A quantitative method has been developed for the analysis of
the reaction of flavan-3,4-diols with thioglycolic acid (also referred
to as mercaptoacetic acid). The flavan-3,4-diols were prepared
from dihydroquercetin (2,3-trans-3,3',4',5,7-pentahydroxyflavanone)
which was isolated in crystalline form from a crude extract of
Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] bark. Dihydro-
quercetin was methylated to yield crystalline 2,3-trans-3-hydroxy-
3',4',5,7-tetramethoxyflavanone. Two diols, 2,3-trans-3,4-trans-
3',4',5,7-tetramethoxyflavan-3,4-diol and 2,3-trans-3,4-cis-
3',4',5,7-tetramethoxyflavan-3,4-diol, were isolated in crystal-
line form from the reduction of the C-4 carbonyl group of 2,3-trans-
3-hydroxy-3',4',5,7-tetramethoxyflavanone with sodium borohydride.

The quantitative method consisted of two sequential reactions.
The synthesized flavan-3,4-diols were reacted with thioglycolic acid
to produce the (flavan-4-ylthio)acetic acid derivative. The (flavan-4-ylthio)acetic acid derivative was subsequently hydrogenated to tetra-\(\text{O}\)-methylcatechin (2, 3-trans-3-hydroxy-3', 4', 5, 7-tetramethoxyflavan) with Raney nickel catalyst. Gas-liquid chromatography was used to detect and identify tetra-\(\text{O}\)-methylcatechin and to collect quantitative data. The data showed that the reaction sequence resulted in a 60.7% yield of tetra-\(\text{O}\)-methylcatechin.

The gas-liquid chromatographic conditions were also applicable to the resolution of tetra-\(\text{O}\)-methylcatechin and tetra-\(\text{O}\)-methylepicatechin (2, 3-cis-3-hydroxy-3', 4', 5, 7-tetramethoxyflavan).
Gas-Liquid Chromatographic Analyses of the Thioglycolic Acid Reaction of Catechin-Type Compounds

by

Robert Joseph Colella

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To Linda Barlow I express a special thanks for typing the rough draft of this thesis.
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GAS-LIQUID CHROMATOGRAPHIC ANALYSES OF THE
THIOGLYCOLIC ACID REACTION OF
CATECHIN-TYPE COMPOUNDS

I. INTRODUCTION

Thioglycolic acid (also referred to as mercaptoacetic acid) is a well known reagent in lignin chemistry and has been used as an investigative tool to help elucidate the structure of lignins from various species of wood. Acting as a nucleophile, thioglycolic acid has been shown to react with benzyl alcohols and benzylic ethers in a nucleophilic substitution reaction. Recently, thioglycolic acid has been shown to rupture an acid labile carbon-carbon bond, such as the bond found in condensed tannins. With this discovery, thioglycolic acid has been applied to flavanoid compounds, and especially condensed tannins, as a tool for structural elucidation.

To date, the use of thioglycolic acid has been limited to a qualitative identification of the reaction products of thioglycolysis. The present work outlines a procedure for the quantitative analysis of the reaction products of flavanoid-type compounds with thioglycolic acid.

A flavan-3,4-diol was used as a model compound to develop the quantitative method. The 3,4-diol was reacted with thioglycolic acid and the resulting (flavan-4-ylthio)acetic acid derivative was hydrogenated with Raney nickel to produce tetra-O-methylcatechin.
(2, 3-trans-3-hydroxy-3', 4', 5, 7-tetramethoxyflavan). Gas-liquid chromatography was employed to detect and identify the tetra-0-methylcatechin generated and to collect quantitative data. The conditions of gas chromatography resolved the epimeric flavans, tetra-0-methylcatechin prepared from (+)-catechin and tetra-0-methylepicatechin (2, 3-cis-3-hydroxy-3', 4', 5, 7-tetramethoxyflavan) prepared from (-)-epicatechin. The success of the application of this quantitative analysis scheme to the flavan-3, 4-diol model, along with the successful separation of the methyl ethers of (+)-catechin and (-)-epicatechin suggests the method presented in this work may be useful in the study of natural condensed tannins.
II. HISTORICAL REVIEW

Thioglycolic acid was first shown to undergo nucleophilic substitution reactions with benzyl alcohols and benzylic ethers by Holmberg (21) in 1930. Working with spruce (Picea pungens) lignin (I), Holmberg reacted thioglycolic acid under acidic conditions to produce the (benzylthio)acetic acids (II) which he used to characterize the lignin (36, page 351).

Working with model lignin compounds, Lingren (26) found that for a diveratryl system (III), thioglycolic acid at pH 1.0 would react with the benzylic ether linkage to produce the S-veratryl thioglycolic acid derivative (IV) (26; 36, page 544).

Betts, Brown, Brown, and Pike (1) were the first to utilize thioglycolic acid in the study of the interflavanoid linkages of condensed tannins. In an attempt to determine the mode of linkage in
a condensed tannin from common heather (*Calluna vulgaris*, Salisb.),
Betts and coworkers (1) used thioglycolic acid to degrade the condensed
tannin. The degradation with subsequent permethylation gave the
methyl S-benzylthioglycolate derivative (V), which led them to con-
clude that the condensed tannin was comprised of flavanoid units
attached by C-4 benzylic ether linkages (VI) since thioglycolic acid
was known to cleave such bonds (26).

The procedure represented a milestone in condensed tannin
chemistry. For the first time, not only could a position of inter-
flavanoid linkage be established, but the stereochemistry at the C-2
and C-3 positions was also preserved in a simple derivative.

Subsequent isolation of numerous polymeric proanthocyanidins
(7, 15, 40, 41) provided evidence to support the concept that the most
commonly occurring condensed tannins were comprised of flavan-3-ol nuclei in which the linkage was from the C-4 position of one unit to the C-6 or C-8 position of the others (VII).

\[ \text{VII} \]

Thioglycolic acid had not been reported to cleave carbon-carbon bonds. However, Sears and Casebier (37, 38) showed that the acid labile carbon-carbon linkages of proanthocyanidins could be ruptured with this reagent. Sears and Casebier (37) prepared the synthetic proanthocyanidins (VIII) and (IX) in a condensation reaction of a flavan-3,4-diol with phloroglucinol and (+)-catechin respectively according to the methods of Geissman and Yoshimura (16) and Jurd and Lundin (22).
Cleavage of the synthetic proanthocyanidins followed by permethylation resulted in the expected methyl S-benzylthioglycolate (X).

The success in cleaving the model proanthocyanidin compounds with thioglycolic acid led Sears and Casebier (38) to the application of this technique as a structural tool to identify the condensed tannin from western hemlock (*Tsuga heterophylla*).
Because of the proven value for determination of the structure and stereochemistry of condensed tannins, Betts, Brown, and Shaw (2) investigated in detail the reaction of thioglycolic acid with some flavanoids. They found that when both flavan-4α-ol (2,4-trans) (XI) and flavan-4β-ol (2,4-cis) (XII) were reacted with thioglycolic acid, after methylation, only methyl (flavan-4α-thio)acetate resulted (XIII).

\[
\begin{align*}
\text{flavan-4α-ol} & \quad \xrightarrow{\text{thioglycolic acid}} \quad \text{methyl (flavan-4α-thio)acetate} \\
\text{flavan-4β-ol} & \\
\end{align*}
\]

(XI) (XIII) (XII)

The production of a 2,4-trans compound irrespective of the stereochemistry of the starting material led Betts, Brown, and Shaw (2) to conclude that the nucleophilic addition of thioglycolic acid to 4-hydroxyflavans followed an $S_{N}^1$ mechanism. This conclusion was supported in further work by Brown and Shaw (4) in which they noted that as expected for an $S_{N}^1$ mechanism, progressive introduction of methoxy groups into the 7 and 5 positions of flavan-4-ols enabled the reaction to be conducted at progressively lower acid concentrations. Likewise, the stereochemistry of the resulting thioethers was the
same, 2,4-trans, regardless of the stereochemistry of the starting material.

Compared to flavan-4-ols, Betts, Brown, and Shaw (2, 4) found that 2,3-trans-flavan-3,4-diols required more vigorous conditions for replacement of the 4-hydroxy group. In addition, they found the reaction of 2,3-trans-3,4-cis-flavan-3,4-diol with thio-glycolic acid gave, after methylation, a mixture of the 3,4-cis and 3,4-trans-isomers of methyl 2,3-trans-(3-hydroxyflavan-4-ylthio)acetate in a ratio of 2:3 to 1. The mixed 3,4-stereochemistry was believed to be the result of steric hinderance by the neighboring 3-hydroxy group (4).

Introduction of a 4'-methoxy group (XIV) enabled Brown and Shaw (4) to conduct the nucleophilic substitution reaction at milder conditions than for unsubstituted flavan-4-ols (XV).

\[
\text{(XIV) } R = \text{OCH}_3 \\
\text{(XV) } R = \text{H}
\]

These milder conditions controlled the unwanted opening of the heterocyclic ring to which Betts, Brown, and Shaw (2) found 4'-methoxyflavanoids to be prone.

Brown and Shaw (4) also found the replacement of a 4-hydroxy
group in nucleophilic substitution reactions with sulfurous nucleophiles was much easier in the presence of a 4'-methoxy group. They interpreted this observation in terms of the assistance given by enhanced π-bonding from the B ring in stabilizing the carbonium ion intermediate (XVI).

Further, Brown and Shaw (4) claimed this π-bonded carbonium ion intermediate also explained the 2,4-trans-stereochemistry of the products resulting from flavan-4-ols since the incoming sulfurous nucleophile must approach trans to ring B.

Several workers (8, 9, 10, 12) have studied the reaction of thioglycolic acid with naturally occurring flavan-3,4-diols. During the course of these studies, du Preez and coworkers (8, 9) found that the reaction of thioglycolic acid with flavan-3,4-diols was subject to prominent side reactions producing dihydroflavonols (XVII) and flavanones (XVIII).
Determination of the stereochemistry about the C ring (heterocyclic ring) of substituted flavan derivatives was a difficult problem before the advent of nuclear magnetic resonance (n.m.r.) spectroscopy. The application of n.m.r. spectroscopy has afforded a quick and definitive answer to the stereochemical problems in flavan chemistry. Corey, Philbin and Wheeler (6) used n.m.r. to distinguish the cis and trans isomers of a synthesized flavan-3,4-diol cyclic carbonate. Interpretation of the coupling constants of the $H_{2,3}$ and $H_{3,4}$ protons led them to elucidate the structures (XIX) and (XX).

$J_{2,3} = J_{3,4} = 10.6\ Hz$  
(XIX)

$J_{2,3} = 9.7\ Hz \quad J_{3,4} = 6.4\ Hz$  
(XX)

Clark-Lewis, Jackman, and Spotswood (5) published a very thorough account of the applications of n.m.r. in flavan chemistry.
Three general classes: (1) 3-substituted flavanones and flavans; (2) flavan-4-ols; and (3) 3,4-disubstituted flavans were investigated. In this work, Clark-Lewis and coworkers determined the chemical shift and coupling constant data for the 2-, 3-, and 4-protons in 68 flavan derivatives.

Bolger and coworkers (3) investigated the 4-substituted flavans in greater detail, applying a computer program to determine the exact coupling constant values for complex 4-substituted systems in which $H_2$, $H_3'$, and $H_4$ signals were indistinguishable.

Raney nickel, a finely divided powder catalyst, has been used by Betts, Brown, and coworkers (1, 2, 4) to obtain desulfurization of (flavan-4-ylthio)acetic acid derivatives. A solution of 2,3-cis-3',4',5,7-tetramethoxy-(3-hydroxyflavan-4-ylthio)acetic acid (XXI), stirred with Raney nickel for 100 minutes at room temperature, resulted in removal of the thioglycolic acid portion to produce tetra-O-methylepicatechin (XXII).

This reaction was used by Betts, Brown, Brown, and Pike (1) and Weinges and Freudenberg (41) in the characterization of natural
condensed tannins.

Gas-liquid chromatography of flavanoids has received little attention relative to separation by paper and thin-layer chromatography due to the difficulties caused by the low volatility of the hydroxylated flavanoids (33). Narasimhachari and von Rudloff (32) successfully separated 36 flavanoid compounds by preparing the methyl ether and acetate derivatives. A variety of flavone, flavanones, isoflavones, and chalcones as their methyl ethers were separated using SE-30 silicone polymer as liquid phase. A fairly regular increase in the retention time was observed when the number of substituents in the parent compound was increased. Conversion of a free hydroxyl group to the methyl ether decreased the retention time, whereas acetylation increased retention time to a value greater than that of the free phenol. Further, Narasimhachari and von Rudloff (32) found that the use of higher temperatures to shorten the retention time values was undesirable due to the chance of thermal decomposition of the flavanoid samples.

Furuya (14) determined the retention times of 22 flavanoid and related compounds prepared as the trimethylsilyl ether derivatives (39). Using SE-30 silicone polymer as liquid phase, Furuya (14) found that the trimethylsilyl ether derivatives had a much shorter retention time than the methyl ethers with better resolution in most cases. Similarly to Narasimhachari and von Rudloff (32), Furuya (14)
found a regular increase in retention time as the number of hydroxyl substituents in the parent compound was increased. Also, Furuya (14) found that the hydroxyl substitution pattern of the A and B rings affected the retention time, with meta substituted compounds running faster than the corresponding ortho compounds. Retention times were also found to increase with increasing oxidation or hydroxylation in the order flavanones < leucoanthocyanidins < dihydroflavonols < flavonols < flavones (14; 17, page 35). Finally, Furuya (14) observed that the gas chromatographic spectra of some flavanoids did not show a single peak, but usually showed a major peak followed by one or more minor peaks. The results suggested that some flavanoids may undergo dehydration or some other chemical change on the column.

Keith and Powers (25) separated several anthocyanidins as their trimethylsilyl ethers as well as a number of flavonols and their glycosides. In this work, however, the authors noted several difficulties with the use of trimethylsilyl ether derivatives. One problem with the reagents used was that they were corrosive. Syringes were observed to deteriorate far more rapidly, apparently from the corrosive action of the silane materials. The silicone material formed upon burning at the detector also caused problems by coating the detector jets and collecting rings. The sensitivity of the instrument changed so drastically in the course of two to three determinations that quantitative results were not reported.
Nordstrom and Kroneld (33) conducted a comparative study of the retention times of trimethylsilyl ether vs. methyl ether derivatives of flavones and hydroxyflavones. OV-17, a methyl phenyl silicone polymer was used as the liquid phase for gas chromatography. The liquid phase was expected to be selective for aromatic compounds due to the interaction between the flavanoid samples and the phenyl rings of the liquid phase. Separation of the trimethylsilyl ethers was better than the corresponding methyl ether derivatives. The methyl ethers also had longer retention times. The trimethylsilyl ethers were easily prepared but were to some degree sensitive to moisture. The methyl ethers, however, were stable and easy to handle.
III. EXPERIMENTAL

A. Isolation of 2, 3-Trans-3', 4', 5, 7-pentahydroxyflavanone (dihydroquercetin)

Crude dihydroquercetin (40.0 g) obtained from the Weyerhaeuser Company, Longview, Washington was refluxed in 400.0 ml of acetone for 2 hours. The solution was filtered and the residue washed three times with 100.0 ml of hot acetone then discarded. Carbon tetrachloride was added to the combined filtrates until a flocculent, yellow precipitate formed. The addition of carbon tetrachloride was continued until there was no longer a formation of precipitate (total addition of carbon tetrachloride approx. 200 ml). The precipitate was removed by filtration leaving a dark brown, clear filtrate. A second aliquot of carbon tetrachloride (300.0 ml) added to the filtrate caused the formation of a thick, black oil which floated to the surface of the solution. Upon formation of this oil, the solution became light yellow in color. The mixture was poured into a separatory funnel and the clear yellow bottom layer was collected.

The solution was concentrated to dryness on a rotary evaporator (40°) and the residue was redissolved in a minimum amount of acetone. The orange color of the solution suggested that impurities were still present. Carbon tetrachloride (50.0 ml) was added until an oil formed. The mixture was allowed to separate in a separatory funnel and the bottom layer again collected. This procedure—evaporation to dryness, redissolve in acetone, addition of carbon tetrachloride—was repeated until the addition of carbon tetrachloride caused no oil to form.
Heating of the acetone solution upon addition of carbon tetrachloride sometimes aided oil formation. Oil formation was not always spontaneous and at times it was necessary to allow the solution to sit for several hours before the oil separated. The addition of excess carbon tetrachloride was also observed to force dihydroquercetin out of the acetone solution resulting in lower yields. Therefore, care was taken when carbon tetrachloride was added to induce oil formation.

When the addition of carbon tetrachloride no longer produced an oil, the solution was evaporated to dryness and the residue redissolved in a minimum of hot water. The initial crystallizations from water resulted in a product slightly tinged with yellow. The product was subjected to paper chromatography using an irrigating solvent comprised of 3 parts (by volume) of the upper layer of the system chloroform-acetic acid-water (8:12:5 v/v) mixed with 1 part (by volume) of 1-butanol (30, p.24) and diazotized 4-nitroaniline (5.0 ml of 0.5% 4-nitroaniline in 2N hydrochloric acid, 0.5 ml of 5.0% aqueous sodium nitrite, and 15.0 ml of 20.0% aqueous sodium acetate) as the indicator spray reagent. The chromatogram showed a major spot which migrated the same distance as authentic dihydroquercetin and a trace spot which migrated the same distance as authentic quercetin under conditions of co-chromatography.

It was found that as the hot water solution of this yellowish material cooled, a fine, yellow residue precipitated. The residue,
believed to be quercetin, was removed by filtration. This process was repeated at 10 minute intervals until a white crystalline material (dihydroquercetin) began to form. Final purification was achieved by decolorizing a hot-water solution with MCB Activated "Darco" Charcoal 20-40 mesh.

The product was recrystallized four times with water. Dihydroquercetin was collected and dried under vacuum over phosphorus pentoxide at 80°, m. p. 240-241° [lit (20, 35) m. p. 240-241°] (Found: C, 59.50; H, 3.93. C_{15}H_{12}O_{7} requires C, 59.23; H, 3.95%).

B. Preparation of 2,3-Trans-3-hydroxy-3',4',5,7-tetramethoxyflavanone (tetra-O-methyl-dihydroquercetin)

Dihydroquercetin was methylated by the method of Hergert, Coad, and Logan (18). Dihydroquercetin (3.00 g) was dissolved in 100.0 ml of acetone. Anhydrous potassium carbonate (25.00 g) was added and suspended in solution by means of a magnetic stirrer. The solution was brought to reflux and dimethyl sulfate (14.0 ml) was added over a period of 1.0 hour. The mixture was then refluxed an additional 14.0 hours.

The solution was filtered and the potassium carbonate was washed three times with 100.0 ml portions of acetone. The combined filtrate and washings were evaporated to dryness. The residue was recrystallized from 90.0% ethanol. White, needle-like crystals of
2, 3-trans-3-hydroxy-3', 4', 5, 7-tetramethoxyflavanone (tetra-O-methyldihydroquercetin) were obtained, (1.35 g, 45.4%), m. p. 169-170°, [lit (18) 169-170°] (Found: C, 63.02; H, 5.42; methoxyl 34.47, C_{19}H_{20}O_{7} requires C, 63.36; H, 5.55; methoxyl 34.43%). Treatment of the material with alcoholic ferric chloride solution failed to produce the characteristic greenish color indicative of free phenolic hydroxyls.

C. Preparation of the 2, 3-Trans-3'4', 5, 7-tetramethoxyflavan-3,4-diols

1. Diol I

Tetra-O-methyldihydroquercetin (1.50 g) was dissolved in 150.0 ml of methanol. The solution was heated to a gentle reflux and when all of the tetra-O-methyldihydroquercetin had dissolved, sodium borohydride (0.6 g) was added gradually. After the addition of sodium borohydride, the solution was allowed to reflux for 15 minutes.

After cooling (5 minutes), the methanolic solution was poured into 150.0 ml of cold water. The solution was evaporated on a rotary evaporator until a fine, white crystalline precipitate began to form. The solution was then placed in a refrigerator overnight.

The following day, an off-white crystalline product was collected by filtration. The filtrate was saved. Recrystallization of the
off-white crystals from methanol gave colorless prisms (0.23 g, 15%) of the high-melting-point isomer, 2,3-trans-3, 4-trans-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol (Diol I), m. p. 205° [lit (13) m. p. 205°] (Found: C, 62.86; H, 6.05. C_{19}H_{22}O_7 requires C, 63.02; H, 6.08%).

2. Diol II

To the filtrate saved from the collection of Diol I, 10.0 ml of acetic acid was added. A colorless, gelatinous precipitate formed which was collected by filtration. Recrystallization from methanol gave white, felted needles (0.42 g, 28%) of the low-melting-point isomer, 2,3-trans-3, 4-cis-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol (Diol II), m. p. 165° [lit (13) m. p. 165.5-166.5°] (Found: C, 62.79; H, 5.96. C_{19}H_{22}O_7 requires C, 63.02; H, 6.08%).

D. The Reaction of 2,3-trans-3, 4-cis-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol with Thioglycolic Acid

A mixture of 2,3-trans-3, 4-cis-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol (Diol II) (175 mg), 1, 4-dioxane (10.0 ml), water (5.0 ml), and thioglycolic acid (500 mg) was refluxed for 1.0 hr. The solution was cooled, an excess of 5% sodium bicarbonate solution (30.0 ml) was added, and the mixture was extracted with diethyl ether (50.0 ml). The original solution was acidified to a pH of 1 with 2N-hydrochloric acid, then extracted again with diethyl ether (50.0 ml). The
ethereal extracts were combined, dried with anhydrous magnesium sulfate, concentrated to a volume of approximately 15 ml by passing a stream of nitrogen gas over the solution, and stored in a refrigerator at -10°.

Attempts to crystallize the product, 2,3-trans-3,4-cis-(3-hydroxy-3',4',5,7-tetramethoxyflavan-4-ylthio)acetic acid, were unsuccessful. Therefore, the methyl ester was prepared.

E. Preparation of Methyl 2,3-trans-3,4-cis-(3-hydroxy-3',4',5,7-tetramethoxyflavan-4-ylthio)acetate

An ethereal solution of the thioglycolic acid reaction products was methylated with an excess of ethereal diazomethane. Upon evaporation, a yellow syrup remained. The methyl ester was purified by column chromatography.

A column was prepared using "Baker" Silica Gel 7GF (70.0 g) slurried in diethyl ether-n-hexane (1:1 v/v). The syrup from the methylation of the thioglycolic acid reaction products was dissolved in a minimum amount of diethyl ether-n-hexane (3:1 v/v) and applied to the column. Not all of the syrup dissolved in the diethyl ether-n-hexane (3:1) solvent. A thick, bright yellow syrup remained insoluble. This material would not dissolve even when diethyl ether alone was added.

The column was developed with diethyl ether, and seven 100-ml
fractions of eluent were collected. The seven fractions were evaporated to dryness by heating in a hot water bath while passing a stream of nitrogen gas over the solution.

Fractions 2 through 6 gave a residue upon evaporation of the solvent. Each residue was dissolved in a minimum amount of diethyl ether and all the solutions were placed in a refrigerator (-10°) overnight. Small white crystals formed in all five of the flasks. Fraction 4 contained the largest amount. The fractions were combined and recrystallized from n-hexane-dichloromethane (1:1 v/v). Colorless crystals of methyl 2, 3-trans-3, 4-cis-(3-hydroxy-3', 4', 5, 7-tetramethoxyflavan-4-ylthio)acetate were collected and dried at 100° over phosphorus pentoxide, m. p. 121°, [lit (38) m. p. 122.5-123.5°] (Found C, 58.42; H, 5.47; S, 6.95. C_{22}H_{26}O_8S requires C, 58.48; H, 5.77; S, 7.00%).

F. Preparation of Raney Nickel

Raney nickel #28, a finely divided activated catalyst, was obtained from the W. R. Grace Company. This catalyst is similar to the W-2 preparation developed by Mozingo (31) at the University of Wisconsin. The primary difference between Raney nickel #28 and W-2 is the solvent under which they are stored. Raney nickel #28 is stored under water, while W-2 is stored under ethanol. For the
hydrogenation of methyl 2, 3-trans-3, 4-cis-(3-hydroxy-3', 4', 5, 7-tetramethoxyflavan-4-ylthio)acetate, an organic medium was preferred.

The procedure for the solvent exchange of Raney nickel #28 from water to ethanol was followed according to Mozingo (31). The catalyst was suspended in water, allowed to settle, and the supernatant decanted. This process was repeated until the supernatant tested neutral to litmus. The same washing process was next repeated three times with 200-ml aliquots of 95% ethanol, followed by three more washings with 200-ml aliquots of 100% ethanol. The catalyst was then stored under 100% ethanol.

G. Thioglycolysis-Raney Nickel Reduction Reactions

2, 3-trans-3, 4-cis-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol (50 mg) and thioglycolic acid (500 mg) were reacted as described previously. Following reflux, the reaction solution was diluted to 50.0 ml in a volumetric flask with 100% ethanol. The solution was divided into two 25.0 ml portions. Excess Raney nickel in ethanol, prepared above, was added to each. One solution was allowed to stand at room temperature for 2.0 hours while the second solution was refluxed for 2.0 hours.
H. **Gas-Liquid Chromatography**

1. **Determination of Optimum Gas-Liquid Chromatographic Conditions**

Gas-liquid chromatographic analyses were performed on a Hewlett-Packard 5751B Research Chromatograph equipped with dual flame ionization detectors. The conditions were: column 3 ft x 1/8 in, 0. D. stainless steel, packed with 3% OV-17 on Gas Chrom Q 100/120 mesh; injection port 250°; detector 250°; column temperature 220° isothermal; carrier gas helium, flow rate 30 ml/min; hydrogen flow rate 55 ml/min; oxygen flow rate 400 ml/min; attenuation setting 32; range setting 10².

Peak retention times and area data were collected by means of a Hewlett-Packard 3370B Integrator.

2. **Preparation of the Internal Standard**

4'-Methoxy-3', 5, 7-trihydroxyflavanone (hesperetin) (1.00 g) was dissolved in 25.0 ml of methanol. Ethereal diazomethane (25.0 ml) was added and the solution was placed in a refrigerator (-10°) overnight. The next day the solution was concentrated to a volume of approximately 25 ml by passing a stream of nitrogen gas over the solution. A second aliquot (25.0 ml) of ethereal diazomethane was added and the solution stored in a refrigerator overnight. The
procedure was repeated until gas-liquid chromatography showed a single major peak.

At this stage, the solution was concentrated to approximately 25 ml (nitrogen stream) and placed in a refrigerator. Yellow-brown, long, needle-like crystals formed which were collected and recrystallized three times from methanol to give white crystals of the compound used for the internal standard. The crystals were dried over phosphorus pentoxide at 80° for 24 hr m. p. 159-160° (Found: C, 64. 92; H, 5.41; methoxyl, 28.37. \( \text{C}_{19} \text{H}_{22} \text{O}_5 \) requires C, 65.49; H, 5.45; methoxyl 28.17%).

3. Determination of Instrument Calibration Curves

The method described by McNair and Bonelli (29, p. 150) was used to determine the difference in detector response between the internal standard and each of the flavan compounds, 2,3-trans-3',4',5,7-tetramethoxyflavan-3-ol (tetra-\( \text{O} \)-methylcatechin) and 2,3-cis-3',4',5,7-tetramethoxyflavan-3-ol (tetra-\( \text{O} \)-methylepicatechin). Six solutions of various weight ratios of the flavans to the internal standard were prepared. Stock solutions were prepared by dissolving 10 mg of the internal standard in 1.0 ml of dichloromethane, 10 mg of tetra-\( \text{O} \)-methylcatechin in 1.0 ml of dichloromethane, and 20 mg of tetra-\( \text{O} \)-methylepicatechin in 2.0 ml of dichloromethane. The six weight ratio mixtures were prepared by
drawing the appropriate volumes from the stock solutions with a
Yale 1/4 cc Tubercubin syringe. The weight ratio mixtures and the
volumes of stock solutions used in preparing the mixtures are shown
in Table 1.

Table 1. Weight ratio mixtures of the internal standard to determine the instrument calibration
curves.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight Ratio 1/</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Internal standard</td>
<td>0.400</td>
</tr>
<tr>
<td>Tetra-O-methylcatechin</td>
<td>0.100</td>
</tr>
<tr>
<td>Tetra-O-methylepicatechin</td>
<td>0.100</td>
</tr>
</tbody>
</table>

1/ Weight ratio = \frac{\text{weight of flavan component}}{\text{weight of internal standard}}

2/ All volumes are in cc's

Four sample sizes 1 µl, 2 µl, 2.5 µl, and 3 µl from each of the six
weight ratio solutions were injected into the gas chromatograph.

The areas under the peaks of the resulting spectra were measured
with a Hewlett-Packard 3370B integrator. Each instrument calibration
curve was obtained by plotting the chromatographic peak area
ratios (individual flavan compound area/internal standard area) vs.
the weight ratios (individual flavan compound weight/internal standard
weight).
I. Analysis of the Hydrogenation Product

The solutions from the hydrogenation reaction of 2, 3-trans-3, 4-cis-(3-hydroxy-3', 4', 5, 7-tetramethoxyflavan-4-ylthio)acetic acid with Raney nickel were filtered directly into 100 ml round bottom flasks. A fine sintered glass funnel was required to collect the smallest of the Raney nickel particle fractions. The filtrates were evaporated to dryness on a rotary evaporator.

To each of the residues, 12.5 mg of the internal standard was added. The residue and the internal standard were dissolved in 5.0 ml of dichloromethane and the solution analyzed by gas-liquid chromatography.
IV. RESULTS AND DISCUSSION

A. Isolation of 2, 3-Trans-3', 4', 5, 7-pentahydroxyflavanone (dihydroquercetin)

The method for isolating dihydroquercetin was an improvement upon a method described by Meng (30). This method was found to be preferable to the isolation schemes described by Markham and Porter (28) and Hergert and Kurth (19) due to the shorter time required to obtain the end product, the smaller amount of starting material and solvents needed, and the resulting yield of dihydroquercetin.

The isolation scheme for dihydroquercetin is shown in Figure 1. Of key importance in this scheme is the sequential treatments of carbon tetrachloride to induce the formation of the supernatant oil. This dark oil tends to act as an emulsifier and must be removed before dihydroquercetin will crystallize from solution. Upon addition of carbon tetrachloride, the oil tended to form spontaneously. However, when it did not, heating the solution sometimes aided the oil formation.

The dihydroquercetin obtained from this isolation scheme gave a major spot at $R_f$ 0.70 on paper chromatography, identical to an authentic sample. In addition, the isolated dihydroquercetin was shown to contain quercetin which was identified by a trace spot at $R_f$ 0.55, identical to an authentic sample of quercetin. Final
Repeat until oil no longer forms

Crude Dihydroquercetin
- Reflux with Acetone
- Filter

Filtrate → Discard Residue
- Add CCl₄ (Heat)

Clear Yellow Solution → Discard Oily Supernatant Layer
- Evaporate to Dryness

Residue
- Redissolve in hot H₂O

Solution → Filter Yellow Precipitate
- Treat with Charcoal
- Filter
- Recrystallize
- Dry

Dihydroquercetin m.p. 240-241⁰

Figure 1. Dihydroquercetin isolation scheme.
purification was achieved by decolorizing a hot-water solution of dihydroquercetin with MCB Activated "Darco" Charcoal 20-40 mesh. Filtration and recrystallization resulted in white crystals, m.p. 240-241°C.

Dihydroquercetin was used as the starting material because it possesses a carbonyl group at the number 4 carbon atom. This carbonyl group was useful in manipulating the oxidation state of this position in subsequent steps to achieve the desired results. Also, the stereochemistry about the number 2 and 3 carbon atoms have the same relative configuration as (+)-catechin.

B. Preparation of 2,3-Trans-3-hydroxy-3',4',5,7-tetramethoxyflavanone (tetra-O-methyl-dihydroquercetin)

The four phenolic hydroxyl groups of dihydroquercetin were methylated to prevent self-polymerization and other side reactions in subsequent reactions. These undesirable reactions can occur under both acidic and basic conditions.

Following the method of Hergert, Coad, and Logan (18), 3', 4', 5, 7-tetramethoxydihydroquercetin was obtained in a yield of 45.4% (based on the theoretical). A negative color reaction when treated with alcoholic ferric chloride and a methoxyl content of 34.47% (theoretical 34.43%) indicated that all four phenolic hydroxyl groups had been methylated. The n.m.r. spectrum (Figure 2) also
Figure 2. NMR spectrum (CDCl₃) of 3', 4', 5, 7-tetramethoxydihydroquercetin.
integrated for 12 methoxyl protons.

A problem with this methylation technique was the recovery of the methylated product when crystallization did not readily occur from the mother liquor. When other flavanoid compounds [i.e. (+)-catechin, (-)-epicatechin, and hesperitin] were methylated by this method, crystallization from the mother liquor did not occur. It was necessary to use thin-layer chromatography (benzene-acetone 9:1 v/v) to collect the tetra-O-methyl derivatives of (+)-catechin and (-)-epicatechin.

The tri-O-methyl derivative of hesperitin (internal standard) for undetermined reasons, was not collected from either the mother liquor or from thin-layer chromatography. It is believed, as noted by Hergert, Coad, and Logan (18), that the unsubstituted C-3 position of hesperitin weakens the resistance of the carbonyl to undesirable side reactions relative to flavanones such as dihydroquercetin, substituted in the C-3 position.

Also, attempts to methylate (+)-catechin and (-)-epicatechin by the method of Hergert and coworkers (18) have resulted in penta-O-methyl derivatives. Gas chromatography-mass spectroscopy spectra shown in Figure 3 show a sample taken from the mother liquor of a methylation reaction of (+)-catechin. The mass spectrum of peak A shows a molecular ion peak at m/e 360, the molecular weight of penta-O-methyl catechin. The compound responsible for peak A was
Figure 3. Upper. Computer printout of a gas-liquid chromatographic spectrum of the dimethylsulfate-potassium carbonate methylation products of (+)-catechin. Peak A is penta-O-methylcatechin; B is tetra-O-methylcatechin; C is unidentified. Conditions: Varian GC; column, 3% OV-17 on Gas chrom Q 100/120 mesh, 3 ft x 1/8 in O.D. stainless steel; injection port 250°; detector 250°; column temperature 220° isothermal; helium flow 30 ml/min; range setting 10^2; attenuation setting 32. Lower. Computer printout of mass spectrum of peak A.
collected by thin-layer chromatography and a subsequent methoxyl content determination confirmed the penta-0-methyl derivative (methoxyl content, 42.98%; theoretical, 43.04%).

An alternative to methylation with dimethyl sulfate - potassium carbonate was methylation using diazomethane. Used with success on flavanoids, diazomethane provides a milder form of methylation. Full methylation of phenolic hydroxyl groups was obtained with no effect upon C-ring aliphatic hydroxyls. Crystallization of the tetra-0-methyl derivatives did occur from the diazomethane methylation solutions. However, recovery by thin-layer chromatography was preferred to obtain pure samples. A drawback to the use of diazomethane as a methylating agent is the time required for tetra-0-methylation of the sample, often taking from three days to one week.

C. Preparation of the 3', 4', 5, 7-Tetramethoxyflavan-3, 4-diols

Figure 4 shows the reactions used for the synthesis of the 3', 4', 5, 7-tetramethoxyflavan-3, 4-diols from dihydroquercetin. The carbonyl on the 4-position of tetra-0-methylidihydroquercetin was reduced to an alcohol using sodium borohydride. This reduction reaction created a good leaving group (-OH) at the 4-position, therefore, producing the reactive center for subsequent reactions with thioglycolic acid.

A new asymmetric center at the C-4 position was also created
Figure 4. Reaction scheme for the synthesis of the 3', 4', 5, 7-tetramethoxyflavan-3, 4-diols.
by the reduction reaction. Because of this, two diols displaying
different physical properties were collected. The configuration about
the C-4 position of the diols was elucidated by nuclear magnetic
resonance spectroscopy.

1. Diol I

The first diol collected crystallized from the methanolic reac-
tion solution after 50.0 ml of water had been added and the solution
was concentrated to approximately half of its original volume.

The crystals were colorless prisms, m.p. 205°. This was in
agreement with the work of Fujise, Adachi, and Hishida (13). Figure
5 shows the n.m.r. spectrum of Diol I. The protons on carbons 2,
3, and 4 had chemical shifts of 5.26, 5.84, and 4.94, respectively.
The coupling constant between the protons on C-2 and C-3 (J_{2,3}) was
10 Hz, indicating a trans configuration (5, 6). This configuration is
in agreement with that known for the starting material, dihydro-
quercetin. The coupling constant between the protons on C-3 and
C-4 (J_{3,4}) was 7.9 Hz, also indicating a trans configuration as
confirmed by Corey, Philbin and Wheeler (6) and Clark-Lewis,
Jackman, and Spotswood (5). Coupling constant data have shown that
for the 2, 3-trans-flavan-3, 4-diols the conformations in which the B
ring on C-2 is equatorial are strongly favored (17, p. 515). Therefore,
in Diol I (2,3-trans-3, 4-trans-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol) the
Figure 5. NMR spectrum (CDCl₃) of 2, 3-trans-3', 4-trans-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol (Diol I). x - solvent peak
preferred conformation would place the B ring on C-2, the hydroxyl group on C-3, and the hydroxyl group on C-4 all in equatorial positions and the protons on these respective carbon atoms would occupy axial positions. The coupling constants of the protons on C-2 and C-4 were negligible and therefore are not of importance in outlining the stereochemistry.

2. **Diol II**

The Diol II isomer was obtained from the mother liquor of the Diol I isomer crystallization. Upon acidifying the mother liquor with acetic acid a thick gel formed. The gel, collected by vacuum filtration, was recrystallized from methanol to produce white, silky needles of the Diol II isomer, m. p. 165°, in agreement with Fujise, Adachi, and Hishida (13).

Although the configuration about C-4 of the Diol II isomer could simply be interpreted as the opposite of the Diol I isomer, Figure 6 lends proof to the matter. The n. m. r. spectrum shows the chemical shifts of the protons on carbons 2 and 4 to be τ 5.08 and 4.95, respectively. The peak for the proton on C-3 is lost under the methoxyl peak. The coupling constant between the protons on C-2 and C-3 again is 10 Hz, indicating a *trans* relationship. The coupling constant between the protons on C-3 and C-4 however, is 4 Hz, suggesting a *cis* relationship. Therefore, C-4 has the proton in the equatorial
Figure 6. NMR spectrum (CDCl₃) of 2, 3-trans-3, 4-cis-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol (Diol II). x - solvent peak
position and the hydroxyl group in the axial position.

D. Reaction of 2, 3-Trans-3', 4'-cis-3', 4', 5, 7-
tetramethoxyflavan-3, 4-diol with
Thioglycolic Acid

Nucleophilic substitution reactions of flavan-4-ols and flavan-
3, 4-diols have been well documented (1, 2, 4, 8, 10, 12). The nucleo-
philic attack of thioglycolic acid on the C-4 position follows an \( S_N^1 \)
mechanism with substitution of the thioglycolic acid in the equatorial
position being favored (2). Attempts to crystallize the (flavan-4-
ylthio)acetic acid derivative were unsuccessful. The product was
more easily purified as an ester than as an acid. Therefore, the
derivative was methylated using diazomethane.

1. Purification of the Reaction Products
by Column Chromatography

Upon evaporation of the methylation solution of the (flavan-4-
ylthio)acetic acid derivative a yellow syrup remained. Column chro-
matography was employed to separate and collect the methylated
product. The silica gel column was eluted seven times with 100 ml
aliquots of diethyl ether. Of the seven 100 ml fractions collected,
small white crystals of methyl 2, 3-trans-3', 4'-cis-(3-hydroxy-
3', 4', 5, 7-tetramethoxyflavan-4-y1thio)acetate were obtained from
fractions 2 through 6 with fraction 4 containing the largest amount.
A total of 45.0 mg of product was collected resulting in a 21% yield (calculated from the theoretical). Betts, Brown, and Shaw (2) have reported yields of 25-35% after methylation.

Figure 7 shows the n.m.r. spectrum of the product methyl 2, 3-trans-3, 4-cis-(3-hydroxy-3', 4', 5, 7-tetramethoxyflavan-4-ylthio)acetate. The stereochemistry about carbons 2, 3, and 4 was determined from the coupling constants of the C-ring protons. The configuration about carbons 2 and 3 was trans as evidenced by the coupling constant between protons 2 and 3 ($J_{2,3}$) equalling 10 Hz. Likewise, the proton 3 and 4 coupling constant ($J_{3,4}$) equal to 4 Hz suggested a cis relationship. Therefore, thioglycolic acid was found to react with a flavan-3,4-diol to produce a main product with a 3, 4 cis relationship. In addition, the thioglycolic acid reaction preserves the stereochemistry of carbons 2 and 3.

E. Gas-Liquid Chromatography

1. Preparation of Authentic Tetra-O-methylcatechin and Tetra-O-methylepicatechin

As mentioned previously, the phenolic hydroxyl groups of flavanoids were methylated to inhibit undesirable side reactions. These methyl ether derivatives also had the advantage of being volatile under conditions of gas-liquid chromatographic analysis.
Figure 7. NMR spectrum (CDCl$_3$) of methyl 2, 3-\textit{trans}-3, 4-\textit{cis}-\textit{(3-hydroxy-}
3', 4', 5, 7-tetramethoxyflavan-4-ylthio) acetate. $\times$ - solvent peak
Samples of authentic tetra-\text{O}-methylcatechin and tetra-\text{O}-methylepicatechin were kindly supplied by Dr. Joseph J. Karchesy (24, p. 82). These compounds were prepared by methylation of (+)-catechin and (-)-epicatechin with diazomethane. The mixed methylated products were separated by preparative thin-layer chromatography (Silica Gel 7GF; benzene-acetone 9:1 v/v). The band containing the desired tetra-\text{O}-methyl derivative was collected and the tetra-\text{O}-methyl derivative was recrystallized from methanol.

2. \textbf{Preparation of the Internal Standard}

A trimethoxyflavanone was synthesized for use as an internal standard for the gas-liquid chromatographic analyses. The compound was prepared from commercially available 4'-methoxy-3', 5, 7-trihydroxyflavanone, common name hesperitin, by methylation using diazomethane. The course of the methylation reaction was followed by gas-liquid chromatography. Figures 8, 9, and 10 show the spectra of the methylation reaction solution at 24 hour intervals. After 24 hours (Figure 8), only a small amount of the ultimate trimethoxyflavanone compound (Peak A) was present. Partially methylated products, peaks B and C, predominated. By the second day (Figure 9) peak A emerged as a substantial product. Disappearance of peak C together with the large area of peak B, believed to be a partially methylated
Figure 8. Gas-liquid chromatographic spectrum of the methylation solution of hesperitin in diazomethane after 24 hr. Peak A is trimethoxyflavanone; B, C and the remaining peaks are unidentified. Conditions: Hewlett-Packard 5751 B GC; column, 3% OV-17 on Gas chrom Q 100/120 mesh, 3 ft x 1/8 in O. D. stainless steel; injection port 250°; detector 250°; column temperature 220° isothermal; helium flow 30 ml/min; range setting $10^2$; attenuation 32.
Figure 9. Gas-liquid chromatographic spectrum of the methylation solution of hesperitin in diazomethane after 48 hr. Peak A is trimethoxyflavanone; B, C and the remaining peaks are unidentified. Conditions: Hewlett-Packard 5751B GC; column, 3% OV-17 on Gas chrom Q 100/120 mesh, 3 ft x 1/8 in O.D. stainless steel; injection port 250°; detector 250°; column temperature 220° isothermal; helium flow 30 ml/min; range setting $10^2$; attenuation 32.
Figure 10. Gas-liquid chromatographic spectrum of the methylation solution of hesperitin in diazomethane after 72 hr. Peak A is trimethoxyflavanone; B, C and the remaining peaks are unidentified. Conditions: Hewlett-Packard 5751B GC; column, 3% OV-17 on Gas chrom Q 100/120 mesh, 3 ft x 1/8 in O.D. stainless steel; injection port 250°; detector 250°; column temperature 220° isothermal; helium flow 30 ml/min; range setting $10^2$; attenuation 32.
derivative,\(^1\) indicated that the methylation was nearly complete. Finally, after 72 hours (Figure 10), Peak A was the main peak. The solution then crystallized to yield a crystalline trimethoxyflavanone which, when injected into the gas chromatograph, possessed a retention time identical to that of peak A in Figures 8, 9, and 10.

The extent of methylation was confirmed by a methoxyl content of 28.37\% compared with 28.17\% on a theoretical basis for tri-O-methyl derivative.

Figure 11 shows the n.m.r. spectrum of the internal standard, believed to be 5-hydroxy-3', 4', 7-trimethoxyflavanone. The assignment of the C-ring protons was made on the basis of their chemical shifts and their coupling constants in agreement with similar compounds studied by Clark-Lewis, Jackman, and Spotswood (5). The C-5 hydroxyl group was believed to be the unmethylated position because of the stabilization through hydrogen bonding that could occur with the adjacent carbonyl group. Hergert, Coad, and Logan (18) noted that in the methylation of dihydrogenation with dimethyl sulfate, methylation of the hydroxyl groups occurred in the order 3', 4', 7, and 5. They proposed the difficulty in methylating the C-5 hydroxyl

\(^1\)Gas-liquid chromatography-mass spectrometry have been used to help characterize unknown peaks from gas-liquid chromatography. However, in the present methylation reaction the intermediates were not of interest and so no attempt was made to identify unknown peaks.
Figure 11. NMR spectrum (CDCl₃) of the trimethoxyflavanone compound (internal standard), a

\[ x \] - solvent peak

\[ a \] It is believed that the compound is 5-hydroxy-3', 4', 7-trimethoxyflavanone, the structure of which is shown above the spectrum.
group as being due to chelation with the adjacent carbonyl group. It is believed a similar situation occurred in the methylation of hesperitin with diazomethane to produce the 5-hydroxy-tri-O-methyl derivative. No effort was made to scan the n.m.r. spectrum below \( \tau = 0 \) where peaks for intramolecular hydrogen bonded phenolic groups are known to occur (11, p. 90).

The trimethoxyflavanone was selected as the internal standard because it met all the requirements for a good internal standard as specified by McNair and Bonelli (29, p. 151). Under the conditions of gas-liquid chromatography, the trimethoxyflavanone compound was well resolved from all other peaks, eluted close to the peaks of interest, could be prepared in the approximate concentration of the unknowns, and was structurally similar to the unknowns. In addition, the trimethoxyflavanone was structurally dissimilar enough from the compounds of interest to be independent of any derivatives produced from the reaction scheme of this analysis.

3. **Determination of Optimum Gas-Liquid Chromatographic Conditions**

There are many parameters which can be varied in gas-liquid chromatography. The resolution of peaks can be affected by changes in carrier gas flow, length of the column, and changes in the column temperature. Peak heights and peak areas can be changed by changes
in injection port and detector temperatures. Several conditions were systematically changed until the optimum conditions, reported in the Experimental Section, were determined.

Identification of the compounds by gas-liquid chromatography was realized by comparing the time required for the unknown material to pass through the column with the time required for a sample of the authentic material to pass through the column. These times are called "retention times."

The condensed tannins of western species conifers are known to contain primarily (-)-catechin and (-)-epicatechin (23, 38). Therefore, authentic crystalline tetra-\(\text{O}\)-methylcatechin and tetra-\(\text{O}\)-methylepicatechin were prepared to positively determine the retention times and instrument response for quantitative analyses. Each of the authentic samples was passed through the gas chromatograph and the retention time for each was determined. The authentic samples were then mixed and passed again to ascertain resolution. The resulting spectrum is shown in Figure 12. Retention times for the internal standard, tetra-\(\text{O}\)-methylcatechin, and tetra-\(\text{O}\)-methylepicatechin, measured at the center of the peak were 14.00 minutes, 18.50 minutes, and 21.25 minutes, respectively.

4. Determination of Instrument Calibration Curves

Gas chromatographic detectors respond differently to different compounds. These response factors must be known to obtain
Figure 12. Gas-liquid chromatographic spectrum showing the resolution of the internal standard peak A; tetra-O-methyl catechin, peak B; and tetra-O-methylepicatechin, peak C. Conditions: Hewlett-Packard 5751B GC; column, 3% OV-17 on Gas chrom Q 100/120 mesh, 3 ft x 1/8 in O.D. stainless steel; injection port 250°; detector 250°; column temperature 220° isothermal; helium flow 30 ml/min; range setting 102; attenuation 32.
quantitative results. In addition, the recorder is also a possible source for error when the chromatogram is used for quantitative results. A good way to reduce these sources of error is to add an accurately weighed amount of an internal standard to the mixture to be analyzed and compare the peak areas of the compounds to be measured against the peak area of the internal standard. In this work, a trimethoxyflavanone was used as the internal standard.

The response of the gas chromatograph to tetra-0-methylcatechin and tetra-0-methylepicatechin was determined by analyzing varying weight ratios of each authentic tetra-0-methyl derivative with the internal standard. An instrument calibration curve for tetra-0-methylcatechin and tetra-0-methylepicatechin was obtained by plotting the ratio of the peak area of each tetra-0-methyl derivative to the area of the internal standard against the ratio of the weights. The results of these plots are shown in Figures 13 and 14.

In both cases, the overall response of the gas chromatographic system was not linear over the entire range of weight ratios tested. Only between the weight ratios of 1.00 through 2.00 were the calibration curves linear. Between weight ratios 0.25 through 1.00 the calibration curves fit exponential equations.

Linear regressions were run on the data points in the linear region of both calibration curves. The resulting regression equations and correlation coefficients were as follows:
Weight of tetra-O-methylcatechin

Weight of the internal standard

Figure 13. Instrument calibration curve of the relative detector response of tetra-O-methylcatechin to the internal standard.
Figure 14. Instrument calibration curve of the relative detector response of tetra-\(O\)-methylepicatechin to the internal standard.
Tetra-O-methylcatechin: \( A_{tmcat} = 0.30 + 0.51 W_{tmcat} \) \( r = 0.98 \)

Tetra-O-methylepicatechin: \( A_{tmepi} = 0.27 + 0.71 W_{tmepi} \) \( r = 0.98 \)

Where \( A_{tmcat} \) = Area ratio of tetra-O-methylcatechin/internal standard

\( A_{tmepi} \) = Area ratio of tetra-O-methylepicatechin/internal standard

\( W_{tmcat} \) = Weight ratio of tetra-O-methylcatechin/internal standard

\( W_{tmepi} \) = Weight ratio of tetra-O-methylepicatechin/internal standard

An attempt was made to fit the data points in the weight ratio regions from 0.25 through 1.00 to an exponential equation using linear regression from the equation:

\[ \ln y = \ln b + ax \]

The resulting equations showed good fits, both having correlation coefficients of 0.99.

\[ A_{tmcat} = 0.19 e^{1.50 W_{tmcat}} \]

\[ A_{tmepi} = 0.23 e^{1.43 W_{tmepi}} \]

These instrument calibration curves were used in subsequent calculations.

5. **Quantitative Analysis of the Thioglycolic Acid-Hydrogenation Reaction**

The main concern of this research was the quantification of the
reactions of flavan-3,4-diols with thioglycolic acid and subsequent hydrogenation of the resulting (flavan-4-ylthio)acetic acid derivatives to produce either tetra-\text{-}\text{O}-\text{methylcatechin} or tetra-\text{-}\text{O}-\text{methylepicatechin} depending upon the stereochemistry of C-2 and C-3. In the present work the flavan-3,4-diol was derived originally from dihydroquercetin which has the same stereochemistry about C-2 and C-3 as (+)-catechin. This stereochemistry remains intact since thioglycolic acid and hydrogenation with Raney nickel affect only the C-4 position.

The intention of the quantitative scheme was to start with the flavan-3,4-diol and proceed to tetra-\text{-}\text{O}-\text{methylcatechin} without isolating the (flavan-4-ylthio)acetic acid derivative. The advantage of this method would then be that the entire reaction sequence could be carried out in a single reaction vessel. Since no intermediates need to be isolated, no loss of material would occur and a more accurate quantification of the reaction could be made.

To begin the quantitative study, it was necessary to determine if the flavan-3,4-diol (2,3-trans,4-cis-3',4',5,7-tetramethoxy-flavan-3,4-diol) would react with Raney nickel to become directly hydrogenated to tetra-\text{-}\text{O}-\text{methylcatechin}. The flavan-3,4-diol was dissolved in dioxane-water (2:1 v/v), the solvent used in the thioglycolic acid reaction. The solution was equally divided among two flasks and excess Raney nickel was added to each. One flask was left at room temperature for two hours while the second flask was
refluxed for two hours. Gas-liquid chromatography showed that the solution left to react at room temperature contained no tetra-\( \text{O} \)-methylcatechin. However, the solution that was refluxed for two hours did produce tetra-\( \text{O} \)-methylcatechin.

The quantitative conversion of the flavan-3,4-diol to tetra-\( \text{O} \)-methylcatechin was determined by taking the refluxed solution to an accurately measured volume. A known amount of internal standard was added to make a 0.005 g/ml concentration (total volume of 2 ml). Four samples were injected into the gas chromatograph. The areas of the peaks were obtained with an integrator and the average area ratio (tetra-\( \text{O} \)-methylcatechin to internal standard) was calculated. The average area ratio was 0.83. Knowing this, the graph in Figure 13 was used to determine the actual weight ratio. Starting from the area ratio axis, it can be seen that 0.83 corresponds to a weight ratio of 0.98. Since the internal standard was present in a 0.005 g/ml concentration, the amount of tetra-\( \text{O} \)-methylcatechin present can be calculated by the following formula:

\[
\text{Weight of product} = (\text{Weight ratio}) \times (\text{Concentration of internal standard}) \times (\text{Volume of solution})
\]

Knowing this, the % conversion can be determined:

\[
% \text{ Conversion} = \frac{\text{Weight of product}}{\text{Weight of starting material}} \times \frac{\text{Molecular weight of starting material}}{\text{Molecular weight of product}} \times 100
\]
For the reaction of 3,4-diol in dioxane-water refluxed for two hours with Raney nickel, the following calculations can be made:

\[
\text{Weight of tetra-O-methylcatechin} = 0.98 \times (0.005 \text{ g/ml}) (2\text{ml}) = 0.0098 \text{ g}
\]

\[
\text{% Conversion =} \frac{0.0098 \text{ g}}{0.0200 \text{ g}} \times \frac{362.12 \text{ g/mole}}{346.13 \text{ g/mole}} \times 100 = 51.3\%
\]

All the quantitative data to follow were determined in a similar manner.

Figure 15 shows the gas-liquid chromatogram of the flavan-3,4-diol and Raney nickel reflux reaction solution. An unexpected peak appeared in the spectrum. The occurrence of this peak suggested that at reflux conditions undesirable side reactions were occurring. Gas-liquid chromatography-mass spectrometry was used in an attempt to identify the unknown, peak A, but the results were inconclusive.

The results of the study of the flavan-3,4-diol with Raney nickel in dioxane-water suggested that the hydrogenation reaction should be run at room temperature. At room temperature, the flavan-3,4-diol is not converted to tetra-O-methylcatechin and therefore quantitative results will not be affected. Refluxing not only causes a 51.3% conversion of the diol to tetra-O-methylcatechin, but causes a side reaction.

Once the conditions for the hydrogenation reaction were
Figure 15. Gas-liquid chromatographic spectrum of the reaction products of the flavan-3,4-diol and Raney nickel refluxed for 2 hr. in dioxane-water (2:1 v/v). Peak A is unidentified, B is internal standard, C is tetra-O-methylcatechin. Conditions: Hewlett-Packard 5751B GC; column, 3% OV-17 on Gas chrom Q 100/120 mesh, 3 ft x 1/8 in O.D. stainless steel; injection port 250°; detector 250°; column temperature 220° isothermal; helium flow 30 ml/min; range setting 10^2; attenuation 32.
determined which prevented direct hydrogenation of the flavan-3, 4-diol to tetra-0-methylcatechin, it was necessary to determine the conversion of the (flavan-4-ylthio)acetic acid derivative to tetra-0-methylcatechin under these conditions. Methyl 2, 3-trans-3, 4-cis-(3-hydroxy-3', 4', 5, 7-tetramethoxyflavan-4-ylthio)acetate was dissolved in dioxane-water (2:1 v/v) and excess Raney nickel was added. The solution was allowed to sit at room temperature for two hours. Gas-liquid chromatographic analysis was performed as described previously. The quantitative data obtained showed that the conversion of the (flavan-4-ylthio)acetate methyl ester to tetra-0-methylcatechin was essentially 100%.

The methyl ester of the (flavan-4-ylthio)acetic acid derivative was used for this reaction because the (flavan-4-ylthio)acetic acid derivative could not be collected in crystalline form. It was not expected that the presence of the ester rather than the free carboxylic acid would affect the results obtained.

The fact that the conversion of the (flavan-4-ylthio)acetate methyl ester derivative to tetra-0-methylcatechin was completely quantitative was of important consequence. Because of this, the percent conversion data obtained from the gas-liquid chromatographic analyses could be interpreted solely as the result of the reaction of thioglycolic acid with flavanoid-type compounds. In addition, it would
not be necessary to attempt to quantify the amount of (flavan-4-ylthio)acetic acid derivative produced, which would be a difficult task since this compound cannot be detected under the present gas-liquid chromatographic conditions.

Having established conditions for the hydrogenation reaction to prevent interference with quantitative results from the direct conversion of the flavan-3,4-diol to tetra-\(\text{O}\)-methylcatechin, and having determined that the conversion of the (flavan-4-ylthio)acetic acid derivative to tetra-\(\text{O}\)-methylcatechin was completely quantitative, it was possible to quantify the reaction of thioglycolic acid with the flavan-3,4-diol through a scheme of two sequential reactions.

Figure 16 shows the reaction scheme. An accurately weighed amount of 2,3-trans-3,4-cis-3',4',5,7-tetramethoxyflavan-3,4-diol was reacted with thioglycolic acid under reflux for one hour. After allowing the solution to cool, excess Raney nickel was added and the solution was allowed to react for two hours at room temperature. The solution was filtered directly into a round-bottom flask. The solvent was evaporated on a rotary evaporator and the remaining residue redissolved in a carefully measured volume of dichloromethane. The internal standard was added, followed by gas-liquid chromatographic analysis.

Figure 17 shows a gas-liquid chromatographic spectrum of the solution from the resulting reaction scheme. The average area ratio
Figure 16. Reaction scheme for the quantitative analysis of the reaction of thioglycolic acid with a flavan-3,4-diol, subsequent hydrogenation to produce tetra-O-methylcatechin, with quantitative data collected by gas-liquid chromatographic analysis.
Figure 17. Gas-liquid chromatographic spectrum of the solution resulting from the quantitative reaction scheme. Peak A is internal standard, B is tetra-O-methylcatechin. Conditions: Hewlett-Packard 5751 B GC; column, 3% OV-17 on Gas chrom Q 100/120 mesh, 3 ft x 1/8 O.D. stainless steel; injection port 250°; detector 250°; column temperature 220° isothermal; helium flow 30 ml/min; range setting 102; attenuation setting 32.
of six sample injections was 0.96. From the calibration curve, Figure 13, an area ratio of 0.96 corresponded to a weight ratio of 1.15. The percent conversion, calculated as previously shown, of the reaction of thioglycolic acid with 2, 3-trans-3, 4-cis-(3-hydroxy-3', 4', 5, 7-tetramethoxyflavan-3, 4-diol) was found to be 60.7 percent. This is in good agreement with the results of Betts, Brown and Shaw (2) who reacted 2, 3-trans-3, 4-cis-(3-hydroxyflavan-3, 4-diol) with thioglycolic acid and after methylation, obtained methyl 2, 3-trans-3, 4-cis-(3-hydroxyflavan-4-ylthio)acetate in a 59 percent total yield on a crystalline basis.
V. SUMMARY AND CONCLUSIONS

The purpose of this study was to develop a method for the quantitative analysis of the reaction of thioglycolic acid with flavan-3, 4-diols. The diols used in this study were synthesized from dihydroquercetin which was isolated from a crude extract of Douglas-fir bark.

The synthesis of the flavan-3, 4-diols was begun by methylating the four phenolic hydroxyl groups of dihydroquercetin to produce the methyl ethers. Methylation was necessary to prevent undesirable side reactions such as self-condensation and autoxidation which can occur among free phenolic flavanoids under both acidic and basic conditions. Methylation was also necessary to produce derivatives that were volatile under the conditions of gas-liquid chromatography.

Following methylation, the carbonyl group at the C-4 position of dihydroquercetin was reduced using sodium borohydride. Attack from either side of the planer carbonyl group by the reducing agent produced two crystalline 3, 4-diols which were epimeric about the C-4 position.

The 3, 4-diols thus produced were used in the quantitative study of the reaction of flavan-3, 4-diols with thioglycolic acid. The quantitative scheme developed consisted of reacting a flavan-3, 4-diol with thioglycolic acid immediately followed by hydrogenation with Raney
nickel. The thioglycolic reaction produced (flavan-4-ylthio)acetic acid derivatives of the starting flavan-3,4-diol. The subsequent hydrogenation with Raney nickel effected a desulfurization resulting in the production of tetra-$\text{O}$-methylcatechin. Quantitative determination of the amount of tetra-$\text{O}$-methylcatechin present was made using gas-liquid chromatography.

It was found that the desulfurization reaction of a (flavan-4-ylthio)acetic acid derivative with Raney nickel proceeded to essentially 100% completion. Therefore, the quantitative results obtained from gas-liquid chromatography on the presence of tetra-$\text{O}$-methylcatechin relate directly to the yield of the reaction of a flavan-3,4-diol with thioglycolic acid to produce the (flavan-4-ylthio)acetic acid derivative. In this study, it was found that the conversion was 60.7 percent. Although the development of this quantitative method in itself is an important step in understanding flavanoid chemistry, the applications of this method may prove far more significant.
VI. RECOMMENDATIONS

A logical next step for the application would be the quantitative study of the reaction of thioglycolic acid on a carbon to carbon bond. Sears and Casebier (37) showed that the carbon 4 to carbon 6 or 8 bond of condensed tannins could be cleaved by thioglycolic acid. Starting with a known condensed tannin molecule, the proposed quantitative technique could be performed to determine the effectiveness of thioglycolic acid in breaking this carbon to carbon bond.

Application of the quantitative method to the carbon to carbon bond of a condensed tannin was not within the scope of the present work. However, a procedure was developed and is documented here as a proposal for further study.

Geissman and Yoshimura (16), Jurd and Lundin (22), and Creasy and Swain (7) have synthesized condensed tannins through a condensation reaction of a flavan-3,4-diol with a flavan-3-ol. The result of this reaction has been a condensed tannin dimer linked by a carbon to carbon bond from the 4 position of one monomer to the 6 or 8 position of the second. The condensation reaction is performed under acidic conditions, however, there is a discrepancy in the literature as to the reaction medium and temperature at which the reaction is to be performed. Experimentation would be required to determine the most successful reaction conditions.
Upon determining the reaction conditions, a dimer could be synthesized from a known flavan-3,4-diol and flavan-3-ol. The flavan-3,4-diol produced from tetra-O-methylidihydroquercetin would be useful for this reaction because the stereochemistry about the number 2 and 3 carbons have the same relative configuration as (+)-catechin. This could be condensed with (-)-epicatechin to produce a dimer with a 1:1 ratio of catechin to epicatechin. A methylation reaction would then be performed on the dimer to prepare the methyl ethers of the four phenolic hydroxyl groups of (-)-epicatechin. Again, the reason for this is that the compounds must be volatile under the conditions of gas-liquid chromatography. Unmethylated (-)-epicatechin is used for the condensation reaction because the 6 and 8 positions of the free phenolic form is more reactive relative to the methyl ethers. This fact should help prevent flavan-3,4-diol self condensation reactions under the conditions of dimerization since the tetra-O-methyl derivative is used. Figure 18 shows the proposed synthesis of the condensed tannin dimer.

With the successful preparation of a methylated condensed tannin dimer, the quantitative analysis of the thioglycolic acid reaction on a carbon to carbon bond could be determined. The dimer would be reacted with thioglycolic acid, producing tetra-O-methyl-epicatechin and the (flavan-4-ylthio)acetic acid derivative. Hydrogenation with Raney nickel would produce tetra-O-methylcatechin and
Figure 18. Proposed synthesis of methylated condensed tannin dimer.
the resulting solution could be analyzed by gas-liquid chromatography.

The conditions of gas-liquid chromatography resolve the two remaining tetra-\(\text{Q}\)-methyl flavans (Figure 12) and therefore determination of the desired quantitative results could be obtained from either or both of the two peaks.

Once the quantitative results are determined for the reaction of thioglycolic acid with model condensed tannin dimers, the method can be applied to characterize unknown condensed tannins. Upon completion of the gas-liquid chromatographic analysis, the proposed method could provide qualitative information as to the flavanoids present, determine the relative ratio of these compounds, and specify exact quantitative amounts.
BIBLIOGRAPHY


