Isolation of Black Tea Pigments Using High-Speed Countercurrent Chromatography and Studies on Properties of Black Tea Polymers

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Isolation of theaflavins and epitheaflavic acids from black tea using high-speed countercurrent chromatography (HSCCC) on a preparative scale is demonstrated. HSCCC also enabled the isolation of a polymeric fraction from black tea. According to Roberts' classification, the polymeric fraction mainly consisted of SII thearubigins (TR). HPLC analysis showed that the isolated material is free of any known chromatographically resolved tea constituents and eluted from reversed-phase packings as a convex "hump" (a broad signal). The antioxidant activity of the TR fraction was 3.6 mmol of Trolox equivalents per gram. The total phenolic content of this fraction was determined to be 34.7 g/100 g (as gallic acid equivalents).

Keywords: *Black tea; polymers; thearubigins; theaflavin; epitheaflavic acid; color; high-speed countercurrent chromatography; HSCCC; TEAC values; Folin−Ciocalteu; polyphenols; flavonoids; antioxidants*

INTRODUCTION

Whereas green tea is treated by steam or dry heat to prevent enzymatic oxidation of flavanols (catechins), black tea has undergone a fermentation process inducing flavanol oxidation. This process is initiated by the preferred oxidation of gallocatechin, epigallocatechin, and epigallocatechin gallate catalyzed by the enzyme polyphenol oxidase (PPO) (Balentine et al., 1997). Colorless catechins form a variety of pigments during this process and, as a result, the color of the leaves changes to red-brown. Furthermore, the astringency is reduced. Thearubigin-like substances were shown to be formed in model reactions of individual flavanols (Opie et al., 1995) by a PPO-mediated fermentation. A group of compounds formed during tea fermentation, the so-called theaflavins (TF), exhibit a bright orange-red color in solution. This is a group of well-defined, well-characterized compounds, and numerous HPLC methods for the analysis of TF exist (Anan et al., 1988; Steinhaus and Engelhardt, 1989; Shao et al., 1995). Oxidative coupling of the aromatic B rings of two flavan-3-ol molecules leads to the formation of TF. A similar reaction between a flavanol molecule and gallic acid yields theaflavic acids (Davis et al., 1995). TF and related compounds have been intensively studied using NMR techniques, revealing a benzotropolone functionality in the molecule (Davis et al., 1995). Quantitative data for TF in a wide range of different black teas were reported by Lapczynski (2000). Total TF contents seldom exceed 2.5%, but TF are nevertheless believed to make important contributions to the "brightness" of tea infusions, which is an important marker for the evaluation of tea quality (Balentine et al., 1997). Isolation of TF has been, so far, based on chromatography on lipophilic Sephadex LH-20 and (semi-) preparative HPLC (Davis et al., 1995). The all-liquid chromatographic technique of high-speed countercurrent chromatography (HSCCC) was recently applied to the isolation of polyphenols from tea on a preparative scale (Degenhardt et al., 2000). The theory and operation principle of HSCCC are described in detail by Ito (1986).

Thearubigins (TR) is the name originally assigned by Roberts (1958) to all acidic brown pigments of black tea. Roberts (1958) classified this heterogeneous class of compounds into SII, SI, and SIa thearubigins. SI TR are extractable into ethyl acetate, whereas those remaining in the aqueous phase are named the SIa and SII TR, respectively, with the SIa group being more soluble in diethyl ether. TR chromatographed on paper streak or remained immobile in aqueous acetic acid (Roberts et al., 1957). Separation of TR was attempted on Sephadex LH-20 (Cattell and Nursten, 1977). Moreover, TR elute as a Gaussian "hump" from reversed-phase HPLC packings together with more or less resolved tea polyphenols (Bailey et al., 1991, 1994a,b; Bruschi et al., 1999). A classification, which takes the chromatographic behavior of TR into account, was published by Bailey et al. (1991). TR were classified into three groups, group I being excluded from HPLC columns, group II being resolved, and group III remaining unresolved. Because TR are a heterogeneous class of compounds, no exact method for quantification is available. Roberts and Smith (1963) developed a method to quantify TR based on spectroscopic measurement at 380 nm. This method overestimates the content of TR because flavonol *O*-glycosides contribute to the absorption at 380 nm. Bruschi et al. (1999) evaluated the feasibility using the peak area under the hump for quantification purposes but found unexpected hyperchromic shifts of the UV-active phenolic material in the presence of monomeric flavonoids. Common practice is also an indirect determination of TR: a Folin−Ciocalteu (Singleton and Rossi, 1965) assay gives the content of total phenolics. TR are determined by subtraction of monomeric compounds, that is, flavonoids (flavonol

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10.1021/jf000757+ CCC: $19.00
Published on Web 10/06/2000

O-glycosides, catechins, TF, flavone *C*-glycosides; each determined by HPLC using a separate method) and nonflavonoids (gallic acid, theogallin, and chlorogenic acids, also determined by HPLC), from total phenolics (assayed by using the Folin−Ciocalteu method). One main drawback of this approach is the fact that no response factors of TR to the Folin−Ciocalteu reagent are available. Moreover, compounds such as proanthocyanidins are not included. Hence, all quantifications using this indirect approach may introduce some error.

As for the isolation of TR, Roberts et al. (1957) devised a solvent fractionation procedure, which requires extensive workup. Bailey et al. (1992) developed a method employing chromatography on Solka-Floc cellulose and named the isolated fraction theafulvins. Published chromatograms of Bailey's theafulvins show a convex broad band with some peaks floating on top. However, theafulvins isolated by Solka-Floc cellulose proved to be free of flavonol *O*-glycosides, proteins, and caffeine. Fractionation on Sep-Pak C_{18} cartridges as well as cation-exchange cartridges resulted in the isolation of polymeric fractions from tea (Bailey et al., 1994a). Precipitation with caffeine was found to be effective in the isolation of a "hump" fraction from black tea (Powell et al., 1992). A partial resolution of caffeine-precipitable TR with size exclusion HPLC was published by Clifford and Powell (1996). Wedzicha and Donovan (1989) used normal-phase HPLC and partially separated a derivatized black tea polymeric fraction.

The structural characteristics of TR remain unknown. Bailey et al. (1992, 1994a,b) shed some light on TR composition using plasmaspray and thermospray MS together with NMR studies and suggested that theafulvins are chemically different from proanthocyanidin polymers and could consist of B-ring-linked galloylated flavanol monomers. A different study (Ozawa et al., 1996), using Toyopearl HW-40F column chromatography and chemical degradation of isolated fractions, found that in addition to C4−C8 or C6 interflavonoid linkages, C6′−C6′ (B-ring) linkages are also present. The presence of benzotropolone and *o*-quinone structures of TR could not be confirmed. The elucidation of chromophores in TR remains a challenge.

Many approaches using column chromatography struggled with massive interaction of TR with solid supports. It is widely believed that TR have a great affinity to many solid supports used in column chromatography. This made us think about the use of HSCCC for the isolation of TR. HSCCC is a support-free technique that is based on the partitioning of solutes between two immiscible liquid phases. Hence, possible adsorption on solid supports is eliminated. Wedzicha et al. (1990) used HSCCC to isolate TR fractions from black tea. Results of the application of HSCCC to the isolation of TR and studies on the properties of black tea polymers are presented below. Moreover, this paper deals with the isolation of TF and epitheaflavic acids by HSCCC.

MATERIALS AND METHODS

Tea. A commercial black tea (Sumatra black tea) was used.

Isolation of TF. Twenty grams of black tea was extracted for 10 min with 1000 mL of boiling water. After cooling to ~45 °C, the aqueous infusion was extracted with 2 × 500 mL of ethyl acetate. The solvent was evaporated in vacuo and lyophilized. The yield was 2.2 g of a brown-red powder. The lyophilysate was dissolved in ethanol and applied on the top of a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column (70 cm × 8 cm i.d.). The column was eluted with ethanol and 5% acetone in ethanol (v/v) to elute flavanols (Davis et al., 1995). The elution of TF was carried out with 10% acetone in ethanol (v/v), and all brown-red fractions were collected, pooled, evaporated to dryness, and freeze-dried. Solvent systems for HSCCC separation of TF: hexane/ethyl acetate/methanol/water (2:5:2:5 and 1.5:5:1.5:5; less dense layer as stationary phase) was used. Flow rate was set at 2.8 mL/min.

Isolation of TR. Black tea (2.5 g/100 mL of water) was infused with boiling water for 5 min. This solution was cleaned-up on an Amberlite XAD-7 column (50 cm × 4 cm, Fluka Chemie, Buchs, Switzerland). The column was washed with 2 L of water, and elution of phenolics was carried out with 700 mL of methanol. The methanolic eluate was concentrated in vacuo and freeze-dried. The lyophilysate was separated by HSCCC using ethyl acetate/*n*-butanol/water (2:3:5) in the head-to-tail elution mode with the organic layer as stationary phase. Flow rate was set at 2.8 mL/min.

HSCCC. A high-speed model CCC-1000 manufactured by Pharma-Tech Research Corp. (Baltimore, MD) was used. Briefly, it consisted of three preparative coils, connected in series (total volume = 850 mL). The separations were run at a revolution speed of 1000 rpm. The elution mode was head-to-tail with the less dense layer as the stationary phase. The flow rate was set at 2.8 mL/min and delivered by a Biotronik HPLC pump BT 3020. Lyophilysates were dissolved in a 1:1 mixture of light and heavy phase and injected into the system by loop injection. The amount of sample injected varied from 300 mg to 2 g. Stationary phase retention was in the range of 53−75%. Ten milliliter fractions were collected with a Pharmacia LKB Super Frac fraction collector. Elution was monitored with a Knauer UV−vis detector at 280 nm; chromatograms were recorded on a Knauer L 250 E plotter and digitalized using a scanner.

HPLC with Diode Array Detection (HPLC-DAD). A Beckman System Gold programmable solvent module 126, equipped with a Beckman autosampler 502 and a diode array detector module 168, was used. Peak detection was carried out at 280 and 350 nm. The chromatographic separation was performed on a Nucleosil RP18 column (5 μm, 150 mm × 4.6 mm) from Phenomenex (Aschaffenburg, Germany) at ambient temperature. The mobile phase was a linear gradient of 9% acetonitrile in 2% aqueous acetic acid (v/v/v, solvent A) and 80% aqueous acetonitrile (v/v; solvent B). Conditions: initial, 100% A, 0% B; isocratic at 100% A, 0% B for 15 min; in 20 min to 68% A, 32% B; isocratic at 68% A, 32% B for 5 min; back to initial conditions in 5 min; flow rate, 0.8 mL/min.

Proton Magnetic Resonance Spectroscopy (^{1}H NMR). All spectra of theaflavins and related compounds were obtained on a Bruker AMX 300 spectrometer (300 MHz). Spectra were recorded in CD_3OD or acetone-d_6. Assignments were made on the basis of spectral data published by Collier et al. (1973), Davis et al. (1995), and Lewis et al. (1998).

Electrospray Ionization Ion Trap Multiple Mass Spectrometry (ESI-MS/MS). Bruker Esquire-LC-MS/MS with electrospray ionization in the negative mode was used. The drying gas used to evaporate the solvents in the spray chamber was nitrogen with a gas flow of 4 L/min (350 °C); the nebulizer was set at 10 psi. The parameters were as follows: capillary, 3000 V; end plate, 2500 V; capillary exit, −120 V; skim 1, −40 V; skim 2, −8 V. MS/MS experiments were performed with different fragmentation amplitudes.

Trolox Equivalent Antioxidant Capacity (TEAC) Test [According to Miller et al. (1993)]. Metmyoglobin was prepared by oxidation of commercial myoglobin by potassium ferricyanide and purified prior to use on a Sephadex G-15-120 column. The concentration of metmyoglobin was determined spectrophotometrically, and the solution was diluted to a concentration of 25 μmol/L. For calibration of the assay, Trolox standard was diluted to final concentrations of 0.05, 0.1, 0.15, and 0.2 mmol/L, respectively. One hundred microliters of water for the blank, standard, or sample solutions, respectively, was pipetted into semimicrocuvettes containing 800 μL of phosphate buffer (pH 7.4), 600 μL of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; 300 μmol/L),

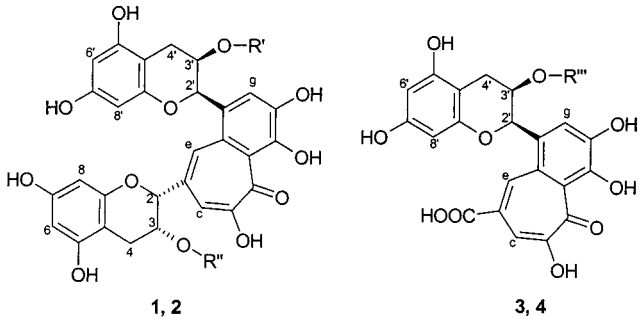

Figure 1. Structures of isolated theaflavins and epitheaflavic acids: **1**, theaflavin (R′, R″ = H); **2**, mixture of two theaflavin monogallates (R′ or R″ = gallate, H); **3**, epitheaflavic acid (R‴ = H); **4**, epitheaflavic acid 3′-monogallate (R‴ = gallate).

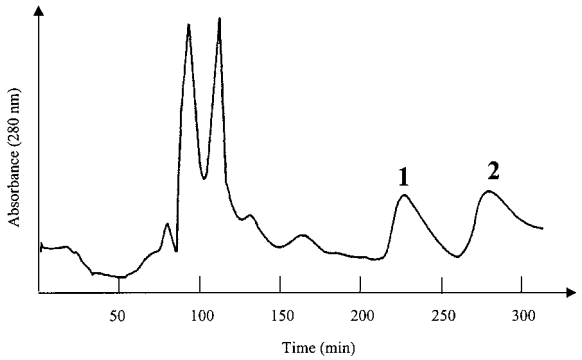

Figure 2. HSCCC separation of theaflavin **1** and theaflavin monogallates **2** using hexane/ethyl acetate/methanol/water (1.5:5:1.5:5; less dense layer as stationary phase).

and 200 μL of metmyoglobin. After mixing, the reaction was initiated by the addition of 300 μL of hydrogen peroxide (0.5 mmol/L). The absorbance at 734 nm was measured exactly after 6 min. Measured values were compared to the results obtained for Trolox standards, which were plotted as a calibration curve.

Folin—Ciocalteu Assay (Singleton and Rossi, 1965). A standard stock solution of gallic acid (Riedel-de Haën, Seelze, Germany) with 1 mg/mL was prepared. A working solution was prepared by dilution of the stock solution: 1 mL with 9 mL of water (= 100 μg/mL). Sample solutions were typically at ~10 mg/mL, and 0.2 mL were diluted with 9.8 mL of water. A calibration curve was measured: 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mL of working solution were pipetted in 10 mL flasks and made up with water to 5 mL. Five milliliters of the diluted sample solution was also pipetted into a 10 mL flask. To all solutions were added 0.2 mL of Folin−Ciocalteu reagent (Fluka Biochemika, Buchs, Switzerland) and 0.5 mL of saturated Na_2CO_3 solution, the volume was made up to the mark with water, and the flask contents were shaken and allowed to stand for 45 min. The absorbance at 725 nm was measured.

RESULTS AND DISCUSSION

Purification of Theaflavins. HSCCC separation of TF **1** and TF-monogallate **2** (for structures see Figure 1) is shown in Figure 2. HSCCC separation was carried out with hexane/ethyl acetate/methanol/water (1.5:5:1.5:5, less dense layer as stationary phase). TF **1** is of good purity, whereas TF **2** was a mixture of the two isomers TF-3-gallate and TF-3′-gallate. HPLC analysis of **2** revealed the presence of two peaks with a UV maximum at 375 nm and the same molecular weight (716 Da as determined by ESI-MS). NMR data show *cis*-2,3 configuration (epicatechin-like; $J_{2,3}$, $J_{2',3'}$ < 1.0 Hz) and a downfield shift of protons H 3 and H 3′ in the case of **2**

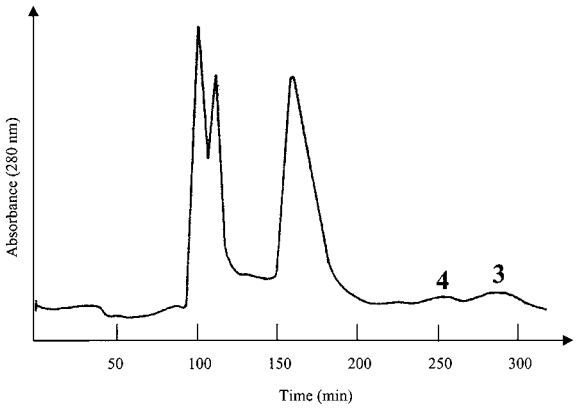

Figure 3. HSCCC separation of epitheaflavic acid **3** and epitheaflavic acid 3′-gallate **4** using hexane/ethyl acetate/methanol/water (2:5:2:5; less dense layer as stationary phase).

shows the presence of both the theaflavin 3-gallate and the 3′-gallate.

Epitheaflavic acid and its 3′-monogallate (for structures see Figure 1) proved to be slightly more hydrophobic than TF and TF-monogallates. An optimized solvent system for the separation of **3** and **4** (cf. Figure 3) is hexane/ethyl acetate/methanol/water (2:5:2:5, less dense layer acting as stationary phase). NMR and MS data of all isolated fractions were in line with published data (Collier et al., 1973; Davis et al., 1995; Lewis et al., 1998; Kiehne, 1997).

Although TF elute after catechins on RP18 HPLC columns, suggesting stronger interaction with C18 chains and therefore greater hydrophobicity, the chromatographic behaviors in HSCCC are identical. The same solvent systems can be used in HSCCC for the separation of catechins and TF, but coelution would occur. For this reason, a cleanup of the black tea infusion on the Sephadex LH-20 prior to HSCCC is necessary.

The maximum sample load of HSCCC with an 850 mL coil volume is ~2−5 g (depending on sample). This sample load can be exploited to purify TF on a preparative scale and will lead to pure compounds on a scale of up to several hundred milligrams (depending on the TF content of the freeze-dried starting material). CCC instruments with larger volumes of liquid phases will become available soon and could be used for the isolation of large quantities of TF because CCC can be predictably scaled up from laboratory to process scale (Sutherland et al., 1998).

Isolation of a TR Fraction from Black Tea. Various methods for TR isolation have been published, but some of them do not produce very constant results (Bruschi et al., 1999). This may be due to the interaction of TR with various solid supports. This made us think about the use of HSCCC for the isolation of TR. One study, published in 1990 (Wedzicha et al., 1990), dealt with the isolation of TR by CCC using ethyl acetate/*n*-butanol/water solvent systems. Analysis of isolated polymeric fractions was done only by paper chromatography.

We started our investigation with the polar solvent system ethyl acetate/*n*-butanol/water (2:3:5) and separated a black tea extract using the organic phase as stationary phase. TR elute with and shortly after the solvent front (cf. Figure 4), whereas all monomers (flavonol glycosides, TF, catechins, and caffeine) remain in the organic stationary phase on the coil. Using this

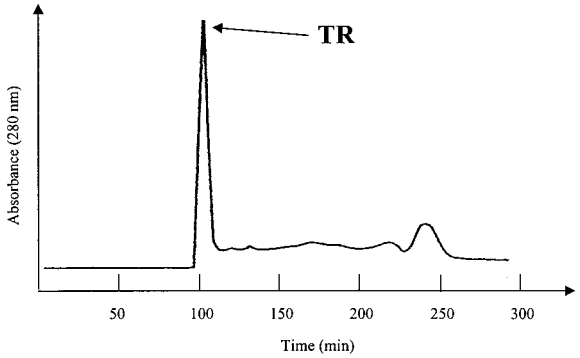

Figure 4. HSCCC separation of a black tea extract for the isolation of polymeric TR fraction using ethyl acetate/*n*-butanol/water (2:3:5; less dense layer as stationary phase).

solvent system, a fractionation into more hydrophilic TR and more hydrophobic monomers is possible. HPLC analysis of the early eluting HSCCC TR fraction showed a broad convex "hump". Analysis of this TR fraction on a polyamide (PA) column showed that a large proportion of this fraction was not absorbed on the PA material (polyamide has a great affinity for polyphenols and should absorb TR). Both ninhydrin and Fehling reactions of the eluate were positive. This means that a large proportion of the early eluting TR in HSCCC probably consisted of proteins and polysaccharides, compounds which are expected to elute in early fractions using these conditions because of their great hydrophilicity. A Folin−Ciocalteu assay of the aqueous PA eluate also showed very little response. According to Jones et al. (1996), tea extracts consist of 30.5% polyphenols, 7.1% caffeine, 26.4% carbohydrates, 12.1% minerals, and 9.6% proteins. A cleanup step prior to HSCCC separation was consequently developed. Amberlite XAD-7 resin turned out to be the material of choice. A black tea infusion was applied to XAD-7 material; all phenolic compounds were retained on the resin, and all nonphenolic material could be removed by rinsing with water. Elution of phenolics can be achieved with methanol. A mass balance (gravimetric analysis) of the methanolic eluate from the XAD-7 column showed that ~35% of the material applied can be found in the methanolic eluate. This correlates with literature data of the sum of polyphenols and caffeine (Jones et al., 1996). HPLC analysis showed that the methanolic eluate contained all UV-active phenolic compounds (chromatogram not shown), whereas the aqueous eluate contained no UV-active phenolic compounds.

A purified black tea extract by XAD-7 was again separated by HSCCC using the ethyl acetate/*n*-butanol/water (2:3:5) solvent system. The early eluting TR fraction was again applied to a polyamide column, and this time only 7.5% of the applied material was not retained on the PA column. Therefore, an enrichment of polyphenols was carried out by cleanup on XAD-7 resin. It is demonstrated by HPLC (chromatograms shown in Figure 5) that the black tea extract (left side) prior to HSCCC separation contains the whole range of flavonoids, whereas after HSCCC separation the UV trace at 280 nm (right side) shows a broad, convex Gaussian hump which is free of all monomers, and no peaks were seen floating on top of the hump. Two smaller peaks shortly after the dead volume of the HPLC column can be seen. These peaks may represent TR that are excluded from the column [group I TR according to the classification of Bailey et al. (1991)].

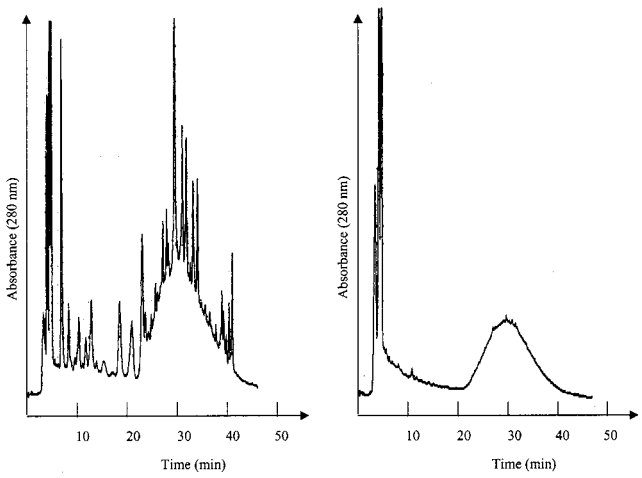

Figure 5. HPLC separation of black tea extract (left side) and HSCCC TR fraction from an XAD-7 cleaned-up black tea extract (right side).

The TR fraction represented ~1.3 g/100 g of tea, corresponding to ~5.4 g/100 g of the dried tea soluble solids. Various HSCCC separations showed constant yields. This figure seems low compared to TR contents published elsewhere (Balentine et al., 1997). This fact suggests that our HSCCC method allows isolation of a specific proportion of total TR only.

Studies of Properties of Isolated TR. To characterize further the isolated fractions, various tests were carried out. Roberts (1958) described TR as weakly acidic, a fact that cannot be confirmed for the HSCCC fraction. In aqueous solution (1.5 mg of TR/3 mL of water) the pH value was 7.6. As a comparison, the pH value of the tea infusion employed was 4.9. As for the color, the freeze-dried TR fraction had a dull gray appearance. In the literature, it is speculated that copigmentation could affect the color of TR (Bruschi et al., 1999). For this reason, we added catechins and caffeine to an aqueous solution of the HSCCC TR fraction. No change in visible color could be observed. A change in color, however, was found after the pH of the aqueous solution was changed. Addition of 1 M HCl resulted in a lighter color; a purple color was observed when the amount of HCl was further increased. Addition of 0.5 M NaOH had the opposite effect. The color intensified and changed to dark brown, olive green. Bailey et al. (1994a) found that an anthocyanidin is present in group I pigments. Kuhr et al. (1994) detected anthocyanidins in a hydrolysate of TR obtained by ultrafiltration. Liberated anthocyanidins from pro-anthocyanidin-like polymers could account for the purple color in strong acidic solutions.

Moreover, the contribution of the HSCCC TR fraction to the total polyphenol content, as determined by Folin−Ciocalteu assay, was measured. Folin data could serve as a measure of free OH groups and will be of use for the quantification of TR using the indirect approach (total of phenolics minus sum of HPLC-analyzed monomers). Setup of response factors for TR is possible using isolated HSCCC TR fractions. The TR fraction from a commercial black tea showed a content of 34.7 g/100 g of gallic acid equivalents; a good response of the Folin reagent to the TR fraction was found. Related to total phenolics of the tea infusion (by Folin), the TR fraction contributes 7.8% of the 25.9% total polyphenols.

The TR fraction was solvent-fractionated according to Roberts's scheme and could be classified as mainly

SII TR (50% SII, 25% of each SI and SIa). The same fractionation was repeated with the original freeze-dried tea extract. SI TR dominate in this extract. This result demonstrates that the HSCCC fraction represents only one portion of the wide TR spectrum.

Determination of Antioxidative Activity of TR. An ongoing discussion about potential health benefits of tea is led by many parties with the argument that black tea provides the same benefits as green tea. Green tea, due to its high flavanol content, has a great portion of antioxidative, radical-scavenging compounds, believed to be beneficial in the prevention of coronary heart disease and cancer (Haslam, 1996; Wiseman et al., 1997; Kuroda and Hara, 1999). Scientific data for black tea polymers' antioxidative and antimutagenic activity is scarce (Catterall et al., 1998; Weisburger, 1999). Data on the antioxidative activity of TR, measured by the TEAC test (Miller et al., 1993) are presented here. The TEAC test measures an in vitro antioxidative activity at a physiological pH of 7.4 and relates results to Trolox, a water-soluble vitamin E analogue. The HSCCC TR fraction exhibited 3.6 mmol of Trolox equivalents/g; ascorbic acid was in the range of 7.8 mmol/g and epicatechin, 8.6 mmol/g. Wiseman et al. (1997) found 3.8 mmol/g for green tea and 3.5 mmol/g for black tea. Consequently, TR are shown to contribute to the antioxidative potential of black tea using the TEAC test. The contribution of this HSCCC TR fraction to the total antioxidative activity of black tea was found to be 5.3%. However, these results are only in vitro results. In vivo studies with the isolated TR are necessary and could become more feasible because the method presented allows isolation of large quantities of TR.

ESI-MS studies of the TR fraction have not been successful as yet. The same is true for HPLC-ESI-MS of TR. No MS signals could be obtained from the hump and the peaks excluded from the HPLC column. Bailey et al. (1992) had similar results and explained the behavior of TR in MS as being typical for a polymeric and polydisperse fraction.

Summary. HSCCC is an ideal tool to isolate black tea pigments because it combines high sample load, relatively short separation time, and high purity of isolated fractions. TF and TR were obtained on a preparative scale. Isolated TR fractions showed a good response to the Folin−Ciocalteu reagent and exhibited antioxidative activity as determined by the TEAC test. The presented Folin−Ciocalteu results may lead in the future to a more exact quantification of TR, because response factors for TR from different teas can be set up. Furthermore, the use of more hydrophilic solvent systems could lead to additional fractionation of TR and is currently being exploited in our laboratory. Better fractionated TR finally could allow structural elucidation. The HSCCC method presented can be used for the isolation of large quantities of TR and has been shown to yield reproducible results.

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Received for review June 21, 2000. Revised manuscript received August 10, 2000. Accepted August 11, 2000.

JF000757+