readily applicable to the analysis of other plant phenolic compounds.

The rather exhaustive extraction procedure employed in this method was developed specifically to remove all free chlorogenic acid from tobacco tissue. Our studies indicate that less extensive procedures commonly reported in the literature[3,7–9] usually leave behind considerable phenolic material, including chlorogenic acid, when used on tobacco. Examination of the residue of tobacco tissue remaining after extraction by the procedure employed in this paper, indicated that essentially all free phenolic compounds are extracted. Equally good results have been obtained in extraction of tomato and bean plant tissue by this procedure, and it seems likely to be generally adequate for extraction of free chlorogenic acid, as well as most phenolic compounds, from plant tissue.

The polyvinylpyrrolidone column chromatography procedure employed in this analytical method accomplishes in a single step preliminary quantitative separation of the chlorogenic acid from other constituents of the complex extract which might interfere with its analysis by gas chromatography. Direct chromatography of the whole extract without preliminary clean-up by solvent extraction or similar procedures eliminates losses of phenolic compounds which usually occur during such procedures. During early stages of this column chromatography step the columns appear quite irregular because of the concurrent presence in the packing of the immiscible solvents benzene and water. The water is soon eluted, however, and the columns then become normal in appearance, and they yield excellent quantitative results. That no irreversible adsorption occurs on such Polyclar AT columns was shown by the quantitative elution of pure chlorogenic acid when chromatographed by the procedure and elution sequence employed in the quantitative method, and by the quantitative recovery of known quantities of chlorogenic acid added to tobacco leaf extract which was carried through the complete procedure.

Time required for the column chromatography step, from packing the column to elution of the fraction containing chlorogenic acid, is about 4 h. This procedure, or proper modifications of it, may have general applicability for preliminary quantitative separation of phenolic compounds from plant extracts. For instance, fraction 2 [150 ml of benzene–methanol (3:1, v/v)] obtained from tobacco leaf extracts by this method contains 3-O-feruloylquinic acid and other phenolic compounds, while further elution of such columns with 250 ml of DMF after elution of fraction 3 (300 ml pure methanol) which contains chlorogenic acid, results in elution of depsides of the isochlorogenic acid group and flavonoid compounds including the flavonol glycoside rutin.

Extensive studies of the preparation of trimethylsilyl derivatives of phenolic compounds indicated the DMF–HMDS system to be most effective for silylation of chlorogenic acid and related depsides. This system gives somewhat higher yields of the TMS derivative of chlorogenic acid than does the commonly used system of pyridine–HMDS–trimethylchlorosilane, and there is no precipitate of ammonium chloride, as occurs with the latter system. In the DMF–HMDS system at room temperature, maximum silylation of chlorogenic acid is attained in approx. 2 h, and there is then essentially no change thereafter for at least 24 h. Neither addition of TMCS to the DMF–HMDS system, nor heating, improves the yield of TMS derivative; in addition, TMCS produces a two phase system which makes quantitation difficult.

The liquid phases UCW-98, SE-30, and SE-52 were all found to be satisfactory for quantitative gas chromatography of the TMS derivative of chlorogenic acid. UCW-98, however, gave better resolution of 4-O- and 5-O-caffeoylquinic acid in our studies, and hence was chosen as the liquid phase for use in the analytical procedure.

The non-linear relationship between peak area and quantity of sample chromatographed for quantities of chlorogenic acid below 0.8 μg likely results from loss of TMS derivative of chlorogenic acid during the gas chromatography step. That this non-linearity is due to conditions of chromatography and not to some anomaly in the silylation reaction was shown by the observation that trimethylnaringenin and benzoquinone, which could be chromatographed without prior silylation, gave similar non-linear response when subjected to gas chromatography using the system described here. Employment of glass columns and on-column injection did not increase linear response range, indicating that non-linearity at low sample levels probably is not a result of decomposition of TMS derivative on hot metal surfaces. It appears most probable that non-linearity can be attributed to irreversible adsorption of sample on the column packing. Efforts to alleviate this situation by increasing percentages of liquid phase on column packings have not been successful, and have also resulted in decreased column efficiencies and increased analysis time. Open tubular columns might be useful, but have not yet been tried. Extension of the linear range to lower levels of TMS derivative of chlorogenic acid would be helpful in further increasing the sensitivity of the method.

This method for quantitative determination of chlorogenic acid illustrates the potential of combined polyvinylpyrrolidone and gas chromatographic procedures for the determination of plant phenolic compounds. Current availability of new silylation reagents and liquid phases and improved column technology and instrumentation for gas chromatography should permit further exploitation of this potential.

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