CHROM. 5281

# THE ESTIMATION OF FLAVANOLS IN TEA BY GAS CHROMATOGRAPHY OF THEIR TRIMETHYLSILYL DERIVATIVES

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SUMMARY

A method for separating and estimating the flavanols of tea by temperature-programmed gas–liquid chromatography of their trimethylsilyl derivatives is described. Columns of 3 % OV-1 on 100–120 mesh Diatomite CQ give optimum separation, and used with a quercetin internal standard, give results reproducible to within ±5 %. Both black and green tea contain a species (thought to be 3-galloylquinic acid) interfering with the estimation of epigallocatechin: this difficulty is avoided by selective extraction of the flavanols into ethyl acetate. Conditions necessary for complete derivatisation are discussed, and a method is described for preparation of samples from unfired leaf.

## INTRODUCTION

The characteristic colour and organoleptic properties of black tea result directly and indirectly from the enzymic oxidation of flavanols, the major polyphenolic constituents of fresh green tea leaf[1,2]. A rapid, accurate method for separation and estimation of flavanols is necessary to establish the rates and routes of these reactions, and their dependence on tea processing conditions. The flavanols concerned are (+)-catechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG)[3,4]. Investigation of the gas–liquid chromatographic (GLC) separation of the trimethylsilyl (TMS) derivatives of these flavanols[5] has shown this technique to hold more promise than paper and column chromatographic methods. We wish to report a detailed investigation of the method, the aims being:

(1) To develop a temperature-programmed method of separation, so that complete analysis requires only one run and one internal standard. (The method[5] currently uses two standards and complete analysis requires isothermal runs at two temperatures.)

(2) To establish derivatisation conditions in which flavanols are completely and rapidly derivatised, even when (as in black tea) large amounts of other derivatisable species* are present. (Complete derivatisation has been directly proven to date[5] only for flavanols reacting in a relatively non-competitive environment.)

* Carbohydrates, theaflavins, thearubigins, phenolic acids and glycosides, amino acids.

(3) To develop a selective extraction process for the flavanols, so that the chromatogram profile might be simplified, and the possible co-incidence of flavanol and non-flavanol peaks might be revealed. (The current pyridine–water extraction[5] is unselective, and no investigation of the retention times of non-flavanol tea constituents has been made.)

## EXPERIMENTAL

### *Reagents*

N,O-Bis-(trimethylsilyl)acetamide (BSA), trimethylchlorosilane (TMCS) and Silyl 8 column conditioner were supplied by the Pierce Chemical Company. Quercetin (Analar) was supplied by Koch-Light Laboratories Ltd. and recrystallised from aqueous methanol. EC and (+)-catechin (puriss.) were supplied by Koch-Light Laboratories Ltd. We gratefully acknowledge the gift of samples of EGC, ECG and EGCG from T. J. Lipton, Inc., Englewood Cliffs, N.J. 07632, U.S.A.

### *GLC equipment and conditions*

A Pye series 104-24 dual column instrument fitted with inlet heaters and flame ionisation detectors was used. 5-ft. coiled glass columns (I.D. 4 mm) were filled with the required packings, injected several times with Silyl 8 column conditioner and left overnight at 310° before use. The (nitrogen) carrier gas flow rate was 50–100 ml/min, depending on the column packing, and the injection block temperature was 290°.

Several liquid phases and solid supports were used in preliminary experiments: 3 % OV-1 on 60–80 mesh acid-washed Chromosorb W; 3 % OV-17 on 60–80 mesh Gas-Chrom Q; 3 % OV-1 on 80–100 mesh Gas-Chrom Q; 3 % SE-30 on 60–80 mesh Gas-Chrom Q; 3 % OV-1 on 80–100 mesh Chromosorb G; 3 % OV–1 on 100–120 mesh Diatomite CQ or Gas-Chrom Q.

The columns giving the best resolution between EC and (+)-catechin were 3 % OV-1 on 100–120 mesh Diatomite CQ or Gas-Chrom Q. Optimum rapid separation was achieved under the following conditions: Nitrogen flow rate: 60 ml/min. Temperature programme: 235° isothermal for 22 min, then 24°/min to 300° (coronene standard) or 48°/min to 310° (quercetin standard), isothermal hold till emergence of EGCG. Chart speed: 15 in./h for 22 min, then 30 in./h until emergence of EGCG.

### *Extraction*

(a) as previously described[5] using aqueous pyridine.

(b) Freeze-dried water solubles of whole black tea (1 g) were dispersed in water (50 ml) and extracted with 3 × 100 ml ethyl acetate. The extracts were (i) combined, dried ($Na_2SO_4$), and evaporated to dryness under reduced pressure at 30°, or (ii) dried and evaporated to dryness separately. The solids so obtained were dissolved in redistilled dry pyridine (5, 10 or 25 ml) and aliquots derivatised as described below.

(c) Freeze-dried water solubles of whole black tea (1 g) were dispersed in water (25 ml), extracted with 4 × 100 ml ethyl acetate, and the extracts prepared for derivatisation as described in (b).

### *Derivatisation*

(a) As previously described[5], using N,O-bis-(trimethylsilyl)acetamide (BSA).

(b) The dry pyridine solution of acetate extract solids (2 ml) is injected into

a septum-sealed tube containing a known weight of internal standard (coronene or quercetin). BSA (2 ml) is injected, and the reaction mixture left for the desired period at room temperature.

(c) As in (b), but trimethylchlorosilane (TMCS, 0.5 ml) is used in addition to the BSA as a silylation catalyst.

*GLC trapping and mass spectrometry*

The freeze-dried solids obtained from acetate-extracted aqueous dispersions (1 g in 25 ml) of tea solubles were taken up in dry pyridine (25 ml) and an aliquot (1 ml) derivatised with BSA (2 ml)–TMCS (0.5 ml) for 30 min. Solvent and excess reagents were distilled off at 45° under vacuum, and the residue taken up in AR carbon tetrachloride (0.5 ml). Aliquots of this solution (5 × 5 $\mu$l) were injected onto a flavanol analysis column fitted with a 100:1 stream splitter. Column temperature, 235° isothermal; carrier gas flow rate, 75 ml/min. EGC and EC were used as reference compounds. Material corresponding to the relevant peak was collected in a U-tube trap cooled in a cardice–acetone slurry. The collected material was washed from the trap with carbon tetrachloride, and the solvent removed at room temperature in a stream of dry nitrogen. Spectra of the materials trapped from black- and green-leaf solubles at EGC's retention time were obtained by direct introduction onto the probe of an AEI MS 902 mass spectrometer operating at 70 eV.

*Experiment B1*

Fresh leaf from green-house plants was ground with solid $CO_2$, extracted with aqueous pyridine as previously described[5], and two equal volumes of the filtered extract solution evaporated to dryness at 40° under vacuum. One sample of the extract (1A, 1 g) was taken up directly in dry pyridine (25 ml) and aliquots (2 ml) derivatised (BSA/TMCS) and analysed for flavanols. The other sample (1B, 1 g) was slurried in water (25 ml) and extracted with ethyl acetate (4 × 100 ml). The combined dried extract was taken up in dry pyridine (25 ml) and aliquots (2 ml) derivatised (BSA/TMCS) and analysed for flavanols. The aqueous layer was freeze-dried, the residual solids taken up in dry pyridine (10 ml) and aliquots (2 ml) derivatised and examined on the flavanol GLC analysis column.

*Experiment B2*

Fresh leaf was divided into a number of samples. One sample (2A) was prepared for analysis by extraction with aqueous pyridine[5] and partitioning into ethyl acetate as described in B1. A second sample (2B, 50 g) was macerated (Ultra-Turrax TP 18/2 *ex* Janke and Kumgel KG) in cold *n*-propanol (200 ml) for 1 min, filtered through a No. 2 glass sinter, then turraxed with 3 × 100 ml cold and 1 × 100 ml boiling 80 % aqueous methanol (filtered after each extraction). The alcohols were evaporated from the combined extracts at 30° under vacuum, and the aqueous solution freeze-dried. An aqueous dispersion of these solids (1 g in 25 ml) was extracted with ethyl acetate (4 × 100 ml) and the evaporated acetate extract analysed for flavanols. The residual leaf fibre was vacuum oven dried, extracted with aqueous pyridine, and the extract analysed for flavanols. A third sample (2C, 50 g) was turraxed in boiling propanol for 1 min, then treated as described for 2B. Further samples of leaf (10 g) were dosed with known amounts of EC, EGC and EGCG prior to treatment with hot propanol (40 ml), then treated as described for 2B.

*Experiment B3*

Fresh leaf was divided into four 50 g samples. Two samples (3A and 3B) were turraxed in boiling propanol (200 ml, 1 min), transferred to lacquer-lined 4-oz. cans, and the cans sealed after topping up with propanol. The canned samples were left at ambient laboratory temperature (18–21°) for the desired period, then opened and the contents extracted with 80 % aqueous methanol as described in B2. The freeze-dried extract solids were analysed for flavanols. Two other samples (3C and 3D) were similarly treated with boiling propanol and immediately extracted, dried and analysed.

*Experiment B4*

Fresh leaf was treated with boiling propanol and extracted with aqueous methanol as described in B2. An aqueous dispersion of the freeze-dried extract (1 g in 25 ml) was extracted with ethyl acetate (4 × 100 ml), and the four extracts separately analysed for flavanols.

## RESULTS AND DISCUSSION

(*A*) *Analysis of flavanols in the water solubles of fired black tea*

(*1*) *Separation of flavanols and internal standards.* Temperature-programmed separation of the five flavanols, coronene[5] and quercetin was best accomplished with a (compensated) 5-ft. column of 3 % OV-1 on 100–120 mesh Gas-Chrom Q or Diatomite CQ. Optimum conditions were as follows: Carrier gas flow rate, 60 ml/min; temperature programme, 235° isothermal for 22 min, then at 24°/min to 300° (coronene standard) or 48°/min to 310° (quercetin standard, Fig. 1).

Calibration data obtained from known mixtures of pure flavanols and coronene (derivatisation as previously described[5]) are presented in Table I. Plots of weight of flavanol *vs.* weight of standard ($W_f/W_s$) against the corresponding peak area ratio ($A_f/A_s$) were linear, least-mean-squares refined values of the slopes ($W_f \cdot A_s/W_s \cdot A_f$) being taken as the detector constants ($F$). (Detector constants on a quercetin standard could not be obtained until derivatisation conditions ensuring complete trimethylsilylation of quercetin were established, see (2) below).

(*2*) *Derivatisation conditions.* Although gas density balance measurements have directly established that milligram quantities of flavanols are quantitatively converted into their TMS derivatives within 15 min by BSA in pyridine[5], no evidence has

TABLE I

FLAVANOL DETECTOR CONSTANTS ($F$)[a] ON CORONENE STANDARD

| | $F$ | *Standard error* (%) |
|---|---|---|
| EC | 0.875 | ±3.54 |
| (+)-Catechin | 0.811 | ±2.96 |
| EGC | 0.814 | ±5.15 |
| ECG | 0.858 | ±5.24 |
| EGCG | 1.006 | ±3.88 |

[a] Found to be the same for both detectors and both Diatomite CQ columns.

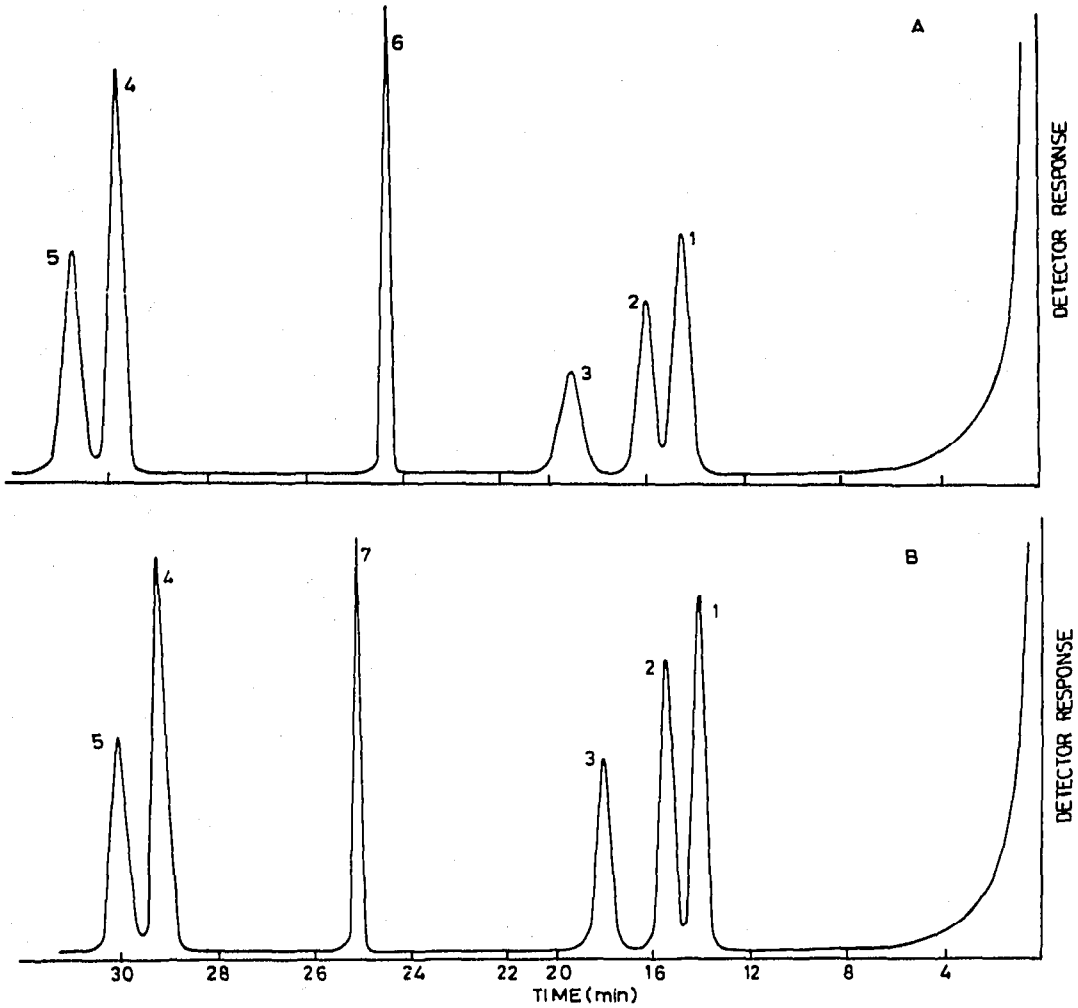


Fig. 1. Separation of flavanol TMS derivatives. 1 = EC; 2 = (+)-catechin; 3 = EGC; 4 = ECG; 5 = EGCG; 6 = quercetin; 7 = coronene. (A) 3 % OV-1 on 100–120 mesh Diatomite CQ; (B) 3 % OV-1 on 100–120 mesh Gas-Chrom Q.

been produced to show that the flavanols in whole black tea extracts undergo quantitative conversion under these conditions. Competition for available BSA from the large quantities of other derivatisable species in such extracts was expected to retard derivatisation of the flavanols, which by comparison are minor constituents (< 10 %).

A freeze-dried aqueous extract of black tea (2.0 g) was dissolved in dry pyridine (50 ml) and aliquots (2 ml) injected into septum-sealed tubes containing BSA (2 ml) and 1–2 mg coronene or quercetin. 5 $\mu$l aliquots were withdrawn at intervals and analysed on the temperature-programmed Diatomite CQ columns*. Comparison of the chromatograms obtained after room temperature reaction periods of 15 min, 60 min, and 12 h showed that:

(a) The profile of the first isothermal region of the chromatogram becomes dramatically simpler with increasing reaction time.

(b) Quercetin invariably gives twin peaks: after 15 min reaction the peak eluted

* Coronene-containing samples were analysed on one column, quercetin-containing samples on the other.

first predominates, whilst with increasing reaction time the slower-moving peak becomes dominant. The sum of the two peak areas remains approximately constant.

(c) The ratio ($A_f/A_s$) of the area of each flavanol peak to the (total) area of the corresponding standard peak(s) changes with reaction time (Table II).

TABLE II

VARIATION OF $A_f/A_s$ WITH DERIVATISATION TIME

No silylation catalyst.

| (1) *Coronene standard* | | | |
|---|---|---|---|
| | *15 min* | *60 min* | *12 h* |
| EC | 0.243 | 0.350 | 0.512 |
| (+)-Catechin | 0.157 | 0.205 | 0.208 |
| EGC | 0.660 | 0.934 | 1.181 |
| ECG | 0.632 | 0.665 | 0.732 |
| EGCG | 1.152 | 1.216 | 1.369 |
| (2) *Quercetin standard* | | | |
| | *15 min* | *60 min* | *12 h* |
| EC | 0.132 | 0.184 | 0.314 |
| (+)-Catechin | 0.085 | 0.108 | 0.121 |
| EGC | 0.359 | 0.493 | 0.726 |
| ECG | 0.344 | 0.351 | 0.450 |
| EGCG | 0.626 | 0.641 | 0.840 |

TABLE III

VARIATION OF $A_f/A_s$ WITH REACTION TIME

TMCS catalyst.

| (1) *Coronene standard* | | | |
|---|---|---|---|
| | *30 min* | *60 min* | *105 min* |
| EC | 0.650 | 0.647 | 0.652 |
| (+)-Catechin | 0.212 | 0.208 | 0.211 |
| EGC | 1.332 | 1.336 | 1.327 |
| ECG | 0.795 | 0.805 | 0.790 |
| EGCG | 1.471 | 1.480 | 1.473 |
| (2) *Quercetin standard* | | | |
| | *30 min* | *60 min* | *105 min* |
| EC | 0.425 | 0.431 | 0.422 |
| (+)-Catechin | 0.128 | 0.123 | 0.131 |
| EGC | 0.917 | 0.906 | 0.920 |
| ECG | 0.521 | 0.512 | 0.527 |
| EGCG | 0.943 | 0.946 | 0.929 |

All three effects are symptoms of slow and incomplete derivatisation. The experiment was repeated using trimethylchlorosilane (TMCS, 0.5 ml) as a silylation catalyst. Injections made after reaction times of 30, 60 and 105 min gave chromatograms in which quercetin gave a single peak and $A_f/A_s$ values were independent of reaction time (Table III), thus showing that complete derivatisation of flavanols in whole black tea extracts may be achieved in a short time only by use of TMCS.

After 15–20 injections of samples containing coronene (not derivatisable), the coronene peak became progressively broader and more asymmetric with each successive injection. The flavanol-TMS peak profiles remained satisfactory, and column performance could be regained by replacing the top inch of packing, thus suggesting that irreversible adsorption of the less volatile tea constituent derivatives (or their decomposition products) is responsible for this deterioration. No such effects were observed with samples containing quercetin (a silylatable standard): accordingly the use of a coronene standard was abandoned.

To determine more accurately the minimum reaction time required for complete derivatisation of quercetin, a 10 mg sample was derivatised in dry pyridine (2 ml) with BSA (2 ml) and TMCS (0.5 ml). Samples (5 $\mu$l) were taken at regular intervals and analysed isothermally at 310° (carrier flow rate 60 ml/min). A plot of peak area against reaction time showed that quercetin is completely derivatised after 25–30 min at room temperature (Fig. 2). Confirmation that this reaction time is necessary when smaller quantities are used in the presence of tea solids was obtained as follows. Different weights of quercetin (0.5–2.0 mg) were added to aliquots (2 ml) of a dry pyridine solution of an ethyl acetate extract of black tea solution (see EXPERIMENTAL for extraction procedure). The samples were derivatised with BSA (2 ml)–TMCS (0.5 ml), and 5 $\mu$l aliquots taken at intervals for analysis on the temperature-programmed column. A plot of weight of quercetin against the corresponding quercetin peak area gave a straight line only when the reaction time was greater than 15 min. Reaction times of 25–30 min gave results indistinguishable from those obtained after 1–2 h (Fig. 3). Detector constants ($F = W_f \cdot A_s / W_s \cdot A_f$) obtained by temperature-programmed analysis of known mixtures of pure flavanols and quercetin derivatised

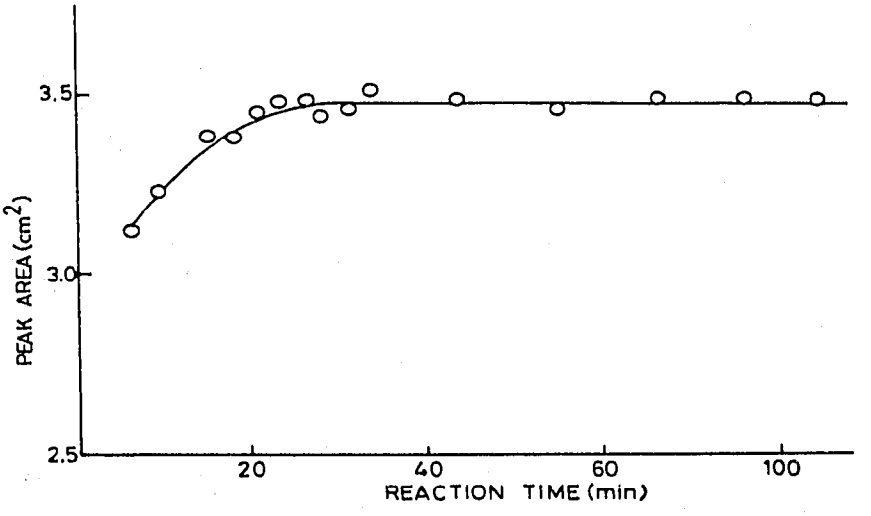


Fig. 2. Rate of derivatisation of quercetin by BSA–TMCS in pyridine.

under these conditions (2 ml pyridine, 2 ml BSA, 0.5 ml TMCS, reaction time 30 min) are given in Table IV.

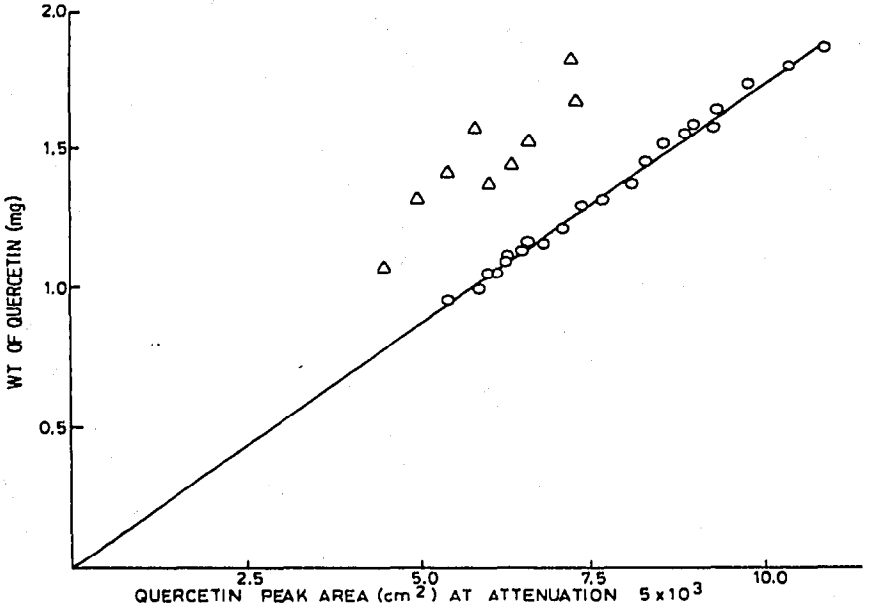


Fig. 3. Rate of derivatisation of quercetin in presence of water-soluble tea solids. △, Reaction time up to 15 min; ○, Reaction time 25–180 min.

TABLE IV

FLAVANOL DETECTOR CONSTANTS ($F$)[a] ON A QUERCETIN STANDARD

| | $F$ | *Standard error* (%) |
|---|---|---|
| EC | 0.848 | ±1.8 |
| (+)-Catechin | 0.808 | ±4.7 |
| EGC | 0.820 | ±4.4 |
| ECG | 0.888 | ±1.4 |
| EGCG | 0.901 | ±2.3 |

[a] Found to be the same for both detectors and both Diatomite CQ columns.

(3) *Extraction with ethyl acetate.* Chromatograms of freeze-dried aqueous extracts of black tea are complex in the low-temperature isothermal region, and it is by no means certain that flavanol and non-flavanol peaks are perfectly resolved. In addition, there is a group of small peaks which slightly overlap that due to quercetin, which can be reliably used as a standard only if a more selective extraction procedure is used. GLC comparison of the whole aqueous extract with the corresponding ethyl acetate extract shows that the acetate extract contains no material interfering with quercetin, and gives a much simpler chromatogram in which flavanol peak areas can be more accurately measured (Fig. 4). To establish conditions for complete extraction of flavanols, the two extraction regimes described under EXPERIMENTAL were used to provide sequential extracts (E1–E4) which were individually derivatised (quercetin

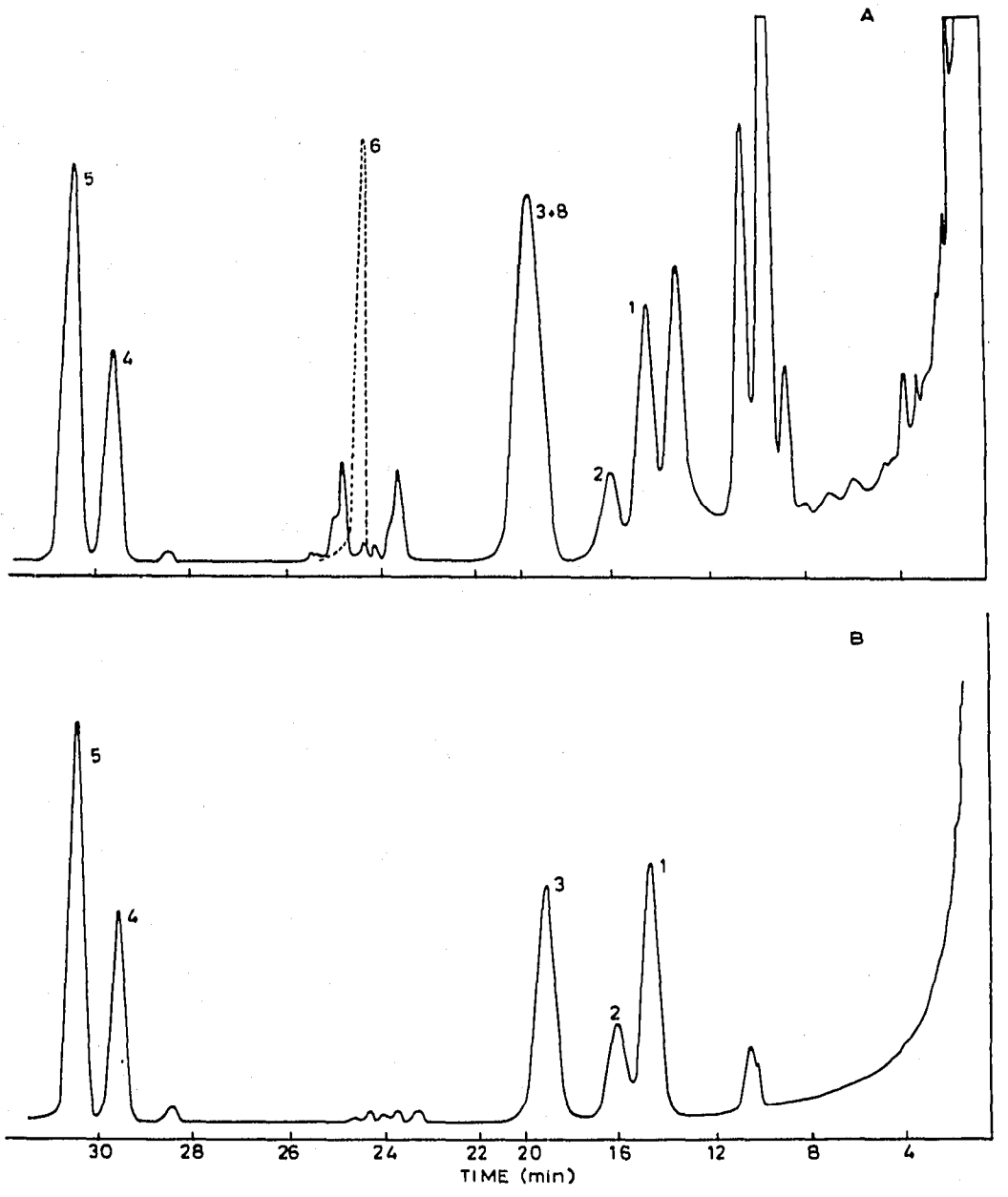


Fig. 4. Chromatograms of whole tea extracts. (A) Whole aqueous extract; (B) corresponding ethyl acetate extract. Peak 8, suspected theogallin.

standard) with BSA–TMCS and analysed on a temperature-programmed Diatomite CQ column. The results (Table V) show that four-fold acetate extraction is necessary. This was confirmed by freeze-drying the acetate-extracted aqueous solutions and analysing the residual solids for flavanols. The residue from four-fold extraction gave (even at ten times the original sensitivity) barely detectable peaks for EC, (+)-catechin, ECG and EGCG. The residue from three-fold extraction gave (at the original sensitivity) discernible peaks for all these flavanols.

More interesting still was the observation that both residues gave chromatograms in which a prominent peak appeared in the position expected for EGC (Fig. 5).

TABLE V

EFFICIENCY OF ETHYL ACETATE EXTRACTION OF FLAVANOLS

| | % Flavanols on tea solubles | | | | |
|---|---|---|---|---|---|
| *(a) 1 g tea solubles in 50 ml water/3 × 100 ml ethyl acetate* | | | | | |
| | *EC* | *(+)-Catechin* | *EGC* | *ECG* | *EGCG* |
| E1 | 0.815 | 0.272 | 0.599 | 1.508 | 2.690 |
| E2 | 0.258 | 0.063 | 0.405 | 0.189 | 0.477 |
| E3 | 0.051 | 0.011 | 0.175 | 0.016 | 0.053 |
| Total | 1.124 | 0.346 | 1.179 | 1.713 | 3.220 |
| | *% of total extracted at each stage* | | | | |
| E1 | 72.5 | 78.6 | 50.9 | 88.1 | 83.6 |
| E2 | 23.0 | 18.2 | 34.3 | 11.0 | 14.8 |
| E3 | 4.5 | 3.2 | 14.8 | 0.9 | 1.6 |
| *(b) 1 g tea solubles in 25 ml water/4 × 100 ml ethyl acetate* | | | | | |
| | *% Flavanols on tea solubles* | | | | |
| E1 | 1.148 | 0.359 | 0.979 | 1.907 | 3.435 |
| E2 | 0.134 | 0.032 | 0.265 | 0.083 | 0.234 |
| E3 | 0.025 | 0.004 | 0.072 | 0.013 | 0.021 |
| E4 | 0.006 | — | 0.013 | 0.003 | 0.002 |
| Total | 1.313 | 0.395 | 1.329 | 2.006 | 3.692 |
| | *% of total extracted at each stage* | | | | |
| E1 | 87.4 | 90.9 | 73.7 | 95.2 | 93.0 |
| E2 | 10.2 | 8.1 | 19.9 | 4.1 | 6.3 |
| E3 | 1.9 | 1.0 | 5.4 | 0.6 | 0.6 |
| E4 | 0.5 | — | 1.0 | 0.1 | 0.1 |

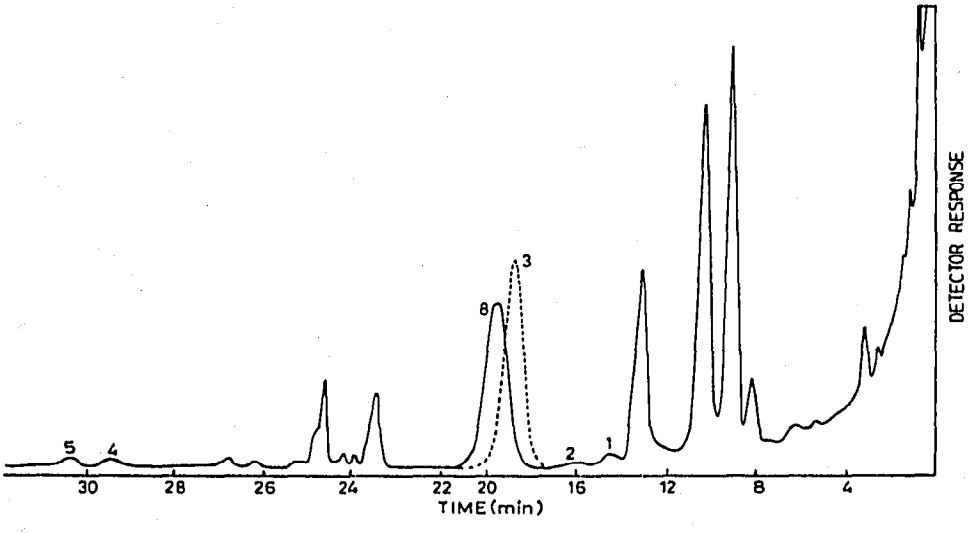


Fig. 5. Chromatogram of residue from ethyl acetate extraction. 3 = EGC standard; 8 = suspected theogallin.

The area of this peak approximated to the combined area of the EGC peaks in the chromatograms of the acetate extracts, thus suggesting that it is not due to EGC. Mass spectral comparison of trapped material corresponding to this peak with the trimethylsilyl derivative of EGC confirmed this (Fig. 6). Thus EGC-TMS shows (as expected) a molecular ion at *m/e* 738, an $(M-CH_3)^+$ ion at 723, and *retro*-Diels–Alder fragments at *m/e* 456 and 283. In contrast, material trapped from acetate-extracted residues at EGC's retention time shows a molecular ion at *m/e* 848, an $(M-CH_3)^+$ ion at 833, and prominent fragments at *m/e* 458, 386, 369, 345 and 255. The first three of this group of fragments are typical of gallate ester fragmentation patterns, and have been observed previously in the mass spectra of TMS derivatives of theaflavin and flavanol gallates[6]. In tea, the most abundant monomeric gallate ester not extracted from water by ethyl acetate is theogallin (3-galloylquinic acid)[7,8]. Its TMS derivative has, like the trapped material, a mol. wt. of 848. The fragments at *m/e* 345 and 255 probably arise from the quinic acid fragment remaining after loss of the gallate group, as outlined in Fig. 6. Smaller fragments at *m/e* 217, 204 and 191 probably arise by ring cleavage, with the possible migration of TMSO groups[9]. Examination of an acetate-

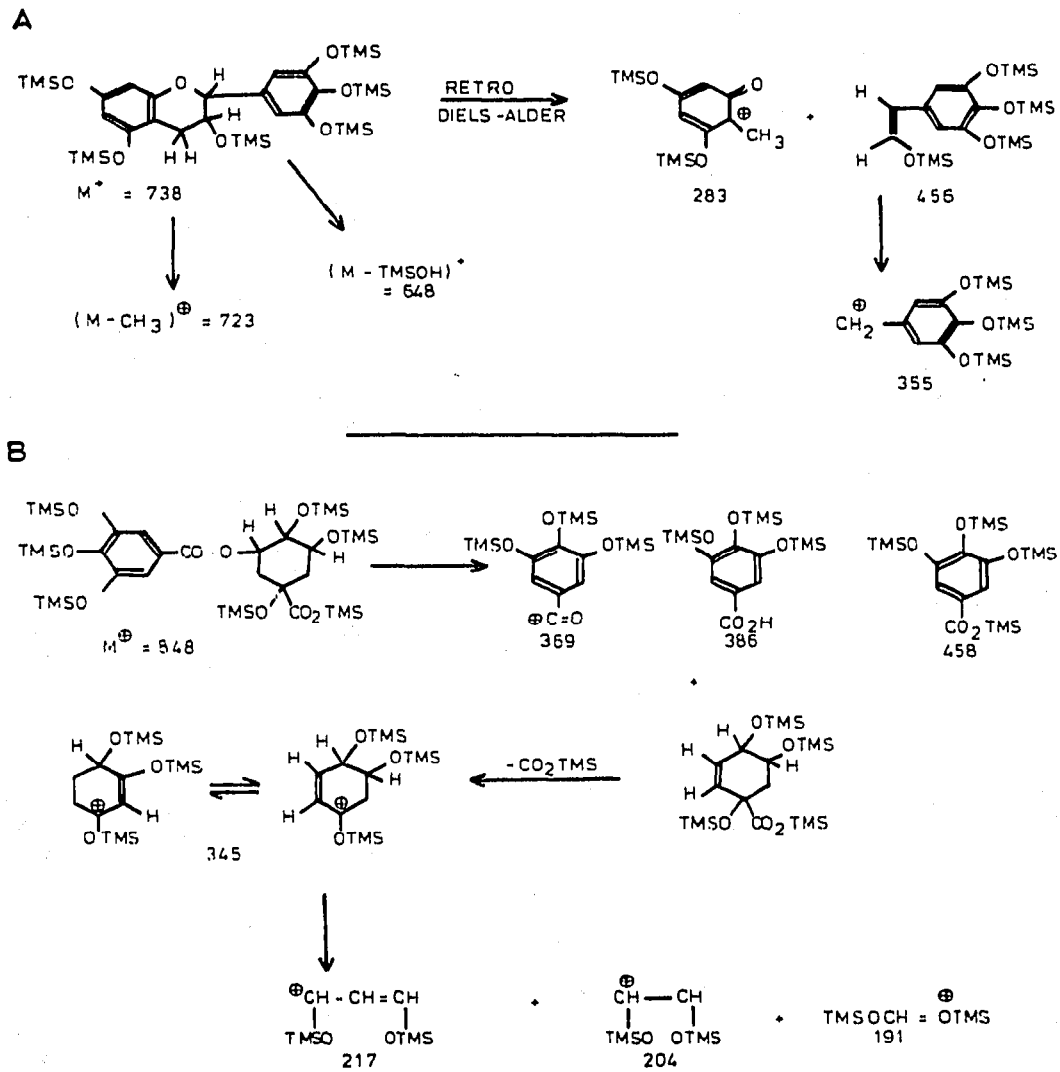


Fig. 6. Fragmentation patterns of TMS derivatives of EGC (A) and theogallin (peak 8, B).

extracted residue obtained from green tea solubles confirmed that it too contains material eluted at EGC's retention time, and that this trapped material is indistinguishable by mass spectrometry from that trapped from black tea. The identification of this species as the TMS derivative of theogallin is at this stage tentative, since insufficient was trapped for NMR spectroscopy, and theogallin itself is neither commercially available nor easily isolated crystalline[8]. Should the identification be confirmed, then GLC of trimethylsilylated acetate-extracted tea solids would provide a convenient method for estimation of theogallin. In view of the partial consumption of theogallin in tea fermentation[10] and its possible role in the formation of high mol. wt. thearubigins[11], such a method would be very useful.

(*4*) *Reproducibility of new flavanol analysis procedure.* We propose that the following standard method be adopted for determining flavanols in black tea solubles.

(a) A dispersion of tea solubles (1 g) in water (25 ml) is extracted with ethyl acetate (4 × 100 ml). The acetate layers are combined, filtered through anhydrous sodium sulphate (wash) and evaporated to dryness under reduced pressure at 30°. The material so obtained is dissolved in redistilled dry pyridine, and the solution made up to 25 ml.

(b) Aliquots of the pyridine solution (2 ml) are injected into septum-sealed tubes containing known weights of quercetin (1–2 mg). BSA (2 ml) and TMCS (0.5 ml) are injected and the mixture allowed to react at room temperature for 30 min.

(c) Aliquots of the reaction mixtures (5 $\mu$l) are injected onto a 5 ft. column of 3 % OV-1 on Diatomite CQ (100–120 mesh) compensated by an identical column and run under the following conditions. Carrier (nitrogen) flow: 60 ml/min. Injection heater temperature: 290°. Temperature programme: isothermal at 235° until emergence of EGC (22 min), then at 48°/min to 310°, isothermal hold until emergence of EGCG. Chart speed: 15 in./h for EC, (+)-catechin and EGC; 30 in./h for quercetin, ECG and EGCG.

To check this method, a sample of black tea solubles was used to provide two pyridine solutions for derivatisation; one prepared from the whole solids, the other from an ethyl acetate extract. Samples of each solution were derivatised under standard conditions with and without added quercetin, and analysed under standard GLC conditions. The results (Table VI) show clearly that omission of the ethyl acetate extraction step leads to serious error in the EGC result, and that the simplicity of the acetate extract chromatograms leads to more reproducible results for EC and (+)-catechin levels.

(*B*) *Analysis of flavanols in unfired tea leaf*

Analysis of flavanols in fired black or green leaf teas (as opposed to dried aqueous extracts) can be conducted using the solids extracted by aqueous pyridine, providing an ethyl acetate extraction step (A3 and 4) is incorporated in the published procedure[5]. To monitor the consumption of flavanols in tea fermentation, the flavanols must be quantitatively extracted from unfired leaf containing active polyphenoloxidase. An extraction technique which also deactivates the enzyme is therefore necessary to ensure that flavanols are not consumed in the extraction step itself. Aqueous pyridine extraction of cryogenically-milled leaf has been used with fresh green leaf[5], but the method is unsuited to dealing with large numbers of samples taken at frequent intervals, nor have results been presented to demonstrate complete

TABLE VI

FLAVANOL LEVELS (%) IN WHOLE BLACK TEA SOLUBLES

| *Derivative* | *EC* | (+)-*Catechin* | *EGC* | *ECG* | *EGCG* |
|---|---|---|---|---|---|
| (*a*) *By analysis of the acetate extract* | | | | | |
| 1[a] | 0.961 | 0.298 | 0.991 | 1.410 | 2.605 |
| 2 | 0.949 | 0.279 | 0.976 | 1.393 | 2.540 |
| 3 | 1.039 | 0.307 | 1.049 | 1.529 | 2.900 |
| Mean | 0.983 | 0.295 | 1.005 | 1.444 | 2.682 |
| Range | ±4 % | ±5 % | ±4 % | ±4 % | ±5 % |
| (*b*) *By analysis of whole solubles* | | | | | |
| 1 | 1.182 | 0.308 | 2.290 | 1.337 | 2.450 |
| 2 | 1.021 | 0.301 | 2.305 | 1.302 | 2.671 |
| 3 | 0.924 | 0.245 | 2.050 | 1.200 | 2.715 |
| Mean | 1.042 | 0.284 | 2.215 | 1.279 | 2.612 |
| Range | ±13 % | ±10 % | ±7 % | ±5 % | ±5% |
| Uncorrected[b] mean values | 1.107 | 0.288 | 2.140 | 1.249 | 2.475 |

[a] Figures quoted are the means from 3 injections.
[b] No correction made for the area of the peak co-incident with that of the quercetin standard.

flavanol recovery. Experiments 1–3 were designed to evaluate both this method and extraction with 80 % aqueous methanol after enzyme deactivation with boiling *n*-propanol (b.p. 97.1°).

(*1*) *Applicability of GLC analysis method to solids extracted with non-aqueous solvents.* The GLC method for flavanol analysis (A4) was developed for water-soluble tea solids, but all the fractions to be examined in this part of the investigation were obtained by extraction with either aqueous pyridine or aqueous alcohols. In view of the fact that even aqueous tea extracts contain species whose TMS derivatives are not resolved from that of quercetin, it was considered desirable to adopt procedures which would check not only for the presence of such species, but also the states of columns and detectors used for GLC flavanol analysis. Two procedures were adopted:

(a) A plot was kept of the weight of added quercetin ($Q_W$) against the quercetin peak area ($Q_A$) for 5 $\mu$l injections of experimental samples.

(b) 5 $\mu$l aliquots of silylated standard mixtures of flavanols and quercetin were injected after each run of ten experimental samples to check detector constants and detector response to quercetin.

Examination of $Q_W/Q_A$ plots for analysis of 80 % aqueous methanol extracts by the standard acetate extraction procedure showed that (Fig. 7)

(i) standard quercetin–flavanol mixtures gave a very good straight line;

(ii) experimental tea samples gave points which invariably fell below the standard line.

These findings imply that the samples contain a species* whose TMS derivative

* The fact that quercetin is more soluble in alcohol and pyridine than in water suggests that the extraction of quercetin naturally present in tea may be responsible for these deviations.

is not resolved from that of quercetin, a point confirmed by chromatography of extracts silylated without added quercetin. Entirely similar results were obtained from pyridine-extracted samples[5]. To correct for these small but experimentally-significant deviations, it is necessary to calculate results using either a corrected quercetin peak area or a corrected quercetin weight read off from the standard line (Fig. 7). An example of the effects of such correction procedures is given in Table VII.

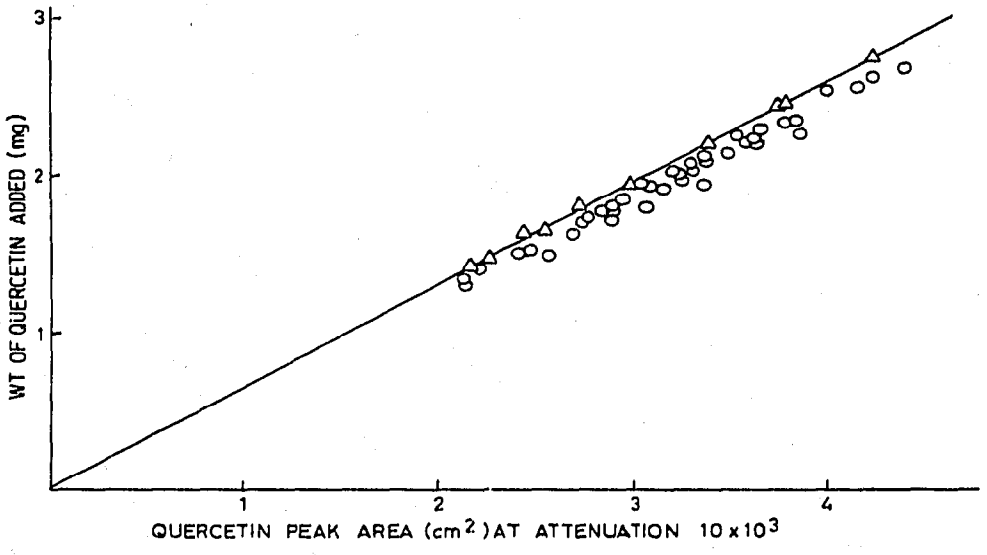


Fig. 7. Quercetin calibration line for analysis of tea solids extracted by aqueous methanol. △, Standard quercetin–flavanol mixtures; ○, quercetin-tea leaf extracts.

TABLE VII

FLAVANOL LEVELS IN 80% AQUEOUS METHANOL EXTRACTS OF GREEN LEAF

| | | *EC* | (+)-*Catechin* | *EGC* | *ECG* | *EGCG* |
|---|---|---|---|---|---|---|
| Uncorrected | A | 2.61 | 0.49 | 9.56 | 5.42 | 19.79 |
| $Q_A$ corrected | A | 2.72 | 0.51 | 9.95 | 5.64 | 20.55 |
| $Q_W$ corrected | A | 2.71 | 0.50 | 9.97 | 5.65 | 20.56 |
| Uncorrected | B | 2.18 | 0.46 | 8.06 | 5.15 | 18.21 |
| $Q_A$ corrected | B | 2.43 | 0.51 | 9.00 | 5.76 | 20.35 |
| $Q_W$ corrected | B | 2.44 | 0.51 | 9.09 | 5.85 | 20.40 |

A preferable solution would be to use a different internal standard, but so far no suitable alternative has been found.

The $Q_W/Q_A$ plots also give warning of fall-off in column or detector performance. If experimental samples give points above the standard quercetin line, either detector response is impaired by fouling with silica, or the TMS derivative of quercetin is being partially decomposed on the column. Such deterioration is less ambiguously revealed by changes in detector constants and by standard flavanol–quercetin mixtures giving points above the original standard quercetin line. In our experience, 5 ft. columns of 3% OV-1 on Diatomite CQ used for analysis of aqueous methanol ex-

tracts of tea leaf have a useful life of 30–40 injections. These checks and corrections were applied to all results reported in this investigation.

(2) *Experiment B1.* Analysis of the aqueous pyridine extract of fresh tea leaf before (1A) and after (1B) ethyl acetate extraction gave the following flavanol compositions for whole leaf solids:

| | *EC* | (+)-*Catechin* | *EGC* | *ECG* | *EGCG* |
|---|---|---|---|---|---|
| 1A | 1.802 | 0.292 | 8.731 | 3.895 | 13.354 |
| 1B | 1.515 | 0.289 | 4.965 | 4.071 | 13.569 |

These results suggest that the whole aqueous pyridine extract (1A) contains species whose TMS derivatives are not resolved from those of EGC and (possibly) EC. Chromatograms of material left in the aqueous layer after acetate extraction (1C) included peaks co-incident with those of EGC (large) and EC (small), thus supporting this view (*cf.* A3).

(3) *Experiment B2.* It is shown that treatment of fresh leaf with cold propanol (2B) does not completely deactivate the tea oxidase (Table VIII). Comparison with results obtained from the aqueous pyridine extract (2A) indicates that substantial amounts of EGC and EGCG are consumed during the extraction of 2B. The low levels of residual flavanols found in the extracted fibres show that four-fold extraction of propanol-treated leaf is more than 99 % efficient. To confirm that no consumption of

TABLE VIII

EXPERIMENT B2

(*a*) *Mass balance in propanol–aqueous methanol extraction*

| | *2B* (*50 g, cold PrOH*) | *2C* (*50 g, hot PrOH*) |
|---|---|---|
| PrOH extract | 3.128 | 3.755 |
| Cold MeOH extract | 1.800 | 1.682 |
| Hot MeOH extract | 0.136 | 0.168 |
| Total extract | 5.064 | 5.605 |
| Oven-dried fibre | 5.200 | 4.971 |
| Total solids | 10.264 | 10.576 |
| % Solids on leaf | 20.528 | 21.152 |
| % Solids on leaf by direct vac. oven drying: | 20.90 | |

(*b*) *Flavanol composition of whole leaf solids and fibre*

| | *EC* | (+)*Catechin* | *EGC* | *ECG* | *EGCG* |
|---|---|---|---|---|---|
| 2A | 1.322 | 0.185 | 4.532 | 3.917 | 10.714 |
| 2B extract | 1.132 | 0.185 | 2.344 | 3.511 | 8.325 |
| 2B fibre | 0.008 | — | 0.049 | 0.013 | 0.087 |
| 2C | 1.421 | 0.189 | 4.469 | 3.884 | 10.902 |
| 2C fibre | 0.006 | — | 0.072 | 0.006 | 0.038 |

flavanols occurs during hot propanol treatment and subsequent extraction, samples of fresh leaf were doped with known amounts of pure flavanols prior to propanol treatment: recovery was invariably better than 95 % (Fig. 8).

(*4*) *Experiment B3.* It is often inconvenient to extract samples immediately after treatment with hot propanol, especially when large numbers of samples are taken at frequent intervals during an experiment. To determine whether propanol-treated leaf samples can be stored overnight without loss of flavanols, two leaf samples (3A and 3B) were sealed in vegetable lacquer-lined cans after treatment with hot propanol, and

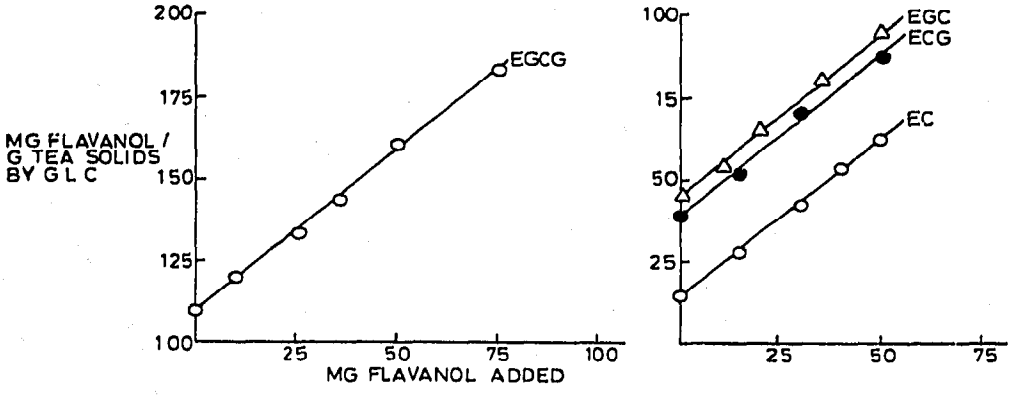


Fig. 8. Recovery of added flavanols in extraction with aqueous methanol. Solid lines, 100 % recovery; points, experimental results.

TABLE IX

EFFECT OF STORAGE ON FLAVANOL COMPOSITION OF PROPANOL-TREATED LEAF

3A, 3B = after storage; 3C, 3D = before storage.

(*a*) *Levels of aqueous methanol extracts (% on whole leaf solids)*

| *3A* | *3B* | *3C* | *3D* |
|---|---|---|---|
| 57.04 | 51.53 | 57.21 | 52.53 |

(*b*) *Flavanol composition of aqueous methanol extract*

| | *EC* | (+)-*Catechin* | *EGC* | *ECG* | *EGCG* | *Total* |
|---|---|---|---|---|---|---|
| 3A | 2.16 | 0.71 | 9.04 | 4.59 | 18.91 | 35.41 |
| 3B | 2.41 | 0.54 | 9.06 | 5.69 | 21.50 | 39.20 |
| 3C | 2.20 | 0.69 | 9.02 | 4.74 | 19.59 | 36.24 |
| 3D | 2.25 | 0.60 | 8.67 | 5.39 | 22.75 | 39.66 |

(*c*) *Flavanol composition of whole leaf solids*

| | *EC* | (+)-*Catechin* | *EGC* | *ECG* | *EGCG* | *Total* |
|---|---|---|---|---|---|---|
| 3A | 1.23 | 0.41 | 5.16 | 2.62 | 10.79 | 20.21 |
| 3B | 1.24 | 0.28 | 4.67 | 2.93 | 11.08 | 20.20 |
| 3C | 1.26 | 0.40 | 5.16 | 2.71 | 11.21 | 20.74 |
| 3D | 1.18 | 0.32 | 4.55 | 2.83 | 11.95 | 20.83 |

compared after 24-h storage at room temperature with duplicate samples (3C and 3D) extracted and analysed immediately after propanol treatment. The results (Table IX) show that flavanol loss during storage is insignificant: further experiments over extended storage periods indicate that flavanol levels remain unchanged even after 1-week's storage.

TABLE X

EFFICIENCY OF ETHYL ACETATE EXTRACTION OF FLAVANOLS FROM AQUEOUS METHANOL SOLUBLES OF FRESH LEAF

| | *EC* | (+)-*Catechin* | *EGC* | *ECG* | *EGCG* | *Total* |
|---|---|---|---|---|---|---|
| (*a*) % *extracted flavanols on aqueous methanol solubles* | | | | | | |
| E1 | 1.720 | 0.328 | 5.503 | 6.602 | 27.79 | 41.943 |
| E2 | 0.255 | 0.045 | 1.681 | 0.480 | 2.553 | 5.014 |
| E3 | 0.035 | — | 0.350 | 0.032 | 0.196 | 0.613 |
| E4 | — | — | 0.082 | — | 0.065 | 0.147 |
| Total | 2.010 | 0.373 | 7.616 | 7.114 | 30.604 | 47.717 |
| (*b*) % *of total extracted at each stage* | | | | | | |
| E1 | 85.57 | 87.94 | 72.26 | 92.8 | 90.81 | 87.90 |
| E2 | 12.69 | 12.06 | 22.07 | 6.75 | 8.34 | 10.51 |
| E3 | 1.74 | — | 4.60 | 0.45 | 0.64 | 1.28 |
| E4 | — | — | 1.07 | — | 0.21 | 0.31 |

(5) *Experiment B4.* The ethyl acetate extraction stage was originally developed for and tested on samples of black tea solubles containing up to 10 % total flavanols. The solids obtained by alcoholic extraction of fresh leaf contain up to 50 % flavanols: it was therefore necessary to check the efficiency of ethyl acetate extraction of flavanols from aqueous dispersions of these richer fractions (*cf.* A3). The four sequential acetate extracts (E1–E4) from the aqueous methanol solubles of propanol-treated fresh leaf were therefore separately analysed for flavanols. The results presented in Table X show that acetate extraction of flavanols from 1 g samples of aqueous methanol solubles is complete after four extractions. This was confirmed by freeze-drying the extracted aqueous solution and analysing the residual solids for flavanols. Even at 50 times the original sensitivity, EC, ECG and EGCG were undetected. However, as previously observed with black tea extracts (A3), there was a large peak in the position expected for EGC. The mass spectrum of the corresponding trapped material suggested it to be theogallin (Fig. 6)[7,8].

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