Phytochemistry, Vol. 22, No. 4, pp. 883–887, 1983.
Printed in Great Britain.

0031–9422/83/040883–05$03.00/0

PRODUCTION AND HPLC ANALYSIS OF BLACK TEA THEAFLAVINS AND THEARUBIGINS DURING *IN VITRO* OXIDATION

ALASTAIR ROBERTSON* and DEREK S. BENDALL

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, U.K.; Tea Research Foundation of Central Africa, P.O. Box 51, Mulanje, Malawi, Central Africa

(*Revised received* 14 *September* 1982)

Key Word Index—*Camellia sinensis*; Theaceae; tea; model fermentation system; HPLC; catechins; theaflavins; thearubigins.

Abstract—A model fermentation system has been designed which utilizes pure catechins and partially purified polyphenol oxidase (EC 1.14.18.1) from green tea shoots. HPLC analysis of the products formed during *in vitro* oxidation has demonstrated a close similarity between this system and *in vivo* oxidation occurring during factory fermentation. Furthermore, changes in theaflavin and thearubigin levels, revealed by time courses of fermentation, show *in vitro* and *in vivo* systems to be qualitatively similar, although the former system produces considerably higher levels of both components. The model fermentation system, therefore, appears to be a suitable experimental system for studying the formation of theaflavin and thearubigin pigments under strictly controlled conditions. In preliminary experiments the theaflavins have been identified on HPLC profiles by enzymic oxidation of the relevant catechin pairs. Similarly, major coloured components other than theaflavins, which are considered to be thearubigins, have been shown to be formed by the oxidation and reaction of two gallocatechins (epigallocatechin and epigallocatechin gallate). The model fermentation system, in conjunction with HPLC as described in this paper, provides a means whereby precise data on theaflavin and thearubigin formation can be obtained and, in the case of the thearubigins, one which could yield additional structural information.

INTRODUCTION

The mechanical maceration of green tea shoots, during black tea manufacture, provides the conditions necessary for the polyphenol oxidase-catalysed oxidation of the green leaf catechins leading to the theaflavin and thearubigin pigments characteristic of black tea. The pigments were named by Roberts [1] and defined according to their respective abilities to partition into the organic and aqueous phases of ethyl acetate–water mixtures [2].

The thearubigin fraction consists of an unstable, possibly polymerizing heterogeneous group of compounds. Their formation, chemical structures and, consequently, their contribution to black tea quality have remained elusive. The theaflavins, on the other hand, are a relatively stable group of compounds, formed by the oxidation and condensation of di- and tri-hydroxylated catechin 'B' rings [3]. The resultant dimers, each containing a benztropolone nucleus, are red in colour and contribute to both the visual brightness and organoleptic astringency [4] of black tea. The theaflavins are, therefore, highly desirable for tea quality [5–7] and, consequently, a considerable amount of research has concentrated on increasing their relative proportions during black tea manufacture.

The accumulation of critical kinetic data on the fermentation (oxidation) process has been impeded by the use of complex macerated tea shoot systems. Results have been difficult to interpret due to the considerable variation in both polyphenol composition and enzyme concentration between different clones, between the same clones under constantly changing growth conditions [8], and even between different parts of the same shoot [9]. Macerated whole shoot systems are also physically heterogeneous, so that not only will air movement and hence oxygenation of the reacting cell contents differ between experiments, but temperature and gaseous measurements cannot be made accurately in a moist particle system.

The use of *in vitro* model fermentation systems has overcome the problems experienced with the heterogeneous tea shoot macerates. However, time of subsequent analysis and/or the large quantities of product required, still presented such difficulties as to make detailed studies using this approach impractical. Of those studies which have utilized such systems, many have involved single catechin oxidations or oxidations of simple mixtures [10, 11]. Others have used catechin and enzyme concentrations considerably different from those occurring physiologically and the results have, therefore, been difficult to interpret on the basis of whole shoot fermentation [12]. All have lacked the necessary reproducible analytical procedure required to produce unambiguous data.

In a recent paper Hoeffler and Coggan [13] reported a method of analysis for the components of black tea using HPLC. The method has been modified and improved for this study and, coupled with an *in vitro* model fermentation system using pure catechins and semi-pure enzyme at concentrations based on the green leaf composition of Malawi teas, a detailed study was carried out to optimize the physical and chemical conditions under which maxi-

*Present address: Department of Chemistry and Biochemistry, Campden Food Preservation Research Association, Chipping Campden, GL55 6LD, U.K.

mum theaflavin levels could be obtained. This paper is the first of a series and describes the model fermentation system, compares its products with those of factory manufactured tea and demonstrates the value of the system with preliminary results which indicate the source of some of the components constituting the thearubigin fraction.

RESULTS AND DISCUSSION

The HPLC elution profile at 375 nm for the oxidation products obtained from the model fermentation system is shown in Fig. 1. Enzymic oxidation of catechin pairs demonstrated peaks 13–16 to be theaflavin, theaflavin 3′-monogallate, 3-monogallate and 3,3′-digallate, respectively. The elution pattern compared favourably with that of Hoeffler and Coggan [13], although improved separation was obtained using Hypersil ODS solid support, and a shorter (20 cm) column. Moreover, these columns were used over a long period without any loss of reproducibility and without any significant reaction of the oxidation products with the column surface, as in the case of μBondapak C_{14}. Columns were repacked occasionally, using the same packing material, when inlet pressures had increased significantly. This was due to the routine use of ordinary grade reagents, since HPLC grade ones were unavailable and blockages usually occurred in the porous Teflon plug at the column inlet. Subsequent use of an in-line precolumn filter reduced column 'plugging' considerably.

Other benztropolone compounds are theoretically possible after the fermentation of a standard catechin mixture. Small amounts of isotheaflavin from condensation of catechin and epigallocatechin quinones were expected and also of epitheaflavic acid, after the release of gallic acid during fermentation. Since the anticipated concentration of these two components was low, little time was spent in characterizing their peaks on the trace. However, peaks 12 or 13a and 11 might well correspond to epitheaflavic acid and isotheaflavin, respectively. Isomers of all the theaflavins mentioned so far are theoretically possible, but this would occur in such low concentrations as to be overlooked. They are expected to chromatograph closely to, or co-chromatograph with, their isomers.

In addition to the benztropolone-based pigments, formed as a result of enzymic catechin oxidation, a number of more polar coloured components were eluted at the solvent front. These were considered to be the thearubigins. However, comparison of the products from a model system with those from a decaffeinated black tea infusion (Fig. 2) showed the presence of additional peaks, 6–10 inclusive, suggesting that the model system was either unable to synthesize all the thearubigins of black tea or that these five components are other than thearubigins. The presence of these peaks in a chromatogram obtained from an infusion of green leaf (Fig. 3) indicated that the latter was the case. Although these five compounds have not been identified it is probable that they are either leucoanthocyanins or flavanol glycosides. In all other respects, apart from the relative amounts of the oxidation products present in the respective systems, the elution profiles presented in Figs. 1 and 2 were similar.

Figure 4 compares the components of a complete standard catechin mixture with components from the oxidation of the gallocatechins singly and in pairs. The acetone concentration of the mobile phase was decreased to 20% for this study in order to resolve further the thearubigin fraction shown in Figs. 1 and 2. Peak 1 in Fig. 1 was, therefore, separated into two peaks (1 and 1a), each of which were formed from the oxidation of epigallocatechin alone. Peaks 2, 3 and 3a were due to the oxidation and combination of epigallocatechin and epigallocatechin gallate. Peaks 4 and 5 of this chromatogram appear to be formed from epigallocatechin gallate alone. The latter also forms a thearubigin component (peak 0) which runs ahead of the solvent front and is, therefore, considerably more polar than the acetone which produces the solvent front peak.

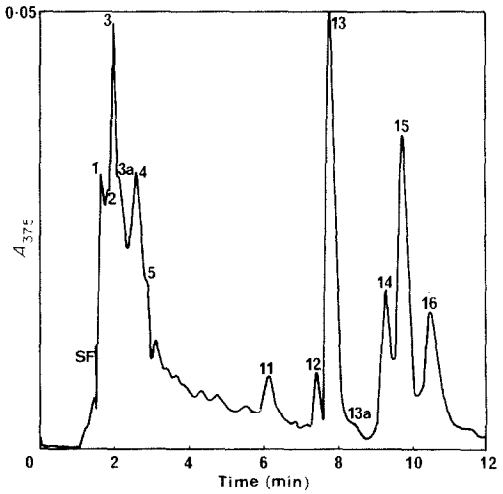

Fig. 1. HPLC elution profile of the products formed after 30 min fermentation of a standard catechin mixture in the model system. Mobile phase: 29% aqueous acetone containing 1% acetic acid; flow rate, 2 ml/min; loading, 5 μl; recorder sensitivity, 0.05 a.u.f.s.; temperature, 20°.

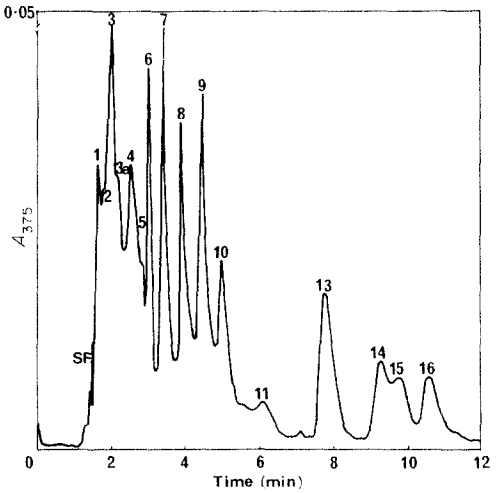

Fig. 2. HPLC elution profile showing the products from an extracted and decaffeinated black tea infusion of Malawi clone SFS 204. Mobile phase: 29% aqueous acetone containing 1% acetic acid; flow rate, 2 ml/min; loading, 10 μl; recorder sensitivity, 0.05 a.u.f.s.; temperature, 20°.

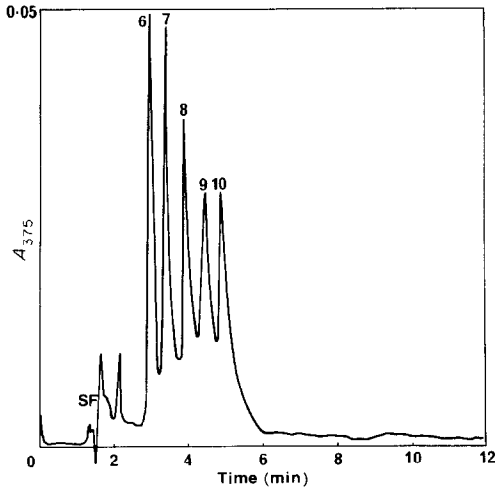

Fig. 3. HPLC elution profile of a green leaf extract from Malawi clone SFS 204. Mobile phase: 29 % aqueous acetone containing 1 % acetic acid; flow rate, 2 ml/min; loading 5 μl; recorder sensitivity 0.05 a.u.f.s.; temperature, 20°.

In Fig. 5, time courses for the formation of total theaflavin and thearubigin fractions, as defined in this paper, are shown for both model system and factory fermentation. Malawi tea clone SFS 204 was chosen for the latter, due to its similarity to the model system in both enzyme and substrate concentrations, and, furthermore, the conditions of fermentation for both systems were similar. In this experiment, samples of LTP* processed leaf were removed at intervals during factory fermentation and the juice extracted by squeezing in a muslin bag. Extract (0.5 ml) was added to an equal volume of 60 % aqueous acetone containing 11 % w/w glacial acid and the precipitate removed by centrifugation at 15 000 *g*. The clarified extract was analysed by HPLC as described in the Experimental.

The similarity of the two time courses is apparent and differs mainly in the quantitative levels of the theaflavins and thearubigins. The high initial levels of the pigments found in the whole leaf fermentation were probably associated with leaf damage during plucking and subsequent handling. As a result of high background theaflavin values, the initial lag phase in theaflavin formation, which is a significant feature of both model and factory fermentations, can often be masked in the latter.

The substantially lower levels of both theaflavin and thearubigin fractions in factory fermented leaf are interesting and most likely reflect the efficiency of a homogeneous aqueous *in vitro* system over the relatively heterogeneous macerated green shoot system, where oxygen diffusion and enzyme and substrate mixing are limited. Complete oxidation of catechins during factory fermentation may occur over an extended time course to produce

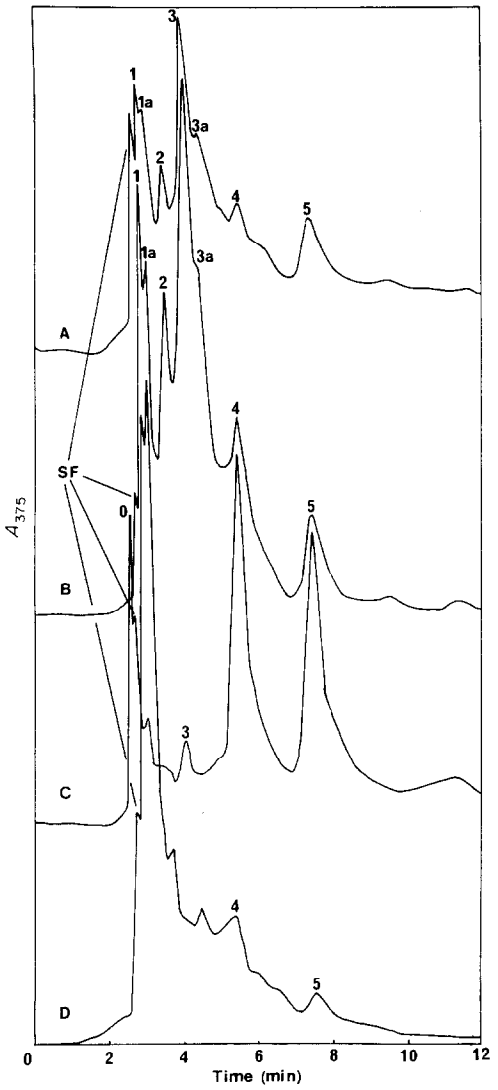

Fig. 4. HPLC separation of thearubigin components. (A) Black tea infusion; (B) enzymic oxidation of epigallocatechin gallate (10 mg/ml) and epigallocatechin (10 mg/ml); (C) enzymic oxidation of epigallocatechin gallate (10 mg/ml); (D) enzymic oxidation of epigallocatechin (10 mg/ml). Mobile phase: 20 % aqueous acetone containing 1 % acetic acid; flow rate, 2 ml/min; loading (A), 10 μl; (B), (C) and (D), 5 μl; recorder sensitivity, 0.05 a.u.f.s.; temperature, 20°.

thearubigin levels similar to, or often greater than, those in a model system. Theaflavin levels, however, continue to fall [14].

Enzyme inactivation, which often occurs in purified systems, was not significantly different in this study to that taking place *in vivo*. The measurement of polyphenol oxidase activity during a 60 min model fermentation demonstrated a decrease of only 10–15 %. Similar and often greater losses in enzyme activity can occur during the fermentation of macerated green leaf [15]. Furthermore, catechin levels remaining after the completion of fermentation were similar to those in manufactured tea.

*LTP (Lowry tea processor). Basically similar to a maize hammer mill. Inner cylinder revolves throwing out large numbers of blunt knives (hammers) under centrifugal force. There is a close tolerance between these and the external casing. Leaves, fed into the top of the LTP, are chopped into fine pieces by the hammers and blown out of the bottom.

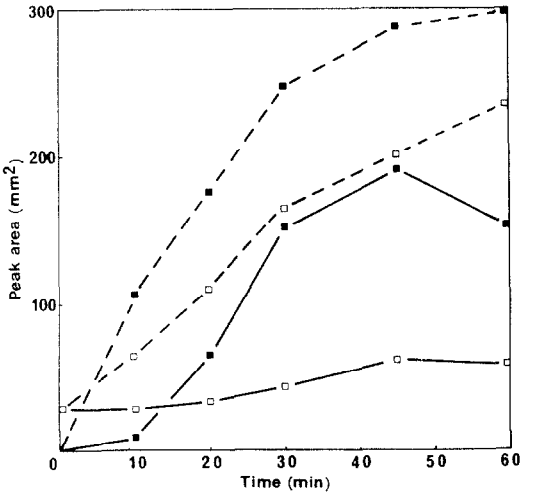

Fig. 5. Time course for the formation of total theaflavin (———) and total thearubigin (----) in a model fermentation system (■) and during factory fermentation of macerated shoots (□). Fermentation conditions: pH 5.6; temperature 25°; Enzyme activity, 30×10^{-8} kat.

The results presented in this paper, therefore, demonstrate that a model system, consisting only of the six major catechins and semi-purified polyphenol oxidase, can essentially simulate the fermentation time course of green leaf to produce all the major pigments of conventional black tea as measured by HPLC. Other enzymes and particularly peroxidase, however, have been reported to affect the levels of the products of fermentation [16]. Since the residual activity of peroxidase in the polyphenol oxidase preparation used in this study was 25% of its initial activity, its effect could be considerable. However, further addition of horseradish peroxidase, hydrogen peroxide, or the addition of considerable amounts of catalase failed to produce any significant change in the levels or ratios of the products formed. It is, therefore, thought that although catechins will act as substrates for peroxidase in low concentrations, their concentrations in green leaf are much too high and peroxidase is possibly inhibited.

The success of the HPLC system as an analytical method for determining and comparing theaflavin levels relies on the fact that the individual theaflavin isomers have similar extinction coefficients at 375 nm [17]. The extinction coefficients of the thearubigins, on the other hand, are unknown and, therefore, similar comparisons between the resolved peaks of these compounds cannot be made. It is, however, considered valid here, due to the heterogeneous nature and large number of compounds contained in the thearubigin class, to compare relative, although arbitrary, increases in total thearubigins with those of theaflavins, as long as the absolute values are not required.

The value of the model system and combined HPLC as a method of studying the products and conditions of black tea fermentation does emerge from this work. The source of some of the thearubigins formed during early fermentation has been demonstrated. The application of these techniques may eventually lead to new structural information on the thearubigins.

EXPERIMENTAL

Model fermentation system. Model fermentations, with a reaction vol. of 1 ml, were carried out routinely in a water-jacketed oxygen electrode, modified to accept a 1 cm diam., 2 ml total vol., glass reaction chamber for improved temp. control. Gases, O_2 and N_2 from cylinders, or air from a diaphragm pump, were introduced to the reaction mixture through a porosity 1 scintered glass bubbler. The gas flow was determined by the reaction chamber dimensions and regulated by low-vol., needle-valve flow meters at 1 l/hr. Resultant frothing and possible surface denaturation of subsequently added enzyme were prevented by the addition of 1 μl octan-2-ol. Once the desired oxygen concn was constant, the reaction was initiated by the addition of polyphenol oxidase and gaseous exchange monitored throughout the expt. The reaction was terminated by removal of the reaction mixture from the electrode and introducing it into an equal vol. of 60% aq. Me_2CO containing 11% HOAc. The ppt. was removed by centrifugation at 15 000 *g* at 4° for 5 min and the supernatant analysed using HPLC.

Standard catechin mixture. Unless otherwise stated, all model fermentations were carried out using a standard catechin mixture containing: epigallocatechin gallate, 75 mM; epigallocatechin, 46 mM; epicatechin gallate, 19 mM; epicatechin, 10 mM; gallocatechin, 6 mM; and catechin, 3 mM. This was based on the average green leaf catechin composition of 12 Malawi clonal teas measured on six occasions distributed evenly throughout the year and adjusted to represent concns after 70% wither. Catechin mixtures were dissolved in 0.1 M citrate–Pi buffer, pH 5.6.

Analysis of black tea components by HPLC. The method used here was based on that of Hoeffler and Coggan [13]. Their use of μBondapak C_{14} reversed phase packing (Waters Associates) was replaced by 5 μHypersil ODS (Shandon). Columns were packed by a high pressure descending slurry packing technique, using an extended tube apparatus (Magnus Scientific). A 10% slurry of packing material in Me_2CO was driven into blank tubes (20 × 0.4 i.d.) with polished int. surfaces (Apollo lc tubing) at pressures of 5000–5500 psi. The column outlet was terminated by two 5 μm stainless gauzes contained in a Parker reducing union of zero-dead-vol. construction. After packing was completed, 3 mm was removed from the column surface and replaced by a porous Teflon plug. A hole 1–1.5 mm depth was drilled into the centre of the plug which, after the fitting of a second Parker reducing union to the inlet end of the column, accommodated the inlet tube. This ensured introduction of the sample close to the column surface and, hence, maximum efficiency.

Analytical columns were connected into a solvent pumping system (Magnus SPS 2296-66), equipped with a Rheodyne 7120 injection valve. Columns were equilibrated directly, using the eventual mobile phase, consisting of 29% (v/v) aq. Me_2CO containing 1% HOAc, at a flow rate of 1 ml/min until all air was removed and a back pres. below 2000 psi was obtained.

Samples of 2–15 μl were injected onto the column and eluted using the mobile phase at a flow rate of 2 ml/min. After initially switching on the system, the first sample of a batch was repeated until the chromatogram was reproducible. This was not carried out because of poor quantitative reproducibility but to gain maximum separation of the theaflavin monogallates.

Preparation of black tea infusions for HPLC analysis. Black tea (18 g) was infused in 375 ml boiling H_2O for 6 min and allowed to cool at 4°. The resultant liquor, containing the tea 'cream', was shaken twice with an equal vol. of $CHCl_3$ to remove the caffeine. The aq. phase was removed and centrifuged at 15 000 *g* at 4° for 10 min and the resultant supernatant used for HPLC analysis.

Preparation of green leaf extract. Freshly plucked green tea leaves (10 g) were ground in a precooled mortar with 20 ml ice-

cold Me_2CO. The resultant macerate was centrifuged at 20 000 *g* for 10 min. The pellet was discarded and the extract used immediately for direct injection into the HPLC column.

Preparation of polyphenol oxidase (*EC* 1.14.18.1). Green tea leaves (250 g), previously withered for 12 hr, were macerated for 4 min in a blender (Waring, 1 gallon), cooled to $-20°$, containing 1200 ml buffer consisting of Na_2HPO_4 (0.05 M), $NaOS_2$ (0.01 M), BSA (0.5 mg/ml), Tween 80 (5%) and NaCl (1 M), pH 7.0. PVP (10% w/v) was added prior to blending. The macerate was squeezed through four layers of muslin and Me_2CO ($-20°$) added slowly to the filtrate, with stirring, to a final concn of 70%. Stirring was continued for 10 min and the ppt allowed to settle under gravity. The supernatant was decanted and the ppt then centrifuged at 6000 g. The pellet was washed twice in 70% Me_2CO and, after being centrifuged at 17 000 *g* was resuspended in 250 ml dilute buffer, containing Na_2HPO_4 (0.02 M) and citric acid (0.01 M), pH 5.6. The ppt was centrifuged at 28 000 *g*, the pellet discarded and the supernatant pumped through a DE-52 ion exchange column (4 × 2.5 cm), equilibrated with the latter buffer. The eluate was collected and Me_2CO added to 30% satn. The pellet was discarded after centrifugation at 28 000 *g* and the Me_2CO concn of this resultant supernatant increased to 65%. The protein ppt was removed by centrifugation, as above, and the pellet dissolved in H_2O, freeze-dried and stored at $-20°$. The freeze-dried powder was redissolved in 0.1 M citrate–Pi buffer, pH 5.6, to give an activity of 36×10^{-4} kat/ml, as measured polarographically by the oxidation of pyrogallol [19], and used in the model system. The enzyme contained peroxidase activity as measured by the method of Gregory [20]. Units of activity are in kats and defined as the amount of activity required to convert 1 mol of substrate/sec.

Preparation of pure catechins. A crude catechin mixture was prepared by the method of Vuataz *et al* [18]. The use of air-dried leaves required an additional purification step, using wet Et_2O and charcoal. This was omitted when using fresh green leaves. Subsequent separation and purification of the mixed catechins was carried out by a modification of the method of Sant [21]. Freeze-dried crude catechin mixture, prepared from 150 g green tea leaves was dissolved in 10 ml MeOH. An equal vol. $CHCl_3$–petrol (1:1) was then added to give a final $CHCl_3$–MeOH–petrol ratio of 1:2:1. The soln was loaded onto a Sephadex LH-20 column (5 × 95 cm), previously equilibrated in $CHCl_3$–MeOH–petrol (1:2:1) and the eluate collected in 25 ml fractions. Catechin and epicatechin remained unseparated and were eluted between 750 and 900 ml. The yield of these catechins was low and, consequently, commercially available samples were obtained (Sigma Chemical Co.). Gallocatechin and epigallocatechin were also unseparated and eluted between 950 and 1150 ml. The ratio of these two catechins (as determined by HPLC) was the same as that required for the model system and, consequently, no attempts were made to purify them further. Epicatechin gallate eluted pure between 1250 and 1500 ml and epigallocatechin gallate between 1600 and 3000 ml. The latter two catechins were recrystallized by the method of Vuataz *et al.* [18]. All the catechins were chromatographically pure as determined by HPLC.

Acknowledgements—This work forms part of a Post Doctoral Fellowship supported by the Tropical Products Institute and carried out at the University of Cambridge and the Tea Research Foundation of Central Africa. Many thanks are due to Mr. P. Cawsey, Dr. G. Howard, Mr. J. Dougan and Mr. J. Johnson of the Tropical Products Institute for their time and experience. A special note of thanks is extended to Dr. R. T. Ellis, not only for the provision of excellent laboratory facilities at the Tea Research Foundation of Central Africa but also for financial aid during the time spent in Malawi, and for his continued interest in the work.

REFERENCES

1. Roberts, E. A. H. (1958) *J. Sci. Food Agric.* **9**, 212.
2. Brown, A. G., Fulshaw, C. P., Haslam, E., Holmes, A. and Ollis, W. D. (1966) *Tetrahedron Letters* **11**, 1193.
3. Bradfield, A. E. and Penney, M., (1944) *J. Soc. Chem. Ind., London* **43**, 306.
4. Wood, D. J. and Roberts, E. A. H. (1964) *J. Sci. Food Agric.* **15**, 19.
5. Roberts, E. A. H. and Smith, R. F. (1961) *Analyst* **86**, 94.
6. Roberts, E. A. H. and Smith, R. F. (1963) *J. Sci. Food Agric.* **24**, 819.
7. Hilton, P. J. and Ellis, R. T. (1972) *J. Sci. Food Agric.* **23**, 227.
8. Hilton, P. J. and Palmer-Jones, R. (1973) *J. Sci. Food Agric.* **24**, 183.
9. Forrest, G. I. and Bendall, D. S. (1969) *Biochem J.* **113**, 741.
10. Roberts, E. A. H. and Myers, M. (1959) *J. Sci. Food Agric.* **10**, 167.
11. Nakagawa, M. and Torii, H. (1965) *Agric. Biol. Chem.* **29**, 278.
12. Hilton, P. J. (1972) *Phytochemistry* **11**, 1243.
13. Hoeffler, A. C. and Coggan, P. (1976) *J. Chromatogr.* **129**, 460.
14. Robertson, A. (1983) *Phytochemistry* **22**, 889.
15. Cloughley, J. B. (1980) *J. Sci. Food. Agric.* **31**, 920.
16. Millin, D. J., Crispin, D. J. and Swaine, D. (1969) *J. Agric. Food Chem.* **17**, 717.
17. Collier, P. D., Bryce, T., Mallows, R., Thomas, P. E., Frost, D. J., Korver, O. and Wilkins, C. K. (1973) *Tetrahedron* **20**, 125.
18. Vuataz, L., Brandenberger, H. and Egli, R. (1959) *J. Chromatogr.* **2**, 173.
19. Gregory, R. P. F. and Bendall, D. S. (1966) *Biochem. J.* **101**, 569.
20. Gregory, R. P. F. (1966) *Biochem. J.* **101**, 582.
21. Sant, D. (1973) PhD thesis, University of Sheffield, U.K.