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QUANTITATIVE ANALYSIS OF DEPSIDES AND RELATED PLANT PHENOLICS

BY GAS CHROMATOGRAPHY

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CHAPTER I

INTRODUCTION

The naturally occurring phenolic compounds include a
large and interesting variety of chemical structures.
Several hundred of the natural phenolic compounds are known,
with the flavonoids and their relatives forming the largest
group. Quinones, xanthones, depsides, and other groups also
exist in considerable number, in addition to many simple
monocyclic phenols (25). Important phenolic polymeric
materials such as lignins, melanins, and tannins are made
from phenolic precursors.

Due to their chemical reactivity and their almost
ubiquitous occurrence in higher plants, these phenolics
have been of interest in botany, chemical taxonomy, agricul-
ture, and industry (25,33,43,46,49). Since the advent of
paper chromatography, much progress has been made in the
area of comparative biochemistry of these phenolics (25).
Nonetheless, upon reading the literature, one is struck by
the lack of a clear understanding of the metabolism of these
compounds in the plant; even less is known about their
metabolism in the animal (including man) who ingests them.

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One of the difficulties encountered in studying the metabolism and function of many phenolics is the lack of a rapid, quantitative means of analysis. A possible solution to this difficulty seemed to be gas chromatography. Its high resolution and short analysis times, coupled with its quantitative potential make it far superior to the present methods of column, paper, and thin layer chromatography (25,41,42). The nature of the compounds of interest, however, presents the first big obstacle to the use of gas chromatography. The highly polar nature and high molecular weight of most plant phenolics make them poor subjects for gas chromatographic analysis. Therefore, the first problem for gas chromatographic analysis is to find a suitable derivative that can be prepared easily and quantitatively. Trimethylsilyl (TMS)[1] derivatives appeared to be the solution. A number of reports (18,19,20,21,22,23,30,37,38) are found in the literature on the silylation of both alkyl and aromatic hydroxyl groups and their use as derivatives for gas chromatography (see especially reference 37). Chapter II is a report of the examination of the use of the TMS derivatives of certain phenolic compounds for quantitative gas chromatographic analysis.

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[1]Abbreviations used in this report are: TMS - trimethylsilyl; TMCS - trimethylchlorosilane; HMDS - hexamethyldisilizane; DMF - dimethylformamide; DMSO - dimethylsulfoxide; THF - tetrahydrofuran; 2,4-D - 2,4-dichlorophenoxyacetic acid.

Typical of the problems involved in phenolic biochem-istry (25) are those encountered when studying the chloro-genic acids[2] in plants. Chlorogenic acid is a depside (a compound consisting of a phenolic acid esterified to a hydroxyl group of an alicyclic acid; see Figure I) which occurs almost universally in higher plants, often in high concentration (42,43). The chlorogenic acid content of unroasted Brazilian coffee beans, for example, is 6.3 per-cent of the dry weight, while sucrose and reducing sugars account for only 6.4 and one percent, respectively. Its relatively high concentration in such commercially valuable plants as the coffee bean, tobacco, potato, and many fruits are chiefly responsible for the continuous interest it has received since its discovery in 1837. Chlorogenic acid has even been reported in a fungus (14). An excellent review of the chemistry and biochemistry of the chlorogenic acids and related depsides, known or surmised up to 1964, has been given by Sondheimer (43).

Isolation of the chlorogenic acids has been achieved by paper chromatography (1,4,27,43,45), column chromatography (24,42), and countercurrent distribution (9,10), with quantitation being accomplished by UV spectrophotometry (1, 24,27), colorimetry (4,51), mocrobiological assay (8), and

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[2]Three isomeric forms of caffeoylquinic acid have been found to occur in nature: chlorogenic acid(3-O-caffeoyl-quinic acid), neochlorogenic acid(5-O-caffeoylquinic), and "band 510"(4-O-caffeoylquinic acid)(see ref. 43). A fourth possible isomer, 1-O-caffeoylquinic acid has, so far, not been reported.

CHLOROGENIC
ACID

Esculetin (R$_1$=R$_2$=H)
Scopoletin (R1=CH$_3$, R$_2$=H)
Esculin (R$_1$=H, R$_2$=glucose)
Scopolin (R$_1$=CH$_3$, R$_2$=glucose)

Cinnamic Acid (R$_1$=R$_2$=H)
Caffeic Acid (R$_1$=R$_2$=OH)
Ferulic Acid (R$_1$=OCH$_3$, R$_2$=OH)

Naringenin (R=H)
Trimethoxynaringen (R=CH$_3$)

Benzophenone

2,4-Dichlorophenoxyacetic
Acid

Maleic Hydrazide
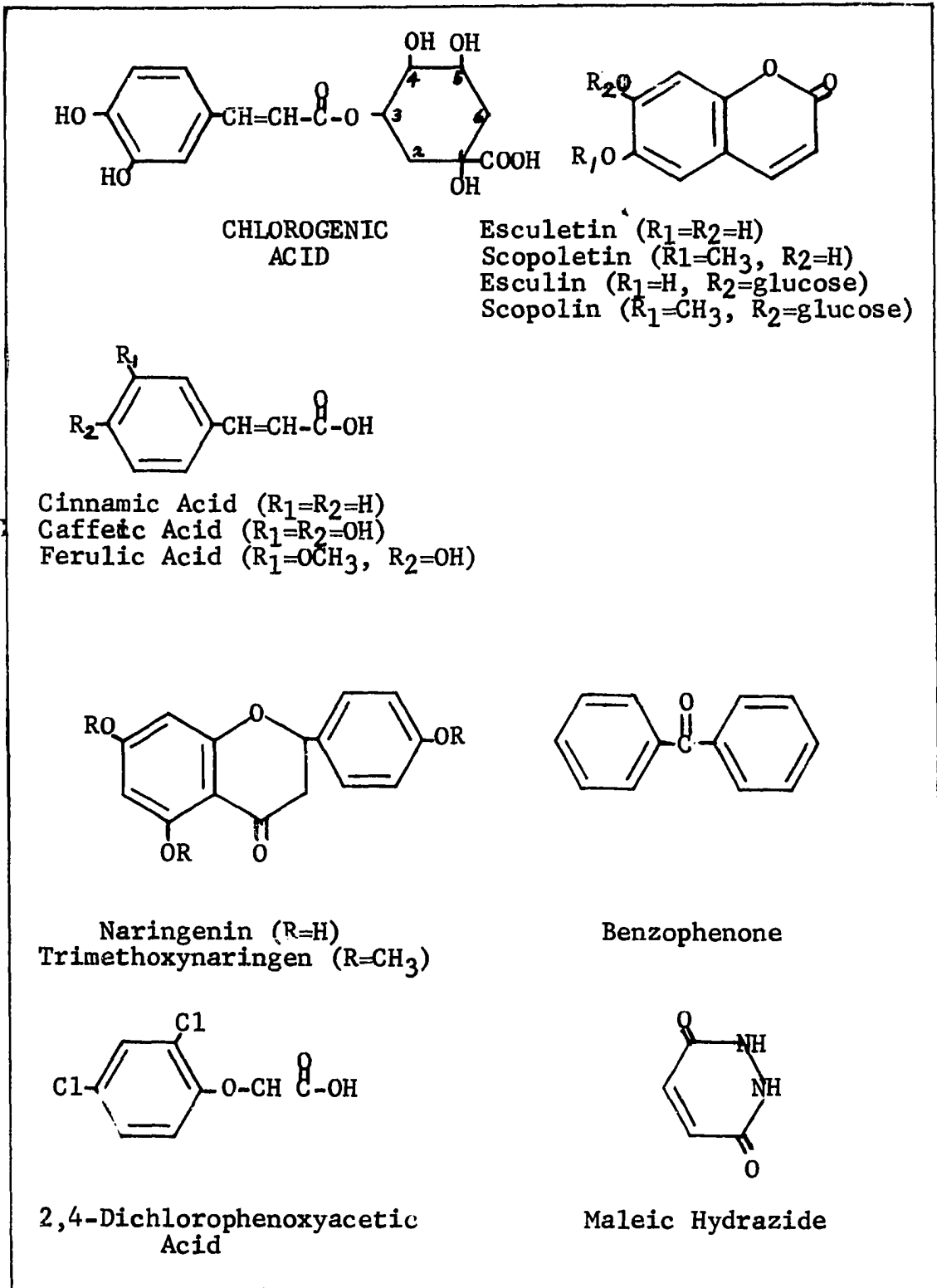
Figure I. Structural Formulas of Some Compounds Discussed in this report.

even polarography (11,12). All of the above methods lack either the desired sensitivity or the necessary rapidity for use in many metabolic studies. In Chapter III the use of polyamide column and gas chromatography to follow quantitatively, changes in chlorogenic acid concentrations in plants grown under various conditions is reported. A discussion of the findings as related to the role of chlorogenic acid in the plant is also presented.

CHAPTER II

A STUDY OF THE USE OF TMS DERIVATIVES OF PHENOLIC COMPOUNDS FOR QUANTITATIVE ANALYSIS BY GAS CHROMATOGRAPHY

Instrumentation

All gas chromatographic work was carried out on an F & M Model 810 Dual Column Research Chromatograph equipped with flame ionization detectors, a Minneapolis-Honeywell one-millivolt recorder, and a Disc integrator. Helium was used as the carrier gas in all experiments, and oxygen was used as the purge gas, to increase the sensitivity of the detector (17).

A six foot by 1/4 inch stainless steel column was selected as the standard for testing purposes. All columns were prepared by the method of Parcher and Urone (35), and cured according to the recommended method for each substrate. Substrates tested were SE30, SE52, UCW-98, XE-60, and OV-17. Of these substrates, only UCW-98, SE30, and SE52 had sufficiently high temperature stability for analysis of depsides. UCW-98 provided the best resolution of the chlorogenic acid isomers, and all quantitative analyses were done on 3% UCW-98 coated on Gas Chrom Q (Applied Science Corporation).

Glass columns were tested and appeared to have no advantages over stainless steel for depside analysis. One-eighth inch columns were also prepared and tested, but none yielded a higher number of theoretical plates than that obtained with the one-quarter inch columns. Coating of substrate could not be reduced much below the 3% level, in our hands, without causing a serious loss in efficiency.

Source and Treatment of Reagents

Hexamethyldisilizane (HMDS) was obtained from Applied Sciences Corporation, State College, Pa., or was prepared from trimethylchlorosilane by the method of Langer, Connell, and Wender (31) at a considerable saving. Technical grade trimethylchlorosilane (TMCS) was obtained from General Electric Silicone Products Department, Waterford, New York. This material, when redistilled, was quite pure (by gas chromatographic analysis) and, if kept free from moisture, could be stored two to three weeks. Since only small quantities of TMCS were used in the silylation reaction, and due to the volatile, corrosive nature of the chemical, only small amounts were distilled at one time and then stored in vials sealed with polyethylene caps. TMCS reacted with all other stopper material, even silicone rubber, and caused glass joints to freeze. The technical grade solvent could be used to prepare HMDS without redistilling. HMDS, (distilling between 124-126°C), if kept dry, can be stored four to five weeks before redistilling.

The solvents pyridine, tetrahydrofuran (THF), dimethyl-sulfoxide (DMSO), and dimethylformamide (DMF) were all reagent grade chemicals purchased from various chemical supply houses and used after a single redistillation. The need for dry solvents (31) was met in the case of pyridine and THF by storing over a column of molecular sieve (4A) after distillation. DMF, which could not be stored over a molecular sieve, was redistilled as needed (two weeks to one month supply each time).

Chlorogenic acid was obtained from Fluka AG, Buchs, Switzerland. Two samples were procured, one labeled "puriss.", the other labeled "purum", and were indistinguishable by thin layer and gas chromatography. However, puriss. grade had small, crystalline impurities that were insoluble in methanol (in which chlorogenic acid is highly soluble) or any other organic solvent, but soluble in water. Most of this material was removed by dissolving the chlorogenic acid in methanol, filtering to remove the insoluble material, and recrystallizing the chlorogenic acid from water. Neochlorogenic acid was obtained from USDA Laboratories in Albany, California, and was chromatographically pure. No 4-0-caffeoylquinic acid was available except for a small spotting standard. Phenolic acids were obtained from various chemical companies and varied in purity from technical to reagent grades.

"Polyclar AT" polyamide, a polyvinylpyrollidone, was obtained from General Aniline and Film Company, Grasselli, New Jersey. The Polyclar AT was washed free of soluble material by the procedure of Mizelle, et al. (34) and stored in distilled water (about twice the volume of the settled polyamide).

Preparation of the TMS Derivatives

Several phenolic compounds were silylated by dissolving 10 mg of sample in 0.5 ml of pyridine and adding 0.2 ml of HMDS, then 0.1 ml of TMCS. This procedure has been used by a number of investigators (2,23,32,37) and, save for variations in the quantities of reagents used, is a generally accepted method for silylating both aliphatic and aromatic hydroxyl groups. In Table I are listed a number of phenolic compounds and their retention times relative to cinnamic acid. Each compound silylated readily and smoothly, yielding a single symmetrical peak upon analysis by gas chromatography. Blakley (2) has also reported the gas chromatography of phenolic acid TMS derivatives, using the same system.

Selected phenolic acids were tested to see what factors affected the silylation of both carboxyl and hydroxyl groups. Cinnamic and p-coumaric acids were dissolved in pyridine; TMCS was added and mixed by slight shaking. After 10 minutes a one μl sample was analyzed by gas chromatography. The solutions were heated to reflux

TABLE I

Relative Retention Times of the TMS Derivatives of
Selected Phenolic Compounds

Compound	Retention time[a]
Cinnamic Acid	1.00
o-Coumaric Acid	2.25
m-Coumaric Acid	2.47
p-Coumaric Acid	2.61
Shikimic Acid	2.40
Quinic Acid	2.63
Ferulic Acid	3.04
Isoferulic Acid	3.02
Scopoletin	2.88
Esculetin	3.08
Caffeic Acid	3.22
Chlorogenic Acid	5.03
4-O-caffeoylquinic Acid	5.22
5-O-caffeoylquinic Acid	5.31
3-O-feruloylquinic Acid	4.98
4-O-feruloylquinic Acid	5.06
5-O-feruloylquinic Acid	5.17
Scopolin	5.03
Esculin	4.98

(a) Relative to cinnamic acid. R_t = 4.41 minutes,
uncorrected

Column: 3% UCW-98 on Gas Chrom Q., 6' X $\frac{1}{4}$" S. S.
Temperature program: inject at 148°, hold 4 minutes, up
10°/minute to 290°
Helium flow rate = 75 ml/minute.

for about two hours, samples being taken about every 20

minutes for analysis by gas chromatography. Comparison of

the peak areas of each sample indicated that cinnamic acid

silylates quite readily at room temperature. No increase

in silylated product was observed with heating or upon

standing for longer than ten minutes. On the other hand,

p-coumaric acid gave very low yields of silylated product

under the above conditions; however, when HMDS was added,

the yield increased markedly, and no significant increase

was seen after heating. It was also found that cinnamic acid will silylate readily using only TMCS, though poor solubility extends the reaction time.

Some compounds of interest (chlorogenic acid, flavonoids) underwent minor side reactions in the pyridine/ HMDS/TMCS system, as evidenced by a yellow color and/or multiple peaks on the gas chromatograph. Other solvents were then tried to reduce or eliminate these symptoms. Replacing pyridine by tetrahydrofuran (THF) eliminated the formation of yellow color and reduced the amount of multiple peak formation. That the yellow color is due to base catalyzed chalconization of flavonoids and to base catalyzed oxidation of ortho-phenols (25) is shown by the fact that refluxing in the pyridine system under dry N_2 eliminates the yellow color. THF was considered undesirable as a silylating solvent because most phenolic compounds are only slightly soluble and quantitative reactions require extended reflux times. Dimethylsulfoxide (DMSO), which had been reported to improve silylation of sterically hindered hydroxyls (20), was found to be a good solvent for all compounds of interest and the reaction appeared to go smoothly. Its disadvantages are that the TMS product is not soluble (20) and TMCS reacts slowly with it.

Dimethylformamide (DMF) had, likewise, been reported as a superior solvent for silylation of sterically hindered phenols (20,21), but it had a disadvantage similar to that

of DMSU in that, when TMCS was added (10 µl), a small
second phase appeared in which most of the silylated
product was dissolved. This, of course, would complicate
any quantitative procedure. However, it was noted that
ammonia was given off when the HMDS was added to the DMF
solution, before any TMCS was added. This indication of a
reaction did not occur in the pyridine/HMDS/TMCS system, so
the DMF/HMDS system was tested for its silylating ability
and compared with the pyridine/HMDS/TMCS system. The
reactions in the two systems are shown in equations (1) and
(2).

$$3 \; ROH + (CH_3)_3Si\text{-}NH\text{-}Si\text{-}(CH_3)_3 + (CH_3)_3Si\text{-}Cl \xrightarrow{\text{pyridine}}$$

$$3 \; RO\text{-}Si(CH_3)_3 + NH_4Cl \qquad (1)$$

$$2 \; ROH + (CH_3)_3Si\text{-}NH\text{-}Si(CH_3)_3 \xrightarrow{\text{DMF}} 2 \; RO\text{-}Si(CH_3)_3 + NH_3 \quad (2)$$
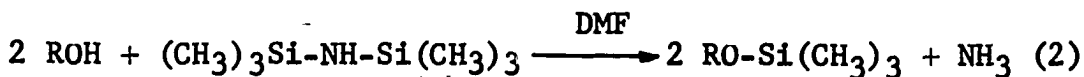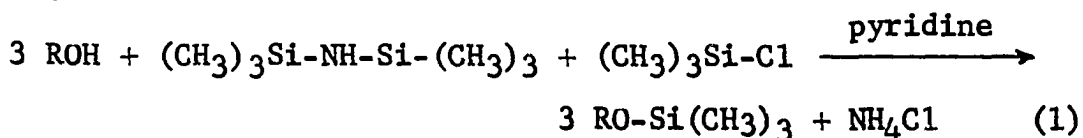
Figures II and III show the degree of silylation of chloro-
genic and caffeic acids, as measured by gas chromatographic
response for each system. The quantity of reagents used in
the DMF/HMDS system was 0.7 ml of DMF and 0.3 ml of HMDS.
According to the stoichiometry shown in equation (2), this
amount of HMDS will silylate 4000 µMoles of hydroxyl groups,
which is equivalent to 236 mg of chlorogenic acid.

Chlorogenic and caffeic TMS derivatives were prepared
in both the pyridine and DMF systems, and the effect of
temperature on the reactions was studied. If the reaction
mixtures were heated to reflux, the solutions became yellow,
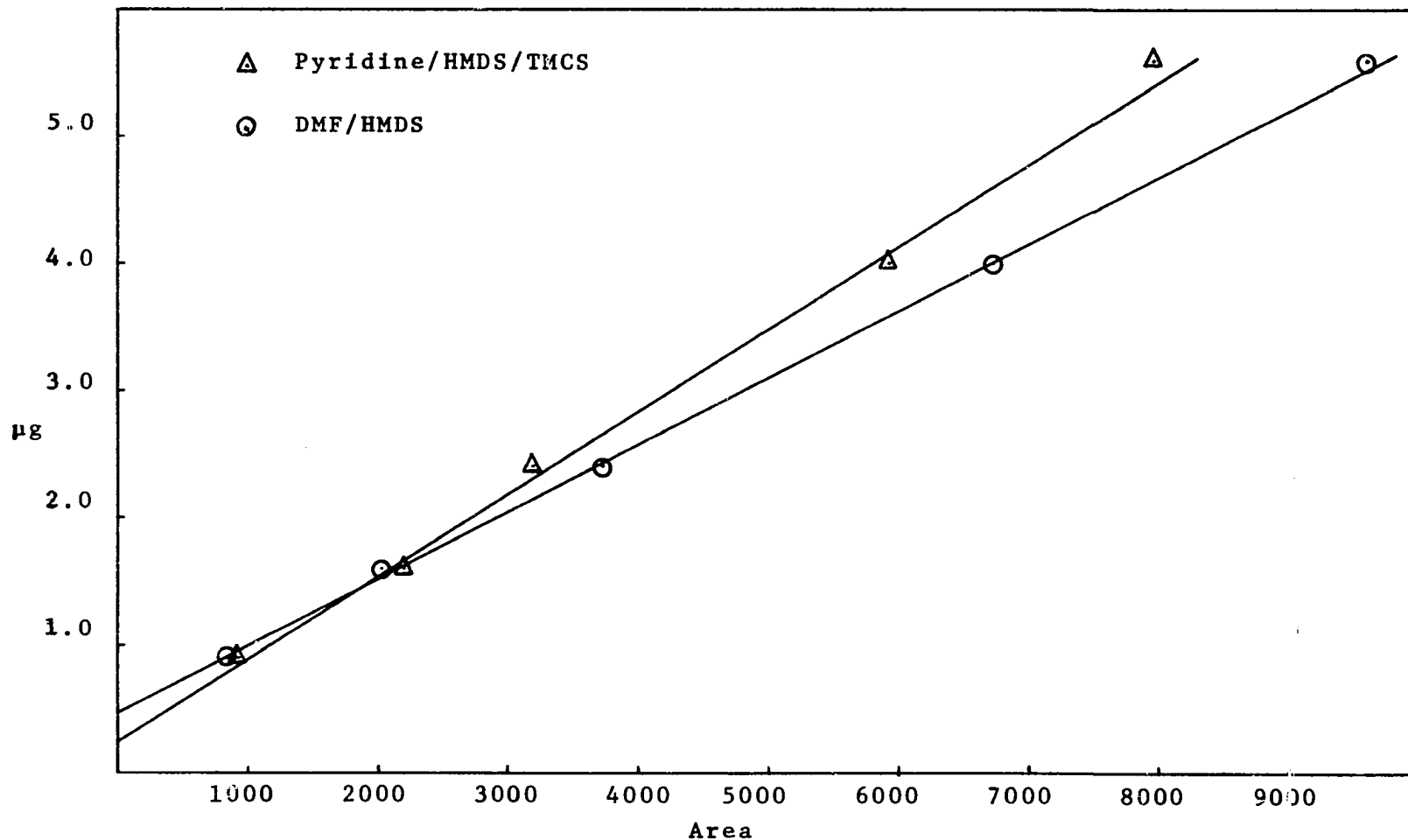
Figure II. Comparison of Degree of Silylation of Chlorogenic Acid in Pyridine/HMDS/ TMCS and DMF/HMDS. Ordinate = μg of chlorogenic acid injected; abscissa = peak area of silylated derivative.
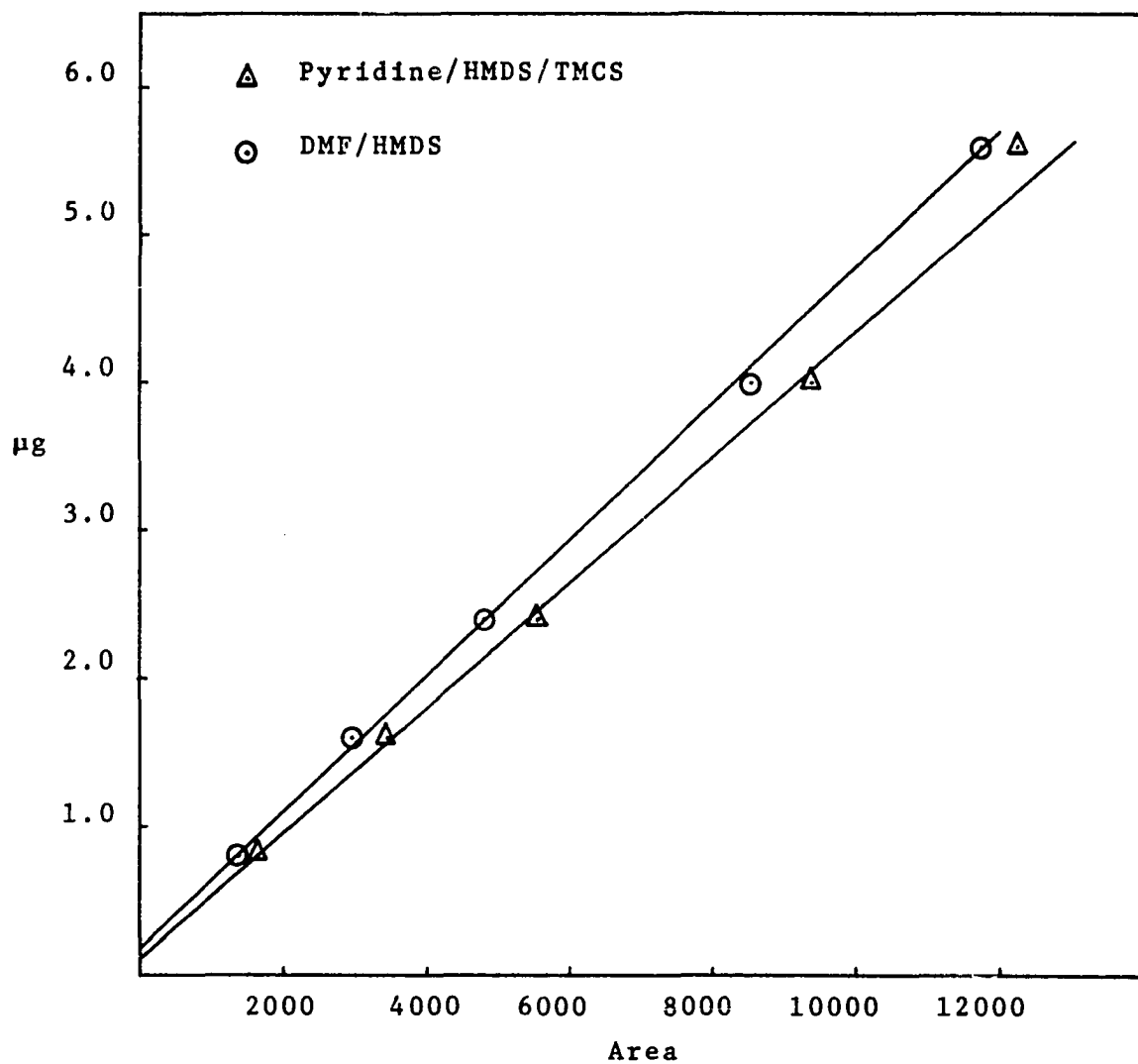
Figure III. Comparison of Degree of Silylation of Caffeic Acid in the Systems Pyridine/HMDS/TMCS and DMF/HMDS.

and analysis by gas chromatography indicated a lower yield of silylated product than could be obtained from the same solution if left at room temperature. The yellowing was eliminated by refluxing under nitrogen but the yield was never greater than from reactions run at room temperature. Heating to $50^{\circ}C$ for thirty minutes under nitrogen also did not improve the yield of silylated product. In the DMF system, maximum response was obtained after two hours at room temperature for chlorogenic and caffeic acids; in the pyridine system it took somewhat longer (32).

Since ammonia is formed during the silylation when using the DMF/HMDS system, the question was raised as to whether a more efficient removal of the NH_3 would increase the formation of the silylated product. A number of samples were again prepared, as described above, and, after addition of HMDS, were treated as follows: one set, after flushing with nitrogen, was placed, loosely capped, in an oven at 50°, immediately after adding the HMDS and left for one hour; the second set had nitrogen bubbled through it for about thirty minutes, then was capped and shaken, while yet another was heated to 50° on a sand bath and nitrogen was bubbled through the solution. And, finally, a set was left to stand at room temperature after shaking and venting. All samples were analyzed at least 24 hours after addition of HMDS. Gas chromatographic analysis of all the above samples indicated that heat or passing nitrogen through the solution gave no

increase in the yield of the reaction, though the rate was increased.

Not all compounds silylate as readily as chlorogenic acid or caffeic acid in the DMF/HMDS system. It had been observed that scopolin (6-methoxy-7-glucosyloxy-coumarin), which has no free phenolic group, silylated only to a very small extent in the DMF system while silylating readily in the pyridine system. If, however, a small amount of TMCS (50 μl) were added to the DMF solution, silylation occurred much more readily. It was thought that perhaps acidic hydroxyls are necessary for silylation to occur in the DMF/ HMDS system. To test this hypothesis, model systems were devised to show the effect on the extent of silylation of aliphatic, non-acidic hydroxyls of (1) acidic groups present on the molecule, (2) acidic groups present on another molecular species in the same solution. Results are shown in Table II.

Collection of the TMS Derivatives from the Gas Chromatograph

The gas chromatograph was set up for collecting samples by attaching a ten to one splitter to the detector end of the column, venting the major portion of effluent through a heated collection port. The column used was 6' X ¼" (OD) stainless steel packed with SE30 (3%), on Gas Chrom Q. Eight microliters of chlorogenic acid TMS derivative solution, containing 10 μg of chlorogenic acid per μl, were injected into the column. After the solvent peak had eluted,

TABLE II

EFFECT OF ACIDIC HYDROGEN ON TRIMETHYLSILYLATION IN THE SYSTEM DMF/HMDS

Model System	DMF/HMDS No. of Peaks	DMF/HMDS/TMCS No. of Peaks	Remarks
Inositol	none	1	Inositol is only slightly soluble in DMF.
Inositol & Resorcinol	none	1	Resorcinol peak is seen in both systems.
Inositol & 3,5-dihy-droxybenzoic Acid	none	1	3,5-Dihydroxybenzoic acid is seen in both systems.
Glucose	6	3	Before adding TMCS, peaks were small and not separated; after TMCS, peaks were larger, well defined with increased R_t.
Glucuronic Acid	4	4	After adding TMCS, one peak had increased at the expense of the others.
α-Methyl Mannoside	trace	1	The very small peaks which appear before TMCS all have lower retention times than the single large peak which appears after TMCS.

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a piece of glass tubing, 138 cm X 0.25 cm (OD), was attached to the collection port through a rubber septum. Three injections were collected in this manner. The tubing was then cut into three pieces and each piece washed out with hexane into a small round bottom flask. The hexane was removed by rotary evaporator and 100 µl of hexane was again added. These three samples were then tested by gas chromatography for the presence of chlorogenic acid. The end and middle pieces of tubing had no chlorogenic acid. The front piece, 50 cm in length, contained approximately 100 µg of chlorogenic acid.

Using the same 50 cm length of tubing with a cold trap at the end resulted in a loss of sample. One could detect an aerosol coming from the end of the tube. Electrostatic precipitation was also tried by inserting a small copper wire inside the open end of the tube for a distance of about five centimeters; a few turns of copper wire were wrapped around the outside of the tubing; and the ends of the wire and the coil were connected to a high voltage source (6-8kV). This procedure increased the recovery to a small extent. Caffeic acid TMS derivative was also successfully collected by the above method.

Hydrolysis of TMS Derivatives and the Determination of the Stability of Chlorogenic Acid under Conditions of Hydrolysis of the TMS Derivatives

Using a hydrolysis solution of methanol, water, acetic acid (80:20:0.1, v/v/v) (33), TMS derivatives of chlorogenic

and caffeic acids were hydrolyzed at reflux temperature for 30 minutes. The solvent was then removed by rotary evaporation, and the residue redissolved in methanol. Aliquots were examined by thin layer chromatography, and it was found that hydrolysis was complete after 30 minutes. In another test the following samples of chlorogenic acid were prepared under the conditions listed: -

1. Chlorogenic acid in hydrolysis solution, allowed to sit at room temperature overnight.

2. Chlorogenic acid, hydrolyzed 30 minutes at reflux temperature.

3. TMS derivative, hydrolyzed overnight at room temperature.

4. TMS derivative, hydrolyzed 30 minutes at reflux temperature.

5. TMS derivative, collected from gas chromatograph, hydrolyzed 30 minutes at reflux temperature.

Examination of these samples by thin layer chromatography showed that there was no breakdown of chlorogenic acid under the conditions of hydrolysis. There was also no breakdown of the TMS derivative other than hydrolysis of the silyl groups. The TMS derivative collected from the gas chromatograph showed an extra spot running close to the solvent front when spotted on a thin layer plate. This material was determined to be column substrate (from a UCW-98 column).

Preparation of Chlorogenic Acid Isomers

Using the method reported by Haslam, et al. (26), a mixture of 3-O, 4-O, and 5-O-caffeoylquinic acids was prepared for analysis by gas chromatography. Sixty milligrams

of chlorogenic acid in approximately one ml saturated potas-
sium carbonate solution was heated on a steam bath for 30
minutes. The solution was cooled and neutralized with 1 N
sulfuric acid and extracted with 3 volumes ethyl acetate.
The ethyl acetate was removed by rotary evaporator and the
water removed by azeotroping with isopropanol. The residue
was then dissolved in five ml of ethyl acetate and filtered
to remove salts accumulated from the water. The filtered
solution was once again taken to dryness, and the residue
converted into the TMS derivatives, using the DMF/HMDS
system. The chromatogram of the clear, yellow solution is
shown in Figure IV. Peak 1 had a retention time correspond-
ing to known chlorogenic acid TMS derivative; peak 3
corresponds to known 5-0-caffeoylquinic acid, and peak 2,
then, must correspond to 4-0-caffeoylquinic acid (26,40).

Analysis of a Compound Obtained from Plant Extracts

When the dried residue of the methanol fraction from
a polyamide column fractionation of a tobacco leaf extract
(see Chapter III) is silylated and analyzed by gas chromatog-
raphy, four peaks are normally observed. Figure V is a
typical chromatogram of such an analysis. Peaks 2, 3, and 4
were identified as chlorogenic, 4-0-caffeoylquinic, and
5-0-caffeoylquinic acids, respectively (note the similarity
to Figure IV), while peak 1 was unknown. That the unknown
peak was a phenolic compound seemed fairly certain since:
(1) the DMF solution, in the absence of silylating reagent,
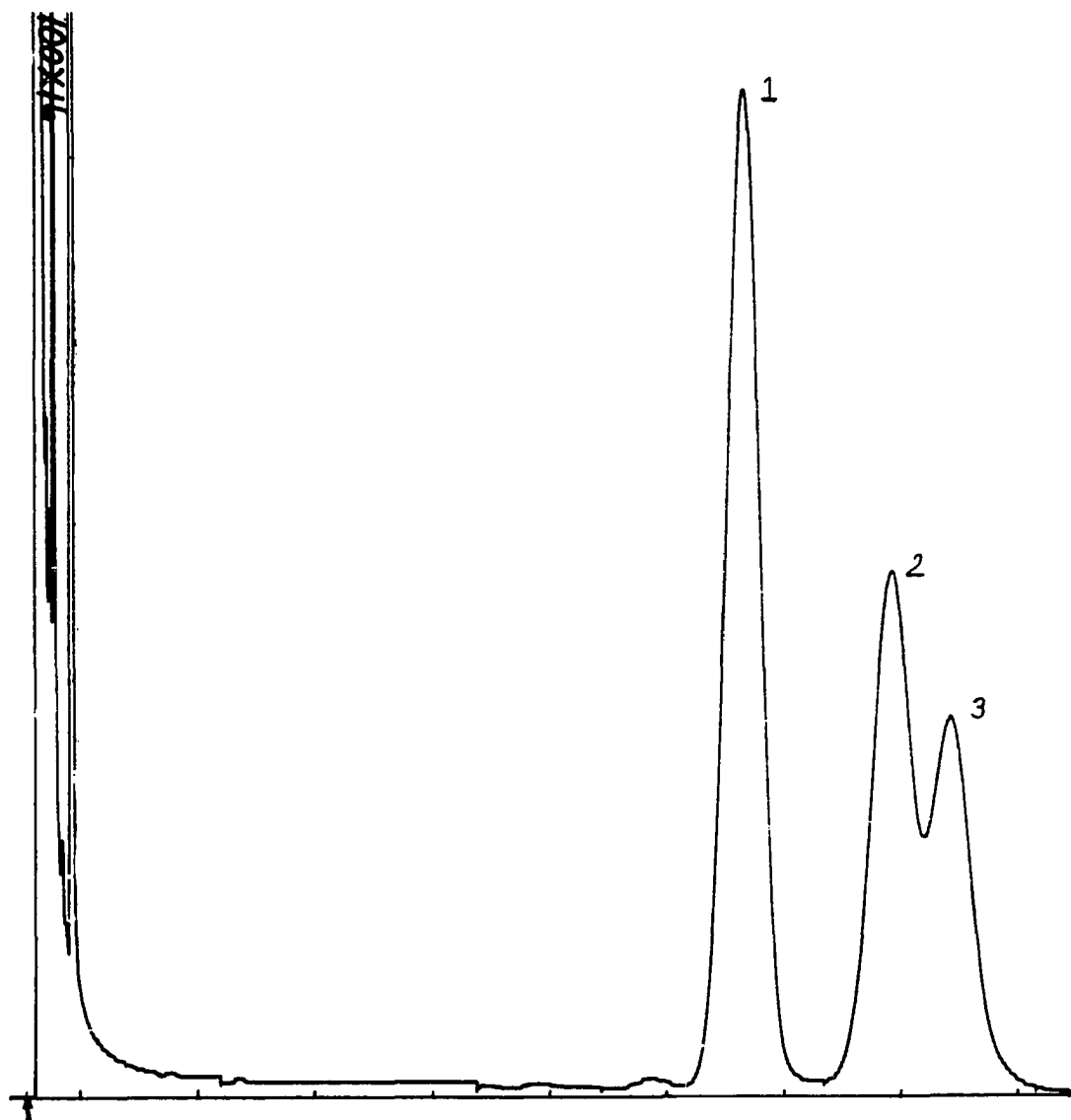
Column: 6' X ¼" Stainless steel; UCW-98(3%) on Gas Chrom Q
          (100-120 mesh)
Column temp.:   240°C
Inject. temp.:   300°C
Detector temp.:   280°C
Helium flow rate: 80 ml/min.

Figure IV.   Gas Chromatograph of TMS Derivatives of
Chlorogenic Acid Isomers.
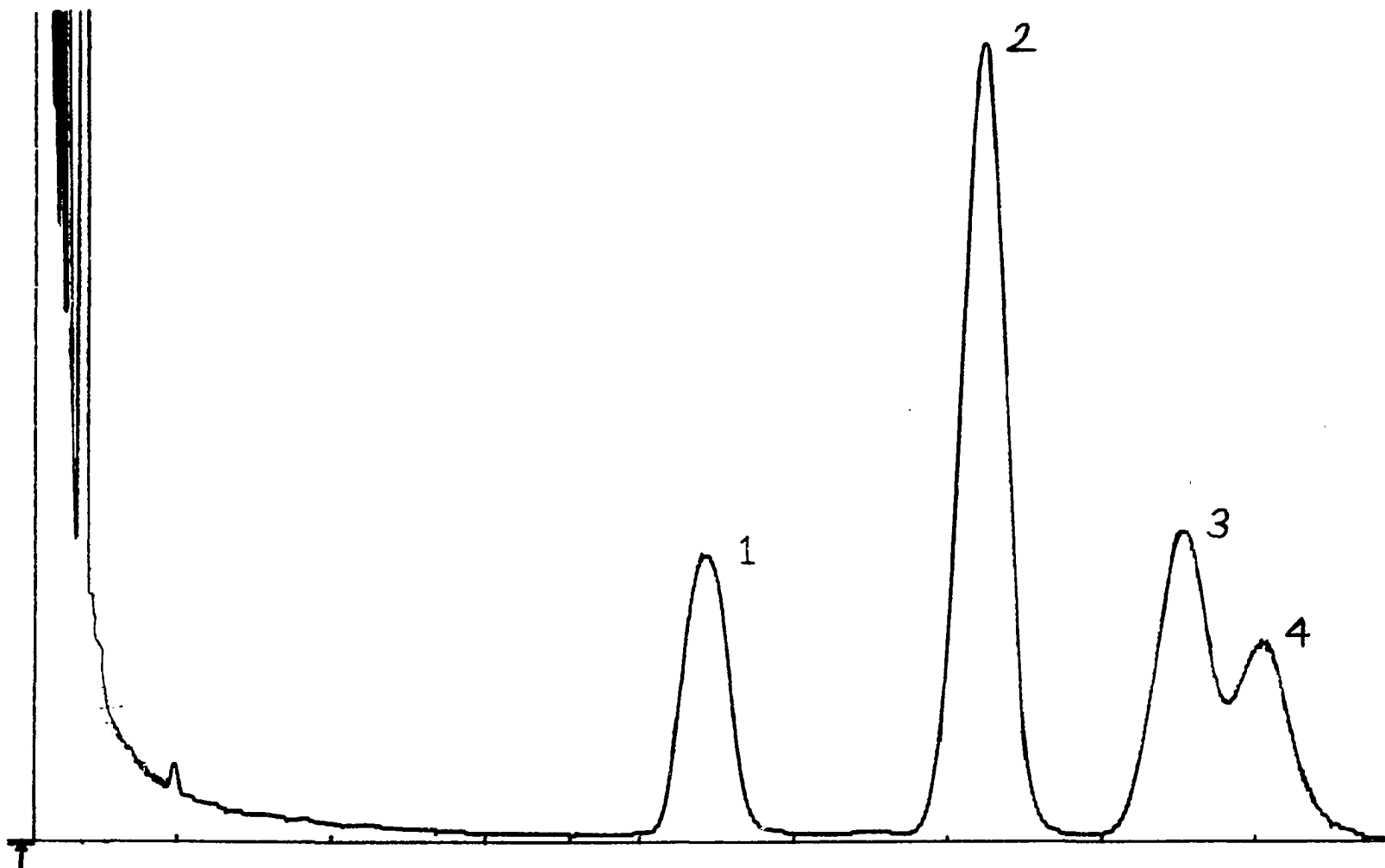
Column: 6' X ¼" S.S., UCW-98(3%) on Gas Chrom Q (100-120 mesh); Column temp. = 240°C;
Inject. temp. = 300°C; Detector temp. = 280°C; Helium flow rate = 80 ml/min.

Figure V. Gas Chromatograph of TMS Derivatives of Polyamide Column Fraction #3,
From Tobacco Leaf Extract.

yielded no peaks when injected into the gas chromatograph,

(2) only one peak was obtained in the DMF/HMDS system,

(3) its elution pattern on the polyamide column suggested a phenolic compound similar to chlorogenic acid.

The TMS derivative of the unknown peak 1 was collected from the gas chromatograph using a DC electrostatic precipitator and was hydrolyzed for 30 minutes in methanol/water/ acetic acid, (80/20/0.1) at reflux temperature. The hydrolysis solvent was removed by rotary evaporator and the residue was redissolved in 100 µl of methanol. Aliquots of this solution were tested in various thin layer systems to compare R_f values with known standards. The results are shown in Table III.

TABLE III

THIN LAYER R_f VALUES OF UNKNOWN TOBACCO PLANT COMPOUND ISOLATED BY GAS CHROMATOGRAPHY

Thin Layer Substrate	Solvent System (v/v)	R_f
Woelm Polyamide	Benzene/methanol, (5/2)	0.80, 0.86
	Benzene/methanol/formic acid (3/2/0.1)	0.92*
Silicar-4	KFW (14/3/2)	0.21*, 0.77
Adsorbosil-1	KFW (14/3/2)	0.21*, 0.85
Avicel SF	Butanol/pyridine/water (14/3/3)	0.77
	Ethyl acetate/pyridine/ water (2/1/2)	0.63*

*Identical with scopolin. All spots give the same blue fluorescence (visual) as scopolin.

Quantitation of Chlorogenic TMS Derivative

To determine the precision of the analysis procedure,
three samples of chlorogenic acid were prepared and analyzed.
Two ml of a standard solution of chlorogenic acid (1 mg/ml,
in methanol) were pipetted into each of three one-dram vials
and the solvent removed by heating at 50°C under a stream of
nitrogen. The three samples were then silylated with DMF/
HMDS and analyzed by gas chromatography. Two injections were
made per sample, injecting approximately one µl each time,
and the average area from all injections was calculated.
Using a one µl syringe (Hamilton, 7101N) the standard devia-
tion was 58.3 with a coefficient of variation equal to 1.7%
and a range of 156 units. Attainable precision was signifi-
cantly less when using a 10 µl syringe for one µl size
injections. Statistical analyses of typical quantitation
runs using varying concentrations of chlorogenic acid are
given in Table IV.

TABLE IV

STATISTICAL ANALYSIS OF STANDARD CURVES OF CHLOROGENIC ACID
TMS DERIVATIVE

Analysis number	Variance	Standard Deviation	Coefficient of Variation	Range
1	2,683	51.8	4.3%	130
2	17,600	132.	12 %	242
3	2,323	48.2	4.0%	185

Each analysis shown in the table consisted of four
weighed samples, varying between one and 20 mg. Each sample

was dissolved in 0.7 ml of DMF, added with a microliter pipette. Three hundred µl of HMDS were added (microliter pipette), the samples stoppered, shaken for 30 seconds, then carefully vented and left open for ten minutes. In analysis #2, DMF was added to bring the final volume to exactly one milliliter. Less than 50 µl were needed for each sample, the reagent volumes being essentially additive. Two injections were made from each sample and the average area obtained. Injections were made with a Hamilton 10 µl syringe with Chaney Adaptor. Total analysis time for four samples was 3.75 hours. Analysis #1 was begun within three hours after addition of HMDS. Analysis #2 was begun one hour after addition of HMDS, and analysis #3 was performed on the same set of samples as analysis #2, but conducted 20 hours later. Plots of the data obtained gave curves similar to the DMF/ HMDS curve in Figure II.

Analysis of the Nonlinear Response of Chlorogenic TMS Derivative in Gas Chromatographic Analysis

The data in Figure II indicate a linear relationship of peak area to µg chlorogenic acid injected over the range tested. It was noted, however, that if the curve were extended back to the point of zero area, the line did not pass through the origin. It was further noted that if quantities of TMS derivative corresponding to less than one microgram of chlorogenic acid were injected, the response fell off rapidly and the areas no longer fell along the line

established by higher concentrations. These observations
suggest a nonlinear response in the zero to one microgram
range. If the response were linear from 0-20 µg's (an
arbitrary upper limit), the area/ug of various sized injec-
tions should be constant. Since the above mentioned obser-
vations did not support such a constant ratio, the variation
in gas chromatographic response was examined by preparing a
set of TMS chlorogenates of varying concentrations, analyzing
by gas chromatography, and plotting area/µg against chloro-
genic acid concentration. The results are seen in Figure VI;
the response is seen to increase rapidly to what appears to
be a maximum value. Another test was conducted in which a
single sample of chlorogenic TMS derivative was prepared and
analyzed by gas chromatography. The sample was then diluted
a measured amount with DMF/HMDS reagent, and again analyzed
by gas chromatography. A series of such dilutions was
analyzed and the area/µg plotted against the concentration.
A curve identical to that in Figure VI was obtained. Varying
the amount injected from a single sample also gave identical
results.

Similar dilution tests were run on trimethoxynarin-
genin and benzophenone (see Figure I), two compounds that
could be chromatographed without need of silylation. Again
a non-constant area/µg was encountered and curves were obtained
like the one in Figure VI. These results suggested that the
nonlinear response was due to the conditions of chromatography
and not to some anomaly in the silylation reaction.
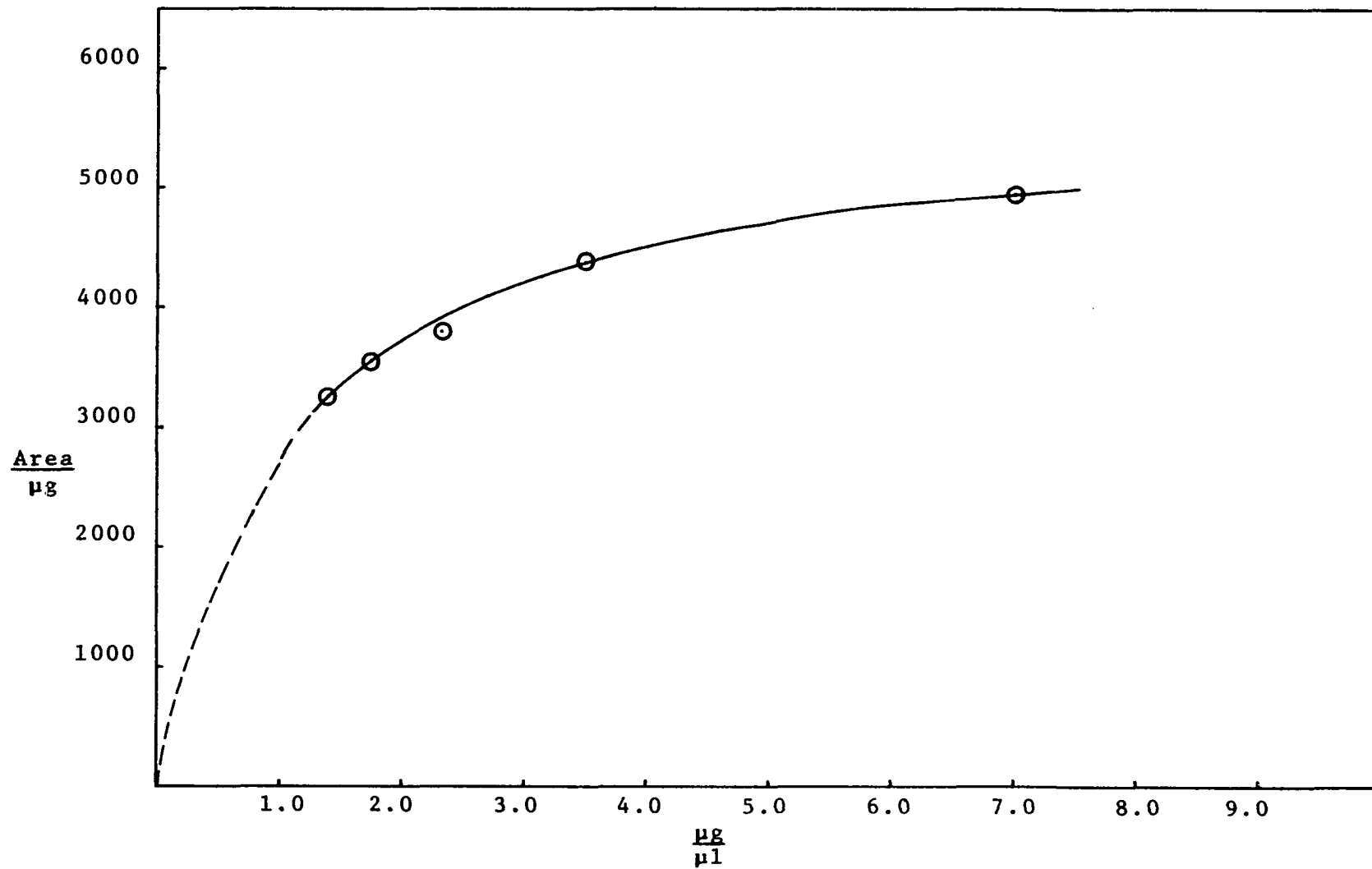
Figure VI.   Variation of Unit Area of Chlorogenic TMS Derivative with Concentration of Sample

Examination of the gas chromatographic components showed that detector, recorder and Disc integrator response were linear over the range desired. The remaining likely sources of nonlinearity were irreversible adsorption and/or decomposition on the gas chromatography column. Such could probably occur at any "active sites" (points of column packing material not covered by column substrate) in the column. These active sites can be reduced by increasing the coating of the substrate; however, increasing the substrate load to as much as 10% did not reduce the nonlinearity. The use of on-column injection and glass columns also did not extend the linearity of response, indicating that loss was incurred neither in the injection port nor by decomposition on stainless steel surfaces.

If a constant amount of sample were lost with each injection, such a loss would affect smaller quantities to a greater extent than larger ones, and one would see a slow increase in area for small quantities, followed by a region where the increase in area/µg is large and constant. Such a situation can be described by the equation

$$y = ax + b(1-e^{-cx}) \tag{3}$$

where x is the area of the chromatographic peak and y is the quantity of sample injected. The constant a corresponds to the slope of the linear region (large x) and is the maximum area/µg response obtained from the gas chromatographic analysis. Constants b and c are related to the amount of

material that is lost on the column. Using data from a
series of chlorogenic acid samples, prepared and analyzed
as previously described, the constants of equation (3)
were calculated and the curve obtained is shown in Figure
VII.

Discussion

The results reported above show the TMS derivatives of
phenolic acids are quite well suited to analysis by gas
chromatography and that these derivatives can be made rapidly
and quantitatively. Further, the experimental results show
that even high molecular weight depsides such as chlorogenic
acid (MW-354) can be quantitatively analyzed by means of
the TMS derivative.

Though the DMF/HMDS system has been demonstrated herein
to be somewhat superior for analysis of caffeic and chloro-
genic acids to the pyridine/HMDS/TMCS system, the latter
system is a perfectly satisfactory one, and has been used in
a number of instances (2,22,23,32,38,48) for quantitative
analysis by gas chromatography. Wells, Pittman, and Wells
(48) have quantitatively analyzed for inositol in rat tissue
by gas chromatography, using a premixed silylating solution
of pyridine/HMDS/TMCS, and report a linear relationship
between mass of inositol and peak area over the range 0.1-
3.0 μg. Lau (32) has studied the factors affecting the
formation of the TMS ethers of steroids in the pyridine/HMDS/
TMCS system. Her findings concerning the effects of heat and
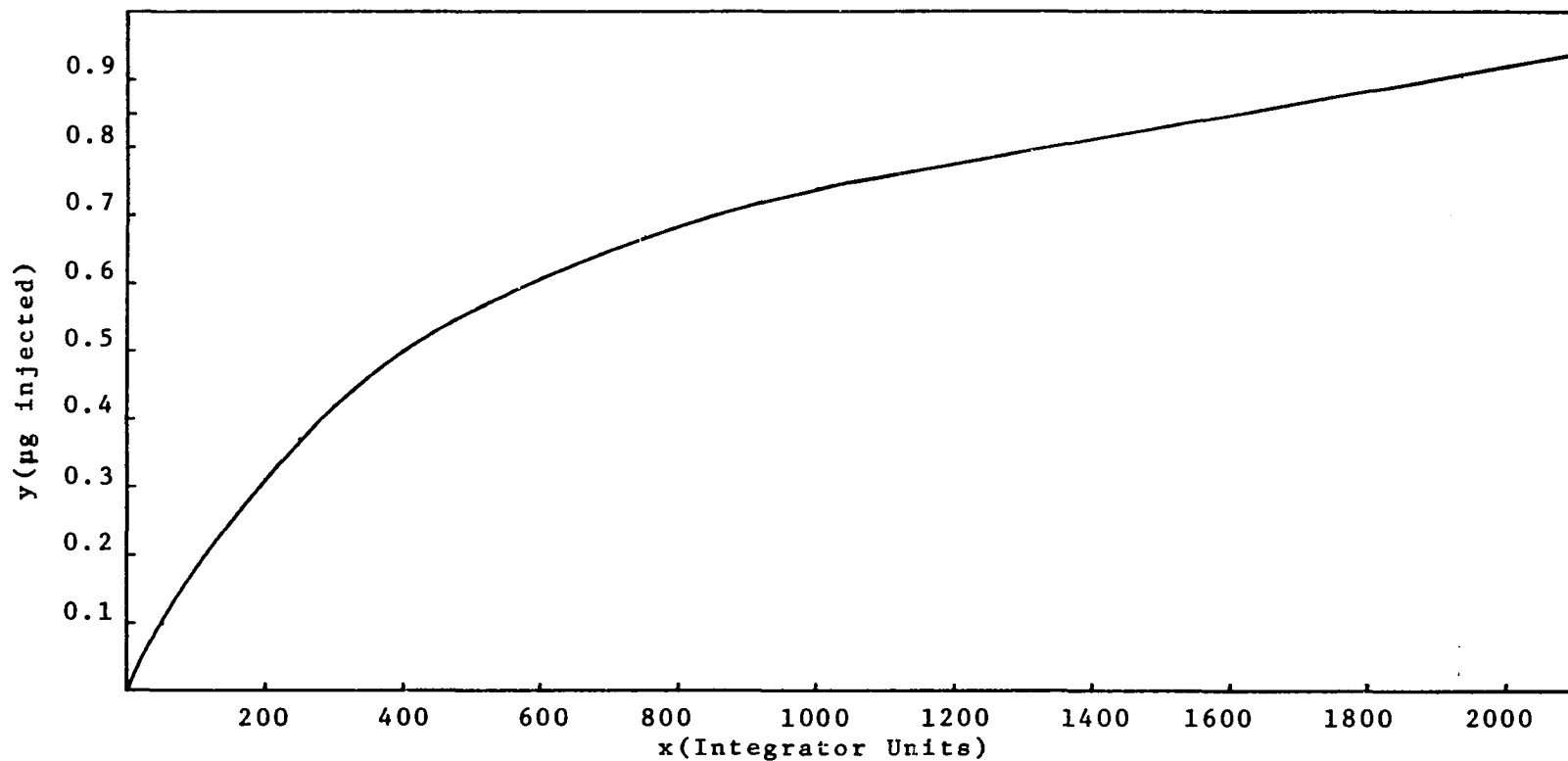
Column: 6' X ¼" S.S., UCW-98(3%) on Gas Chrom Q (100-120 mesh); Column temp. = 250°C; Inject. temp. = 300°C; Detector temp. = 280°C; Helium flow rate = 80 ml/min.

Figure VII. Gas Chromatographic Response Curve for Chlorogenic TMS Derivative.
Equation: $y = (1.6 \times 10^{-4})x + 0.6(1-e^{-(3.2 \times 10^{-3})x})$

moisture on the silylation reaction appear to apply equally well to the DMF/HMDS system. The superiority of DMF over pyridine as a solvent for silylation reactions is shown in the case of sterically hindered hydroxyl groups (21). 2,4,6-Tri-t-butylphenol would not silylate in the pyridine/HMDS/TMCS system, while TMS ether formation proceeded easily when DMF was substituted for pyridine, even in the absence of TMCS. Steric hindrance may well be a factor in the silylation of chlorogenic acid.

In view of the superiority of DMF over pyridine in the case of sterically hindered silylations, it was surprising to find such a poor yield of TMS scopolin in the DMF/HMDS system, compared to essentially quantitative conversion in the pyridine/HMDS/TMCS system. On further examination, this discrepancy can be explained as a kinetic effect. Our experiments were conducted at room temperature over a period of a few hours, while the experiments of Friedman and co-workers (20,21) were conducted under extended reflux. The results of the model system (Table II) seem to indicate that the molecule must have at least one relatively acidic hydroxyl group before silylation will occur in the DMF/HMDS system, to any extent, at room temperature. This is further evidenced by the fact that esculin, a 6-hydroxy-7-0-glucosyloxy-coumarin (Figure I), silylates quite readily in the DMF/HMDS system while scopolin, its 6-methoxy analogue, does not. These results cannot be readily explained on the basis

of simple acid catalysis (18,37). DMSO, like DMF, is also
a good solvent where steric hindrance is a factor (20). It
has a further advantage in the case of flavonoids of being
an excellent solvent for this class of compounds, and being
even less basic than DMF, less likely to promote chalconiza-
tion. The multiple peaks reported by Furuya (22) for certain
flavonoid aglycones were probably due to partial silylation
and/or chalconization, which could occur in the pyridine/
HMDS/TMCS system. In our hands, multiple peaks with naring-
enin could be reduced to one using a solvent system of THF/
DMSO (9/1) to replace pyridine.

An interesting possibility is the preparation of TMS
derivatives without a solvent. Freedman and Croitorn (18)
have described the quantitative preparation of TMS ethers of
low boiling phenols by mixing sample, HMDS, and an acid oxide
(SiO_2, Al_2O_3, MoO_3, TiO_3) and refluxing at $200^\circ C$ for 45
minutes. Basic oxides were reported as being ineffectual
and even inhibitory.

One of the more difficult requirements in the gas
chromatography of phenolic compounds is that of a nonpolar
column substrate having a high temperature stability (5).
Silicone polymers, at present, are the only available sub-
strates satisfying these requirements. SE30 and SE52 have
been used in a number of cases (2,13,17,22,39), and SE30
was used extensively in the development of the gas
chromatographic analysis of depsides. Unfortunately,

resolution of the 4-0- and 5-0-caffeoylquinic acids could not be achieved on SE30. It was later discovered that the isomers could be sufficiently separated on UCW-98, another silicone polymer, differing from SE30 in that the side chains were vinyl groups rather than methyl groups.

Increasing the efficiency of a given substrate can be done in two ways, decreasing the amount of coating, and decreasing the column diameter. Both procedures were tried, without any success. Using three percent coatings, very good efficiencies (400 plates/ft.) were obtained with ¼ inch columns. Reducing the percentage of substrate below this level usually resulted in increased tailing and slight reductions in the number of theoretical plates. These results would support the reported difficulty of preparing low load, non-polar columns (5,16), though a number of researchers (22,23,37,38) have used one percent, and even lower, coatings with success.

The lack of success with eighth inch columns is difficult to explain in view of their theoretical (5,16) and experimental (13,38) superiority to quarter inch columns. Perhaps the degree of technical proficiency gained by preparing quarter inch columns was inadequate for the preparation of a good eighth inch column. Should a commercially prepared column prove to have the desired efficiency (e.g. baseline resolution of the chlorogenic isomers) the linear range would probably be extended to lower levels due to the

drastic reduction in the number of active sites in going from 1/4 to 1/8 inch diameter.

Open tubular (Golay) columns were not used. Should the difficulty of preparing nonpolar columns (16) be overcome, and by using some of the more reactive silylating reagents such as bis-trimethylsilylacetamide (37), one could conceivably analyze quantitatively entire spectrum of phenolic material from a plant extract without any preliminary fractionation.

By the use of temperature programming one can analyze a mixture containing a wide boiling range of phenolic acids in a relatively short time. However, using SE30 is was found to be extremely difficult to maintain a stable baseline over the entire temperature range. Even using closely matched columns, it was not possible to obtain equal bleed rates as the temperature approached the upper limits (300°).

De Maio and Corn (13) also reported this difficulty, using the same instrument and columns. They suggested that the carrier gas flow controllers on the F & M instrument did not give a balanced flow as the pressure increased. Later attempts at temperature programming (Chapter III) were successful when columns were grossly mismatched (1/4" versus 1/8").

The ability to collect chlorogenic and caffeic acids from the gas chromatograph allows one to use the superior resolving powers of the gas chromatograph to separate phenolic

compounds for identification. Using only an analytical sized ($\frac{1}{4}$") column one could collect, within a few hours, sufficient material for analysis by microtechniques, e.g. IR, UV, NMR. The investigation of the unidentified compound found in tobacco samples (page 20) is an example of the combination of gas and thin layer chromatography to identify unknown compounds.

An electrostatic field was used after trapping by cold trap and by solvent proved to be of no value in trapping the gas chromatographic effluent. Kratz, Jacobs, and Mitzner (29) had reported the tendency of high boiling compounds to form aerosols and the use of an electric field to precipitate them. Borka and Privett (3) have reported the use of high intensity electric fields to collect fatty acid methyl esters. They further showed that AC fields caused some degradation of the esters which was not observed in a DC field. Since phenolic acids are more reactive chemically than are fatty acids, similar tests were conducted using chlorogenic acid. No difference was detected between AC and DC fields and no degradation products were found; however, the field strengths obtained in these tests were not as high as those used by Borka and Privett.

In connection with the collection of the TMS derivatives, the recovery of the unsilylated original compound is of course very important. In the case of chlorogenic and caffeic TMS derivatives, the original compounds were easily

recovered. However, with other phenolic compounds, the conditions of hydrolysis may be critical (25,43). Fortunately, the conditions necessary for hydrolysis of the TMS group are much less severe than that used in the hydrolysis of the chlorogenic derivative. Experiments conducted in connection with thin layer chromatography of the TMS derivatives showed that complete hydrolysis in aqueous media could be obtained after one hour at room temperature. That the hydrolysis was too severe in the case of the unidentified compound from tobacco (page 20) is quite probable. If the spots obtained upon thin layer analysis are scopolin (and the evidence is slight; see Table III) then scopolin is only a part of the original molecule, or some molecular rearrangement occurred during hydrolysis.

CHAPTER III

QUANTITATION OF FREE CHLOROGENIC ACID FROM PLANTS

Extraction of Phenolic Material from Plants

Plants were harvested by dividing each plant into separate samples of roots, stem, leaves, and flowers (if present) and enzymic action was stopped immediately by plunging the pieces into boiling methanol. At this point, the material could either be extracted, or stored under refrigeration.

The samples (solid + methanol) were ground thoroughly in a Waring Blendor; then quantitatively transferred to a Soxhlet extraction thimble; and the filtrate collected. Root samples were not ground, but were chopped, since grinding produced a gelatinous mass that could not be efficiently extracted. The solid residues were washed in succession with 3 to 4 volumes of the following hot solvents:

1. isopropanol water (1/1, v/v)
2. isopropanol, benzene, methanol, water (IBMW, 2/1/1/1, v/v/v/v)
3. isopropanol - water azeotrope.

All washings were combined with the methanol and the solvents were removed, using a rotary evaporator. The thimbles were placed in the Soxhlet apparatus, and each residue was

extracted for 24 hours with isopropanol. Analysis of three successive 24 hour extractions by thin layer chromatography indicated that esentially all of the unbound phenolic material had been removed by the end of the second extraction.

The solutions from the two extractions were combined with the residue from the washing procedure, and the combined material was taken to dryness, using a rotary evaporator. The dried residue was cut from the walls of the flask by an emulsion of equal volumes of water and benzene. The emulsion was then brought to one phase by adding a volume of isopropanol equal to the combined volumes of benzene and water. This usually dissolved all solid residue. A volume of methanol equal to the amount of water present was next added, and the solution was transferred to a volumetric flask. More of the solvent mixture (IBMW, 2/1/1/1, v/v/v/v) was added, to bring the extract to the correct volume. Final volumes were adjusted to between two and three times the original fresh weight of the sample.

Preliminary Fractionation of Plant Extracts by Polyamide Column Chromatography

A 14.5 mm (ID) column was packed to a depth of 6.5 cm with Polyclar AT suspended in water. The Polyclar was washed with 50 ml of methanol, and then with 50 ml of IBMW. The column was packed and washed under a pressure of five psig. After washing, the column was charged with five ml of plant extract (in IBMW), using a five ml transfer pipette. The

extract was allowed to run slowly down the sides of the column
so as not to disturb the surface of the polyamide. The walls
of the column were washed with two to three ml of IBMW, and
the extract was forced onto the polyamide under five psig.
Elution was begun with benzene/methanol (9/1, v/v). The
eluting solvents, their volumes, and order of use were

1. Benzene/methanol (9/1, v/v) - 50 ml
2. Benzene/methanol (3/1, v/v) - 150 ml
3. Methanol (100%) ------------- 300 ml
4. DMF (100%) ------------------ 250 ml.

Each solvent was added just as the last of the preceding
solvent drained into the top of the polyamide. All elution
was conducted under five psig. Fractions collected corres-
ponded to the eluting fractions. When such columns were
operated between 22 and 26°C, chlorogenic acid and its isomers
were always found in the third (methanol) fraction.

The fractions were collected in round bottom flasks
and taken to dryness directly on the rotary evaporator. The
dried residue of each fraction was transferred to a four dram
vial with four 10 ml washings of methanol, and the methanol
was evaporated by allowing the solution to stand under a
stream of air, or under a stream of nitrogen when using low
heat (50 - 60°C).

Analysis by Gas Liquid Chromatography

The dried residue in the four dram vial was dissolved in
0.7 ml DMF, then 300 ul of HMDS was added, all by means of
microliter pipettes. The final volume was essentially one

ml (see Chapter II). The vials were stoppered and vigorously shaken for about 30 seconds, then were left to stand loosely stoppered for 30 minutes to one hour. Upon resealing with polyethylene caps, the samples were considered ready for analysis after two to three hours. Two to three injections were made per sample; the areas of the peaks obtained were measured by Disc integrator; and the average area for each component was computed.

Quantitation of Chlorogenic Acid

The gas chromatographic response to the TMS derivative of chlorogenic acid was shown to be linear when the quantity injected was in the range of 0.8 - 20 µg (see Chapter II). It is detectible at even lower levels, but the response becomes too nonlinear to allow for quantitation. Each time a sample, or series of samples, was to be analyzed for chlorogenic acid, two standard samples of pure acid were prepared and analyzed to establish the slope of the response curve in the following manner:

1. A standard sample of chlorogenic acid was prepared in methanol. Only small quantities of standard solution were prepared at one time, as the acid decomposes very slowly in the methanol, even when kept in the freezer. Decomposition could be detected by both thin layer and gas chromatography. Standard solutions were not kept for more than two weeks.

2. Appropriate quantities of the standard solution were transferred to four dram vials and then silylated as described above.

3. The areas of the peaks obtained and the corresponding quantities of chlorogenic acid injected were used to calculate the slope of the standard curve. A one mg and a 10 mg sample were used to determine the slope.

$$\text{Slope} = \frac{\mu g \text{ A injected} - \mu g \text{ B injected}}{\text{Area A} - \text{Area B}}$$

where $\underline{A}$ is the 10 mg standard sample and $\underline{B}$ is the one mg standard sample. The slope thus obtained is used to calculate the amount of chlorogenic acid in an unknown sample,

$$\begin{array}{l}\mu g \text{ chlorogenic} \\ \text{acid in sample}\end{array} = (\mu g \text{ A injected}) - (\text{Slope})\left(\frac{\text{Area}}{\text{A}} - \frac{\text{Area sample}}{\text{injected}}\right)$$

The isomers of chlorogenic acid are quantitated in the same way, using chlorogenic acid as an external standard.

The amount of chlorogenic acid injected into the gas chromatograph must be at least 0.8 μg to lie within the linear range. Two methods were employed to place the analysis within the desired range. In the first, the "standard" injection volume was 0.8 μl, made with a Hamilton one μl syringe (model 7101N). If the area of a given chlorogenic acid sample fell below that of the one mg/ml standard sample, then three μl injections were made using a Hamilton Microliter 75 (five μl capacity). The second method of increasing chlorogenic acid concentration in a given sample was to increase the concentration of the original extract

before fractionation on polyamide. Extracts represented 0.2 - 0.3 g of fresh tissue per ml of extract. Depending on the quantity of solute present, these extracts could be concentrated as much as five fold without precipitation of dissolved material.

Precision of Analysis

Six samples from a single tobacco plant extract were analyzed for chlorogenic acid by the above procedure. The results yielded an average of 1.30 mg in five ml of extract with a standard deviation of 0.05 mg and a coefficient of variance of 3.8%. The combined average yield of 4-0- and 5-0-caffeoylquinic acids was 0.6 mg with a coefficient of variation equal to 9%. A seventh sample was prepared, to which a known amount of chlorogenic acid had been added. Analysis of the sample for chlorogenic acid showed 2.0 mg compared to a theoretical value of 1.9 mg, which is an error of 5%.

Analysis of 2,4-D Treated Tobacco Plants

Set 1 tobacco seeds were planted in sterile chambers on cheesecloth and the resulting seedlings were transplanted to half-gallon jars periodically between 15 and 32 days. The plants were maintained on complete nutrient solution. Sixty days after planting, the seedlings were divided into two groups for spraying with 2,4-D. The 2,4-D solution was prepared by dissolving one g of 2,4-dichlorophenoxyacetic acid +

five g of Carbowax 1500 in 100 ml of water. The pH was adjusted to seven with ammonia, the solution diluted to one liter and two drops of Tween-20 were added. Control plants were sprayed with a similar solution minus 2,4-D. The solutions were sprayed onto the plants in a fine mist until solution dripped freely from the leaves.

Harvesting was begun when pathological conditions became visible in the treated plants. Two plants each from the control group and the treated group were harvested at 4, 8, 20, and 35 days after spraying and analyzed as previously described. Only one treated plant survived until the 35 day harvest and it appeared severely damaged. Control plants continued to grow throughout the harvesting period. Some plants began to flower at 20 days, but only non-budding plants were taken for analysis from this experimental group.

Set 2 (flowering) tobacco seeds were planted in sterile quartz sand. Seedlings were transplanted to sand in No. 29 jars between 30 and 60 days and were maintained on complete nutrient solution. These plants began budding after 84 days and were sprayed with 2,4-D (as above) at 102 days, well after flowering had developed. Plants were harvested at 8, 14,21,28 and 35 days after spraying and analyzed as in Set 1, a single plant being taken at each harvest.

Sets 1 and 2 were grown in growth chambers, using a 16 hour daylight cycle. Average temperatures were 27°C during daylight and 16°C during darkness. The results of the

chlorogenic acid analyses on these sets are shown in Figures VIII through XI.

Analysis of Maleic Hydrazide Tobacco and Tomatoes

Tobacco seeds were germinated in sterile quartz sand and seedlings were transplanted to soil after 60 days. At 84 days the plants were divided into two groups of 45 plants each, and one group was sprayed with a 1% maleic hydrazide solution, prepared as follows: 11.8 ml of triethanolamine, 10 g of maleic hydrazide, and five g of Carbowax 1500 were added to one liter of water and dissolved by heating. After cooling, the pH was adjusted to seven with glacial acetic acid.

The plants were sprayed until solution ran freely from the leaves. Control plants were sprayed with a similar solution lacking maleic hydrazide. Nine plants were harvested from each group at 3, 7, 15, 29, and 47 days after spraying. Between 10 and 15 days after spraying, marked physical differences could be observed between treated and untreated plants. No terminal growth could be detected in the treated plants, while control plants continued to grow throughout the test period. By the 47 day harvest, treated plants were severely chlorotic, and some appeared dead.

Tomato plants were grown in the same manner as the tobacco plants and were sprayed with the maleic hydrazide solution on the 46th day after planting. Harvesting was done on the 3rd, 6th, 9th, 12th, and 15th days after spraying
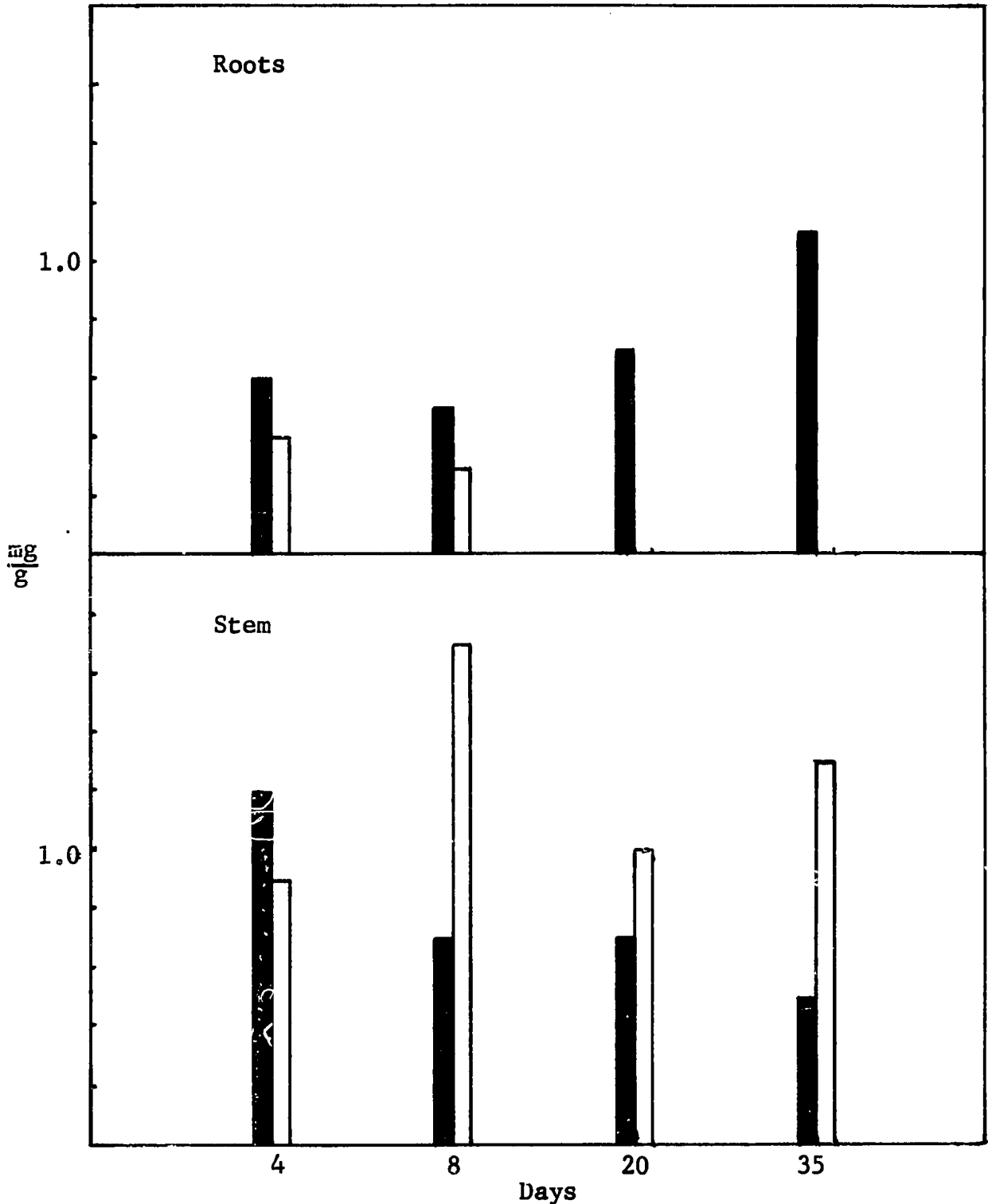
Figure VIII. Variation of Free Chlorogenic Acid in Tobacco
Plants after Treatment with 2,4-D. Set 1. Ordinate: mg
chlorogenic acid/g fresh tissue; abscissa: days after treat-
ment. Black, untreated plants; white, treated.

Figure IX.   Variation of Free Chlorogenic Acid in Tobacco
Plants after Treatment with 2,4-D.   Set 1.   See Figure VIII.

Figure X. Variation of Free Chlorogenic Acid Concentration
in Flowering Tobacco after Treatment with 2,4-D. Set 2.
Ordinate: mg chlorogenic acid/g fresh tissue; abscissa: days
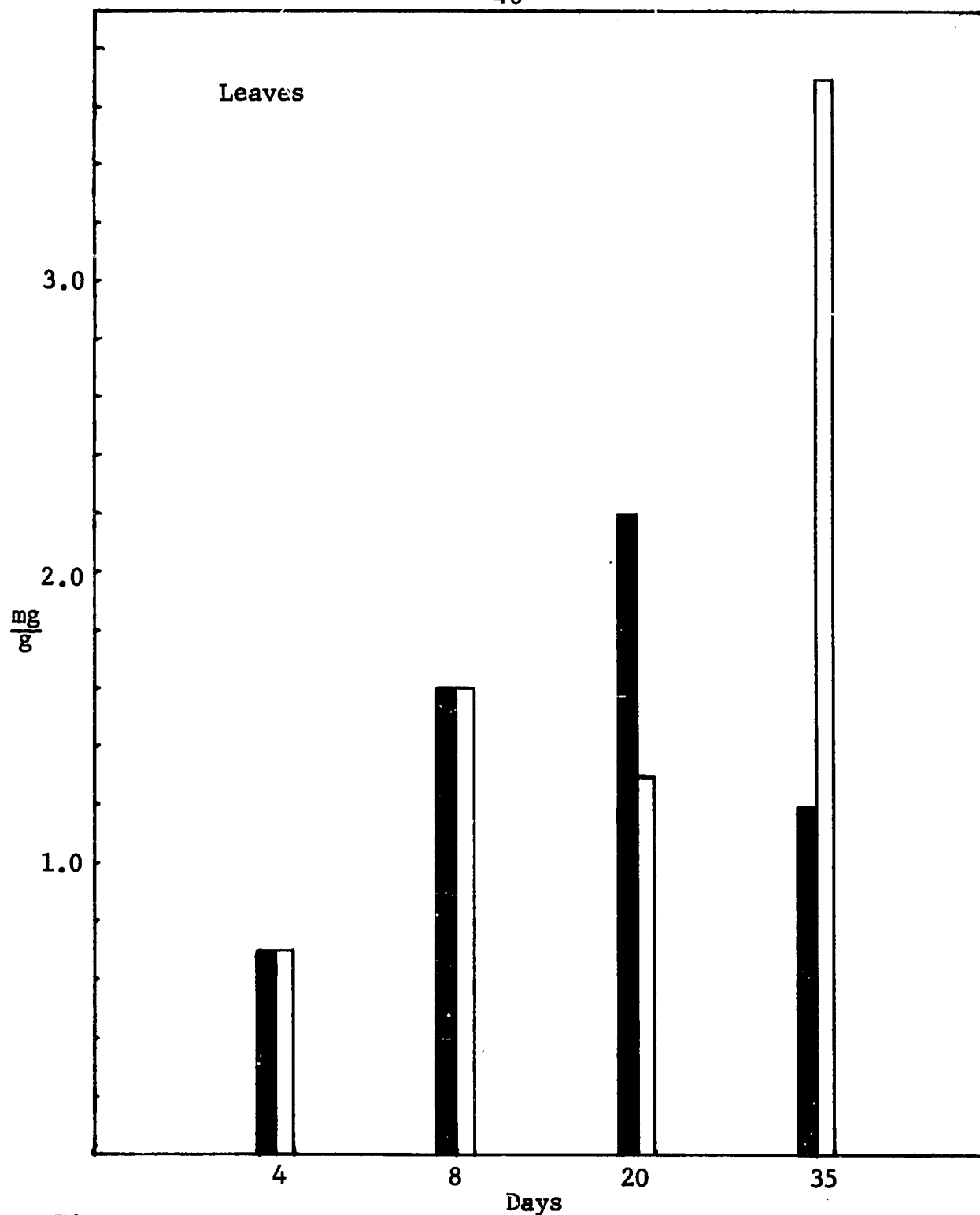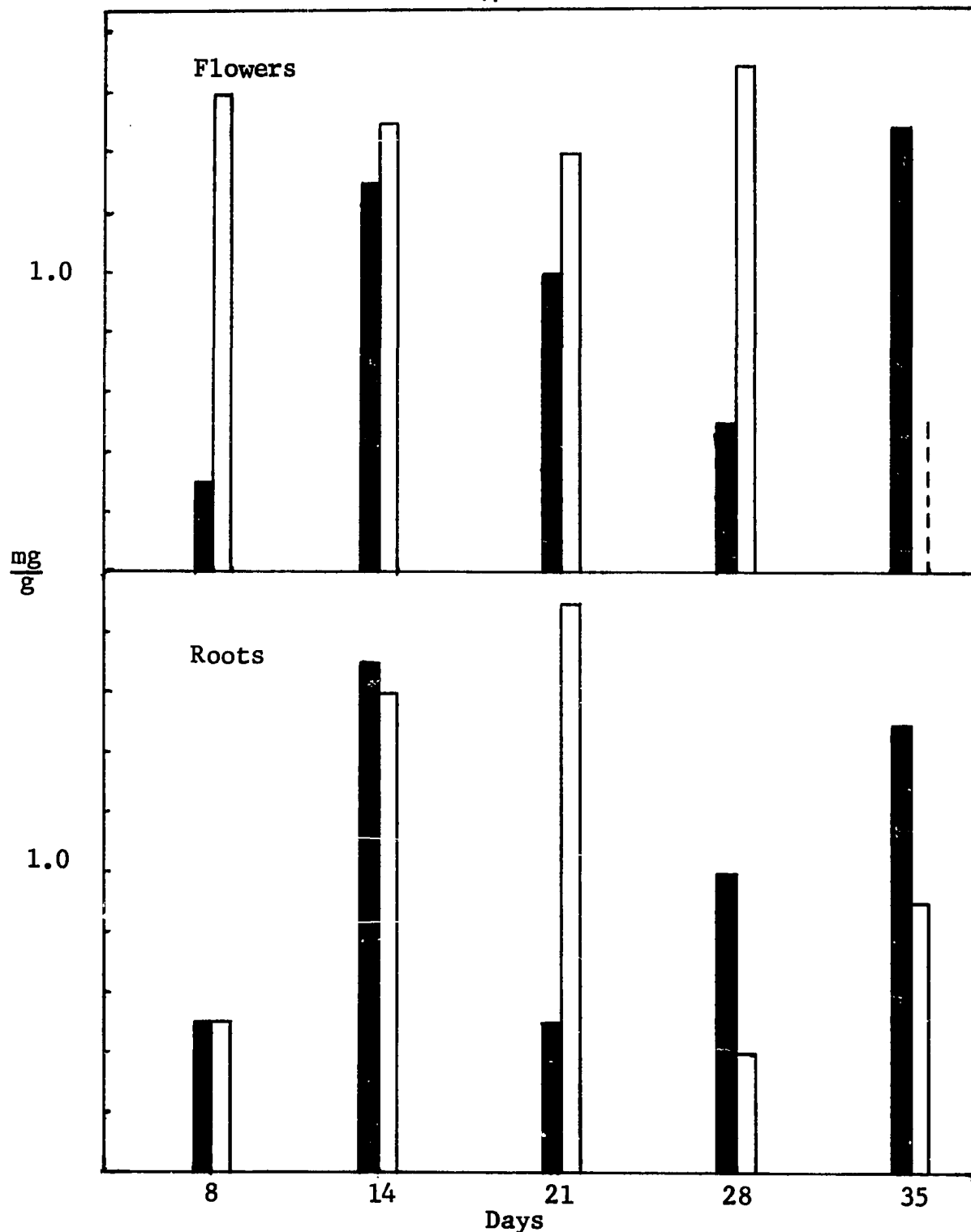after spraying with 2,4-D. Black, untreated plants; white,
treated plants.
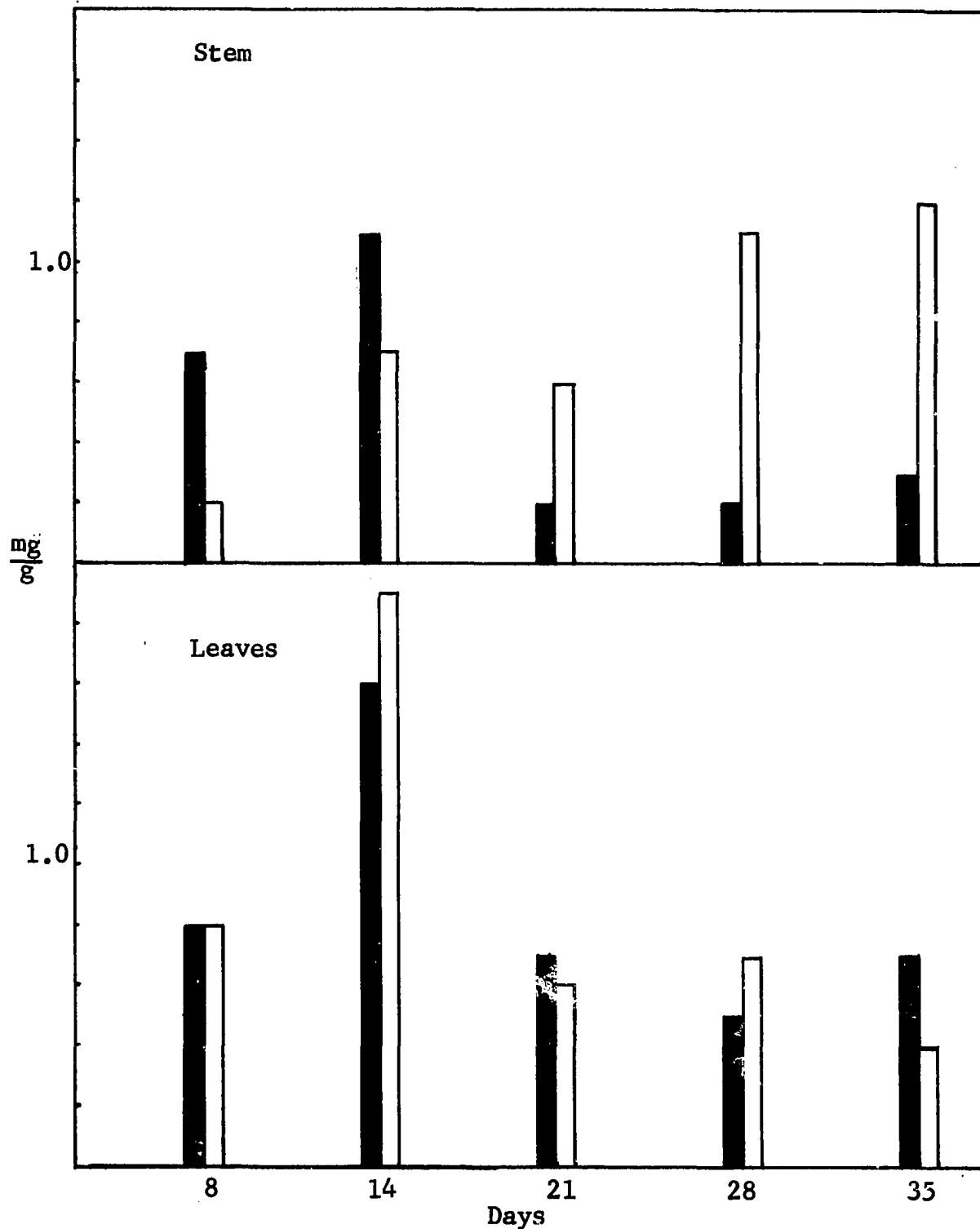
Figure XI.   Variation of Free Chlorogenic Acid Concentration
in Flowering Tobacco after Treatment with 2,4-D.   Set 2
See Figure X.

(only 3 and 6 day samples were analyzed).

Both tobacco and tomato plants were grown in the greenhouse, during winter months (average temperature was 16°C). Results of the chlorogenic acid analyses of these maleic hydrazide treated plants are shown in Tables V and VI.

TABLE V

VARIATION OF FREE CHLOROGENIC ACID CONCENTRATION IN TOBACCO AFTER TREATMENT WITH MALEIC HYDRAZIDE

Days after spraying	Control leaves	Treated leaves	Control stem	Treated stem	Control roots	Treated roots
3	0.2	0.1	-	-	-	-
7	0.2	0.2	-	-	-	-
15	0.2	0.4	0.2	0.2	-	0.04
47	0.4	0.6	0.1	-	0.1	-

Absence of a value indicated only that the amount present was below measurable levels. Data given in mg chlorogenic acid/g fresh tissue.

TABLE VI

VARIATION OF FREE CHLOROGENIC ACID CONCENTRATION IN TOMATOES TREATED WITH MALEIC HYDRAZIDE

Days[a]	CP	TP	CR	TR	CL	TL	CS	TS
3	.03	.03	-	-	.07	.06	.02	.02
6	.04	.03	.03	-	.06	.03	.02	.02

C = control, T = Treated, P = Petiole, L = Leaves, S = Stem, R = Roots. (a) = days after spraying. Data given in mg chlorogenic acid/g fresh tissue.

Tobacco Plants Treated with Ultraviolet Light

Plants were grown in quartz sand using complete nutrient solution. When the plants were 70 days old, one group was transferred to a growth chamber equipped with ultraviolet (UV) lamps. Another group was placed in a separate growth chamber, without UV light. Both groups were grown in a 16 hour light cycle with the UV treated group receiving eight hours of UV light during the middle eight hours of light. The temperature during the light periods was 32°C, and during darkness it was 16°C. Plants grown under UV were divided into sets of high UV (5-8 mwatts/ft^2), medium UV (4-5 mwatts/ft^2) and low UV (1-1.5 mwatts/ft^2). After 21 days of growth under UV, controls and treated plants were harvested and analyzed for chlorogenic acid. Results are shown in Table VII. Figures shown in parenthesis were obtained by a combination paper chromatography-UV absorption analysis procedure.[1] The discrepancies between the UV absorption analysis and the gas chromatographic analysis were found to be due to an improperly washed batch of polyamide. Acetic acid, used in the washing procedure (34) had not been completely removed, which altered the elution pattern of the chlorogenic acid isomers. Under acid conditions the affinity of phenolics for the polyamide is increased (11).

[1]Data contributed from unpublished research by Mr. Dave Koeppe, Department of Botany, University of Oklahoma.

TABLE VII

VARIATION OF FREE CHLOROGENIC ACID IN TOBACCO PLANTS GROWN
UNDER ULTRAVIOLET LIGHT

UV Intensity	Old Leaves	Young Leaves	Roots	Stems
Control	0.3(0.3)	0.5(0.7)	0.2(0.2)	(0.2)
High	(0.4)	1.0(1.1)	(0.1)	0.2(0.2)
Medium	0.2(0.3)	0.5(1.1)	(0.2)	0.3(0.3)
Low	0.2(0.4)	0.9(1.1)	0.2(0.3)	(0.3)

Figures in parenthesis were obtained by paper chromatography
and UV absorption analysis. Data given in mg chlorogenic
acid per g fresh tissue.

Chlorogenic Acid Isomers

The 4-O- and 5-O-caffeoylquinic acids were found in

the tobacco samples, but were usually present at levels too

low for quantitation. Leaves contained barely measurable

levels of the two isomers. Stems contained much less than

the leaves but the ratios appeared to be the same. No

isomers could be detected in the roots. A typical pattern

for leaf extracts is shown in Figure V. Table VIII shows

the ratio of chlorogenic acid to the combined concentrations

of 4-O- and 5-O-caffeoylquinics from leaves of Set 1.

TABLE VIII

RATIO OF CHLOROGENIC ACID CONCENTRATION TO COMBINED CONCEN-
TRATIONS OF 4-O- AND 5-O-CAFFEOYLQUINIC ACIDS IN TOBACCO
LEAF EXTRACTS, SET 1

Days	4	8	20	35
Untreated	1.2	1.8	1.6	2.0
Treated	1.6	1.6	1.3	1.6

More of the 4-O- isomer than 5-O-:isomer was found at every
stage of growth in the untreated leaves, as previously
reported by Zucker, Nitsch, and Nitsch (50) for Nicotiana
tabacum species. However, our findings do not support their
report of a constant ratio of concentrations among the three
isomers in normal tobacco leaves.

Leaves treated with 2,4-D, maleic hydrazide, and ultra-
violet light also contained more of the 4-O- isomer than
5-O- isomer. The ratio of chlorogenic acid to the other
isomers was lower in the maleic hydrazide treated plants
than in untreated plants, as was found with 2,4-D treated
tobacco. In the UV treated plants, the ratio was higher
than that found in the control plants.

Discussion

In devising an extraction procedure for a quantitative
analysis of either a single compound or the entire phenolic
spectrum in a plant, the following points should be considered:
(1) How long an extraction is necessary to quantitatively
remove the compounds of interest?, or (2) Will a given

period of extraction always remove the same percentage of desired material, regardless of variation in concentrations from sample to sample?, and (3) What are the best solvent systems for extracting the compounds of interest?

The extraction procedure described above was developed in an attempt to remove quantitatively the unbound phenolic material present in a given plant. The procedures described were developed using tobacco plants, which are relatively rich in phenolic material, particularly the chlorogenic acids. It was found that a single extraction of 24 hours (or less) with methanol, ethanol, isopropanol, or acetone, a procedure quite commonly reported in the literature (1,4,24, 50), left behind a considerable amount of phenolic material. The amount of phenolic material will, of course, vary according to the species of plant, and it may be that such extensive extractions as are necessary for tobacco are not necessary for some types of plants. Experiments conducted with red kidney bush beans in our laboratory indicate that essentially all phenolic material is removed in a single 24 hour extraction with isopropanol-water azeotrope. These findings suggest that, in the past, too little attention has been given to the problem of removing phenolic compounds from plant tissue.

Polyamide columns were selected for preliminary fractionation of plant extracts because of polyamide's selectivity for phenolic compounds (11) and its reported (34) lack of irreversible adsorption characteristics when used for

separation of flavonoids. The column procedure described was designed solely to separate from the chlorogenic acids substances that would interfere with the analysis by gas chromatography. The entire procedure, from packing the column to the elution of the chlorogenic acids, took about four hours. Preparation of the sample for gas chromatographic analysis took from six to eight hours, while the actual time on the gas chromatograph required less than an hour. The total time required for analysis of a single extract for chlorogenic acid compares favorably with the silicic acid column methods of Sondheimer (42) and Hanson and Zucker (24). Advantages of the polyamide/gas chromatography combination over the silicic acid procedures are

1. Only a small amount (five ml) of extract is used,
2. the extract is applied directly to the column,
3. a number of columns can be run simultaneously, and as many as six gas chromatographic analyses can be conveniently run in a single day.

Other compounds could have been analyzed using the same elution procedure worked out for chlorogenic acid, since the superior resolving power of the gas chromatograph eliminates the necessity for clean column separations of compounds found in the extract. Thin layer analysis of the polyamide fractionation of a tobacco leaf extract is shown in Table IX. Many of the compounds in the plant extract can be separated and quantitated by gas chromatography in a single run.

TABLE IX

FRACTIONATION BY POLYAMIDE COLUMN CHROMATOGRAPHY OF PHENOLIC
COMPOUNDS FOUND IN TOBACCO LEAF EXTRACTS

Fraction	Eluting Solvent	Compounds[a]
1	Benzene/Methanol(9/1, v/v) 50 ml	Chlorophyll, scopoletin, scopolin, unidentified fluorescing compounds
2	Benzene/Methanol(3/1, v/v) 150 ml	Scopolin, 3-O-feruloyl-quinic acid, unidentified fluorescing compounds
3	Methanol (100%) 300 ml	Chlorogenic, 4-O- and 5-O-caffeoylquinic acids
4	Dimethylformamide (100%) 250 ml	Isochlorogenic acids, rutin, unidentified flavonoid

[a]Identified by thin layer chromatography.

At this point, one might question whether any column fractionation is really necessary. Indeed, it was found that in analysis of whole extracts of tobacco leaf, root, or stem many compounds of interest (e.g. chlorogenic isomers) were sufficiently separated from other components for quantitative measurement. Unfortunately, the variation in yield of TMS derivative, in the case of chlorogenic acid, was too great for use in quantitative analysis. Viscous, brown material, left as residue when the IBMW solvent was removed, was not soluble in the DMF used as a silylating reagent. This syrupy mass seemed to trap phenolic material that otherwise would have been silylated and available for injection into the gas chromatograph. For the whole extract, it appears that a more rigorous silylating agent is needed, such as

bis-trimethylsilylacetamide (37), or perhaps silylation in the DMF/HMDS system at reflux temperatures under a nitrogen atmosphere. Another alternative would be to use a preliminary extraction to remove chlorophyll and other lipid material (50) which might interfere with the silylation. However, in our hands, some of the desired phenolic compounds were always removed by such extractions, especially if chlorophyll were present.

As seen in Figures VIII through XI the changes in free chlorogenic acid concentration of 2,4-D treated tobacco plants do not follow any consistent pattern. In the Set 1 samples, the treated tobacco stems had a significantly higher concentration than the untreated, while in the roots, measurable amounts of chlorogenic acid had disappeared from the treated plants by the twentieth day after spraying. The rather high chlorogenic acid concentration found in the Set 1, 35 day, treated leaves may be due more to the loss in fresh weight from dehydration rather than any increased synthesis of the compound. In Set 2 (flowering), no significant difference was found between treated and untreated leaves, while the treated stems show an increase in chlorogenic acid concentration over untreated stems after 20 days, but not as large a difference as was found in Set 1. Set 2 root samples show a marked fluctuation in chlorogenic concentration, quite in contrast to results from Set 1 root samples. It should be noted that, in the Set 1, 35 day treated plants,

only the roots appeared capable of carrying on normal meta-
bolic functions. Grown in water culture, in glass jars,
the roots received some light and contained a noticeable
amount of chlorophyll. Due to the severe damage to the
upper portion of the plant the roots were, in effect, being
grown as root tissue cultures. The decrease in chlorogenic
acid concentration would then be in agreement with the
report of Patil, Zucker, and Dimond (36) that excised potato
root tissue lost its ability to synthesize chlorogenic acid
as the plant aged. The fact that chlorogenic acid is found
in the roots of healthy tobacco plants could be explained
by an active transport of the acid from the upper parts of
the plant to the roots.

In both sets the free chlorogenic acid concentration in
untreated leaf samples fluctuates quite markedly. Zucker,
Nitsch, and Nitsch (50) have reported that chlorogenic acid
increases in tobacco leaves prior to induction of flowering
and drops again after differentiation of the flower
primordia has begun. Other factors that affect the concen-
tration of chlorogenic acid in various plants are age (36,41),
photoperiod (28,44), disease (46), and mineral deficiency (7).

Unlike the nonflowering tobacco, the 2,4-D treated,
flowering tobacco continued to grow throughout the harvest-
ing period and showed little evidence of damage except that
flowering was drastically reduced, compared to untreated
plants. Flower sample weights dropped from 24 g to one g

between eight and 35 days, compared to the untreated samples which fluctuated between 20 and 34 g during the same period. It can be seen that, in the treated flowers, the chlorogenic acid concentration is higher than in the untreated flowers and that the variation is less. Inability to find any chlorogenic acid in the 35 day treated flowers was probably due to the extract's being too dilute.

Maleic hydrazide, used by tobacco growers to prevent sucker formation in the tobacco plant, promotes the growth of the tobacco leaf and is, therefore, desirable from the growers' viewpoint. However, the quality of the maleic hydrazide treated leaf, for smoking purposes, is significantly reduced. Since chlorogenic acid concentration of tobacco leaves correlated well with leaf quality (49), maleic hydrazide treated tobacco plants were analyzed to determine what effect, if any, the chemical had on chlorogenic acid concentration in tobacco plants. When the treated plants began to show visible signs of damage, the amount of chlorogenic acid present was double that found in the untreated plants. The evidence here indicates that maleic hydrazide does not produce a clearcut pattern of effect on the concentration of free chlorogenic acid in the leaf, under the conditions used for field grown tobacco. Therefore, free chlorogenic acid concentration may not be a good indicator of the effects of maleic hydrazide on tobacco leaf quality. The effect of maleic hydrazide on tobacco quality might be correlated more readily with its effects

on certain enzyme systems (15).

The amount of free chlorogenic acid found in the un-
treated plants of the maleic hydrazide set is seen to be
an order of magnitude less than was found in the 2,4-D
controls. This difference is attributed to the difference
in the temperature and the photoperiods of the two groups.
Another contributing factor could be that the 2,4-D test
plants were grown in sand, with nutrient solution added
daily, while maleic hydrazide test plants were grown in
soil. It has been reported that chlorogenic acid is present
in much lower concentration in greenhouse plants than in
field grown plants (49).

Tomato plants contain much less chlorogenic acid than
do tobacco plants, as can readily be seen from Tables V and
VI. It is of interest that a rapid drop in chlorogenic acid
concentration occurs in the leaves, under the test conditions
in contrast to the trend found for tobacco leaves.

The data in Table VII confirm previous reports (49) that
chlorogenic acid concentration rises in tobacco plants
subjected to ultraviolet radiation. It is interesting to
note that the highest concentration of free chlorogenic
acid per plant was obtained under low UV intensity and
decreased slightly from medium to high intensities. All
contained more chlorogenic acid than non-irradiated plants.

The results of the above analyses give a good picture of
the distribution of chlorogenic acid in **Nicotiana tabacum**,

and how that distribution varies with the age of the plant:

1. Chlorogenic acid is present in each part of the plant (root, stem, leaf, flower),
2. The 4-0- and 5-0- isomers are present in the leaves and, to a lesser extent, in the stem,
3. The concentration of free chlorogenic acid is higher in young leaves than in mature leaves (see Table VII),
4. The concentration of free chlorogenic acid is not constant, nor does it slowly decrease with age (45), but seems to fluctuate, especially during flowering,
5. Plants treated with 2,4-D show differences from untreated plants in chlorogenic acid concentration, but no clear, significant trend was noted. Certainly, from these experiments, the visible pathological effects of 2,4-D on tobacco plants cannot be correlated with its effect on the free chlorogenic acid concentration in the plant.

These data give further support to the view that chlorogenic acid is not just an end-product of metabolism, but plays an active metabolic role throughout the life of the plant (43,44).

CHAPTER IV

SUMMARY

Trimethylsilyl derivatives of phenolic compounds were studied and found to be excellent derivatives for use in the quantitative analysis of phenolic material by gas chromatography. With phenolic acids, silylation is complete within a few hours, at room temperature. With more complex phenolic compounds, heating may be necessary, preferably under an oxygen free atmosphere. Various silylating solvents were examined, using hexamethyldisilizane and trimethylchlorosilane as the silylating reagents. It was found that the use of dimethylformamide as a solvent and hexamethyldisilizane as silylating reagent gave higher yields of chlorogenic acid TMS derivative than could be obtained with pyridine, hexamethyldisilizane, and trimethylchlorosilane, a commonly used silylating system. Simpler phenolic acids, e.g. caffeic acid, appeared to silylate as well in the latter system as in the former.

An exhaustive extraction procedure was found to be necessary for quantitative removal of unbound phenolic compounds from <u>Nicotiana</u> <u>tabacum</u> plants. The ground plant

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material was successively washed with hot solutions of isopropyl alcohol/water (1/1, v/v); isopropyl alcohol, benzene, methanol, water (2/1/1/1, v/v/v/v); and isopropyl alcohol-water azeotrope. The ground material was then placed in a Soxhlet extractor and extracted for 24 hours with isopropyl alcohol-water azeotrope, then extracted an additional 24 hours with isopropyl alcohol.

Plant extracts were subjected to a preliminary fractionation on polyamide (polyvinylpyrrolidone) columns before analysis by gas chromatography. Five milliliters of plant extract (solvent: isopropyl alcohol, benzene, methanol, water, 2/1/1/1, v/v/v/v) were fractionated on a 6.5 X 1.45 (D) cm polyamide column. The caffeoylquinic isomers were eluted as a single fraction with 100% methanol, after chlorophyll and other components had been eluted with benzene/methanol solutions.

The gas chromatographic response to the TMS-chlorogenate was determined to be linear between 0.8 and 20 micrograms, and the factors causing non-linearity below 0.8 µg were examined. A procedure for quantitation of the caffeoylquinic depsides, using chlorogenic acid as an external standard, is described.

Tobacco plants sprayed with 2,4-dichlorophenoxyacetic acid were harvested on various days after spraying and their free phenolic material extracted. This extracted material was then analyzed for its chlorogenic acid

concentration. The free chlorogenic acid concentration of both treated and untreated plants was found to fluctuate in roots, stem, and leaves for several weeks after spraying. 2,4-Dichlorophenoxyacetic acid appears to have little net effect on the concentration of free chlorogenic acid in tobacco plants as compared with untreated plants.

The effects of ultraviolet light on chlorogenic acid concentration in Nicotiana tabacum and the effects of maleic hydrazide on chlorogenic acid concentration in tobacco and tomato plants were studied and the results discussed.

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