Centrifugal Precipitation Chromatography − a Novel Chromatographic System for Fractionation of Polymeric Pigments from Black Tea and Red Wine

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A novel chromatographic system was developed and first applied to the fractionation of polymeric pigments from black tea and red wine. Centrifugal precipitation chromatography (CPC) generates solvent gradients through a long separation channel under a centrifugal force field. Tea and wine extracts are precipitated in a hexane- or methyl *tert*-butyl ether-rich environment and are exposed to a gradually increasing ethanol concentration. This causes a repetitive precipitation and dissolution of the biopolymers along the channel. Consequently, they are eluted in the order of their solubility in the organic solvent. It is shown by HPLC analysis of the separated fractions that monomers elute first, whereas fractionated polymers can be found at the end of the chromatographic run. This novel method allows gentle fractionation of polymeric tea and wine constituents and also has potential for use in preparative-scale separations.

Keywords: *Centrifugal precipitation chromatography; high-speed countercurrent chromatography; black tea; red wine; fractionation; polymers; pigments; polyphenols; thearubigins*

INTRODUCTION

Monomeric anthocyanins are the predominant pigments in young red wines. With the aging of red wine, the anthocyanin profile shifts from monomeric anthocyanins to not-well-characterized polymeric pigments by interaction of anthocyanins with colorless phenolics such as (−)-epicatechin and (+)-catechin (*1*). Copigmentation was found to be an initial step in the formation of covalent linkages between anthocyanins and colorless planar copigments (*2*). Confirmation of products resulting from direct anthocyanin−tannin reactions was recently reported (*3*). It was demonstrated that malvidin-3-glucoside is incorporated in polymers and that the pigment is either linked by its C-6/C-8 top or forms bicyclic condensation products with flavanols via the C-4 position. Mainly gel chromatographic techniques have been used so far for the fractionation of polymers from red wine. Johnston and Morris (*4*) succeeded in isolating 4 different fractions of polymers by low-pressure chromatography and provided spectroscopic data on their structural properties. Shoji et al. (*5*) reported the fractionation of red wine pigments by gel permeation chromatography on Toyopearl HW-40 (F). Whereas only a few studies about polymeric pigments from red wine are available, a considerable number of publications deal with proanthocyanidins from grape seed or grape skin. A fractionation of proanthocyanidins based on precipitation was recently reported by Labarbe et al. (*6*), resulting in a quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. The authors deposited precipitated proanthocyanidin polymers in a glass powder column and eluted polymer fractions stepwise with chloroform−methanol gradients. Precipitation with Yb^{3+} was used by Krueger et al. (*7*) to isolate polymeric compounds from a grape seed extract with a good recovery. For structural elucidation the authors used matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry (MALDI−TOFMS) and molecular masses of undecameric proanthocyanidins could be detected. Moreover, separation of grape and wine proanthocyanidins according to their degree of polymerization using C-18 cartridges was reported by Sun et al. (*8*).

Black tea polymers are a heterogeneous class of compounds often referred to as "thearubigins" (TRs) (*9*). TRs are formed from flavanols (catechins) by a polyphenol oxidase-mediated reaction (*10*). Many attempts have been made to separate TRs and elucidate the structures of the compounds incorporated in the polymers. Precipitation of TRs with diethyl ether was found to be an efficient step in Roberts' fractionation workup for the isolation of TRs (*11*). Separation of TRs was later attempted by chromatography on Sephadex LH-20 (*12*), and a partial resolution of caffeine-precipitable TRs with size-exclusion HPLC was achieved (*13*). Wedzicha and Donovan (*14*) reported a normal-phase HPLC method, resulting in a (partial) separation of a derivatized black tea polymeric fraction. In reversed-phase HPLC, TRs elute as a Gaussian "hump" with more or less resolved tea polyphenols "floating" on top (*15*). Preparative-scale fractionations are hampered because of the fact that TR possess a strong affinity to many solid chromatographic supports (*16*).

Most recently, support-free techniques have been successfully applied to the preparative isolation of TRs (*16*). Centrifugal precipitation chromatography (CPC) is another support-free chromatographic technique that was recently applied to the separation of proteins using

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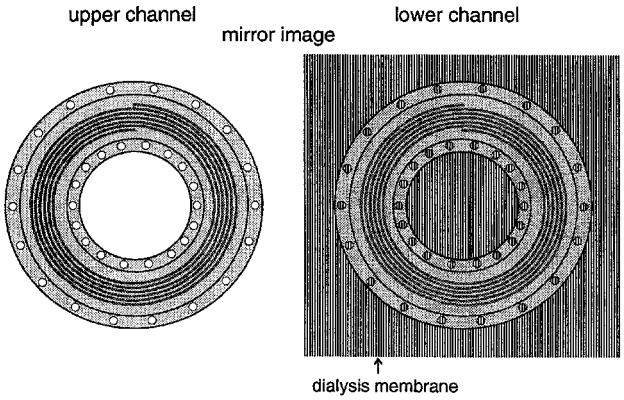

Figure 1. Upper and lower disk with mutually mirror-imaged spiral grooves; dialysis membrane is sandwiched between; disks are carefully aligned to create a single channel.

ammonium sulfate gradients (*17*). In CPC, the separating column consists of a pair of disks equipped with mutually mirror-imaged spiral grooves. A dialysis membrane is sandwiched between the disks to form two identical channels separated by the membrane. The disk assembly is mounted on a sealless, continuous-flow-through centrifuge (*18*). In this paper we report the fractionation of black tea and red wine polymers by organic solvent precipitation using CPC with an open column under a centrifugal force field.

MATERIAL AND METHODS

Reagents. Hexane and methyl *tert*-butyl ether (MTBE) were obtained from Fisher Scientific Co. (Fair Lawn, NJ); absolute ethanol was obtained from Warner-Graham Co. (Cockeysville, MD).

Centrifugal Precipitation Chromatograph (CPC). A sealless, continuous-flow-through centrifuge was obtained from Pharma-Tech Research Corporation (Baltimore, MD). The separation column was fabricated in the National Institutes of Health (NIH, Bethesda, MD) machine shop. It consists of a pair of disks (high-density polyethylene, 13.2 cm diameter and 1.5 cm thickness) with mutually mirror-imaged spiral grooves (1.5 mm wide, 2.0 mm deep, and approximately 2 m long). With a proper alignment, these grooves form a single channel (*17*). A dialysis membrane (or a flat Teflon disk for feasibility studies) is sandwiched between the two disks to form two identical channels partitioned by the membrane (Figure 1). Each channel has a 7.2-mL capacity. The disk is mounted on the sealless, continuous-flow-through centrifuge (Figure 2). Two pairs of flow tubes (0.5-mm i.d., PTFE, Zeus Industrial Products, Raritan, NJ) from the disk assembly are led through the hollow central shaft downward, the hollow idler gear shaft horizontally, and then the tube support upward, and finally exit at the top center of the centrifuge where they are tightly fixed with a pair of clamps. These tubes are bundled, lubricated, and protected with a sheath of Tygon tubing to prevent direct contact with the hard surface. A 2:1 rotation ratio between the disk assembly and the frame prevents the flow tubes from twisting during the revolution (*19*). Thus, rotary seals are obsolete for this type of centrifuge. Details of its operation principle can be found in reference *17*. The revolution speed of the centrifuge is regulated with a speed controller and was set to 2000 rpm for all separations of the present study. Following is the experimental procedure for CPC equipped with dialysis membrane: the column was entirely filled with hexane or methyl *tert*-butyl ether (MTBE). The sample was introduced through a sample loop (1 mL) in the sample channel (lower channel, concentration typically in the range of 5 mg/mL ethanol). The column is rotated at 2000 rpm. The upper channel is then eluted with a linear hexane (or MTBE)/ethanol gradient at 0.5 mL/min with a HPLC pump (Shimadzu SCL-10A and LC-10AD, Shimadzu Scientific Co., Columbia, MD). The lower channel is eluted with ethanol at 0.06 mL/min with a syringe pump (Harvard, model 980532, Harvard Apparatus, South Natick, MA). The sample was introduced from the outer terminal of the lower spiral channel, whereas the MTBE/ethanol gradient was eluted through the upper channel in the opposite direction. Elution was monitored with an UV monitor (UVICORD S, LKB Instruments, Stockholm, Sweden) at 280 nm; the eluent was fractionated into test tubes at 20 min intervals using a fraction collector (ULTRORAC, LKB Instruments; Stockholm, Sweden). The overall elution system is schematically shown in Figure 3. Following is the experimental procedure for CPC, equipped with Teflon disk: the column was completely filled with hexane or MTBE, the sample was injected, the column was eluted isocratically with hexane or MTBE for 30 min, the gradient to 100% ethanol (50% ethanol for red wine extract) was performed in 370 min, the column was eventually flushed with 50% aqueous ethanol to elute ethanol-insoluble material. The flow rate was set at 0.2 mL/min.

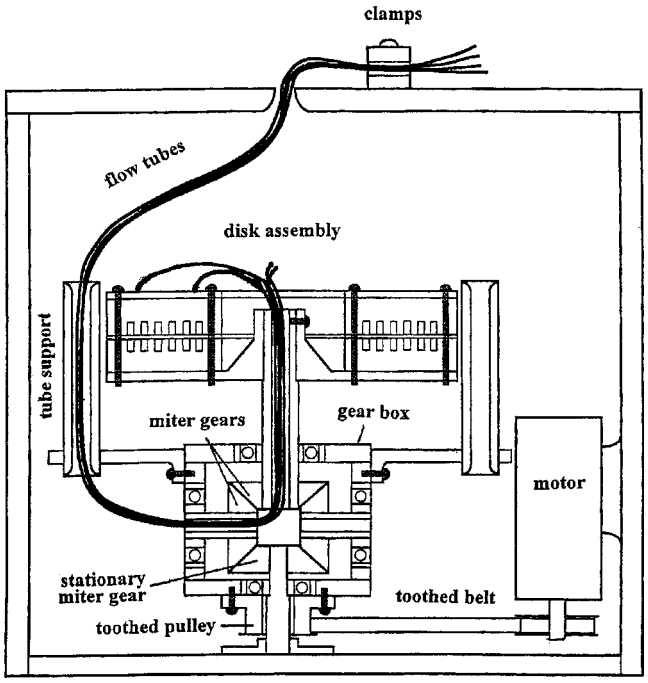

Figure 2. Cross-sectional view of continuous-flow-through centrifuge.

Black Tea Extract. A commercial black tea was used. A 2.5-g portion of black tea per 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, elution of phenolics was carried out with 700 mL of methanol. The methanolic eluate was concentrated in vacuo, and freeze-dried (*16*). The resulting lyophilisate was used for CPC fractionation.

Red Wine Extract. A French Merlot wine from 1990 was used. After evaporation of ethanol in vacuo, the dealcoholized red wine (0.7 L) was applied to an Amberlite XAD-7 column (50 cm × 4 cm, Fluka Chemie, Buchs, Switzerland). The column was washed with 1 L of water to remove sugars and organic acids. The pigments were eluted with 500 mL of a mixture of methanol/acetic acid (19:1, v/v). The solvent was evaporated and the residue was twice partitioned against 300 mL of ethyl acetate to remove less polar compounds. The aqueous phase was concentrated in vacuo and freeze-dried to yield 2.6 g of a dark powder which was used for CPC.

HPLC with Diode Array Detection (DAD) for Red Wine Pigments. A Jasco ternary gradient unit LG-980-02, with degasser and MD-910 multiwavelength detector driven by BORWIN chromatography software was used. Column was a RP-18 5-μm LUNA 150 × 4.6 mm (Phenomenex, Aschaffen-

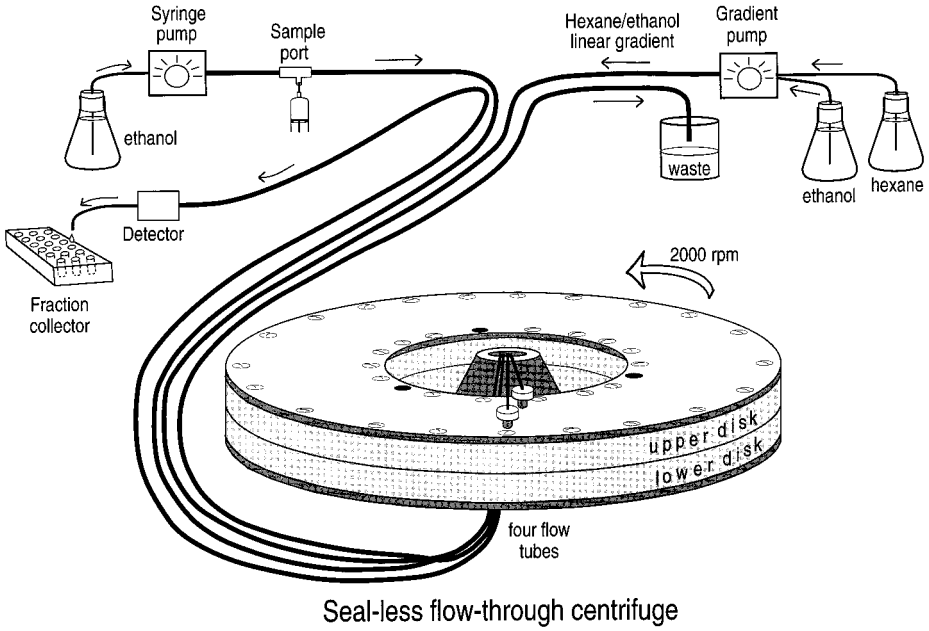

Figure 3. Elution system of the centrifugal precipitation chromatograph.

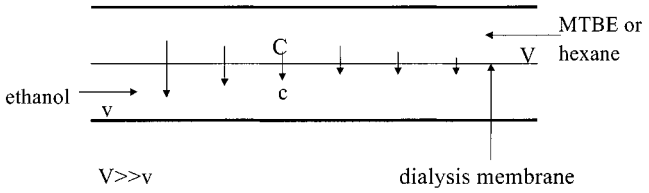

Figure 4. Principle of CPC: flow pattern through channel. V,v = flow rate; C,c = concentration.

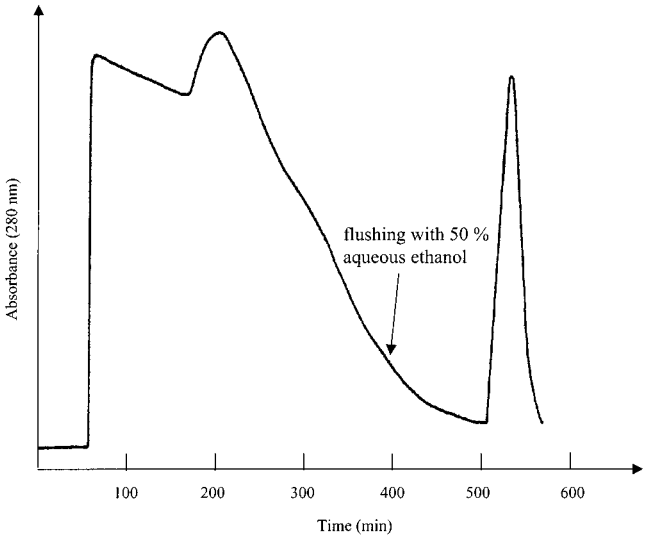

Figure 5. CPC fractionation of black tea extract, Teflon disk sandwiched between the two layers; for experimental conditions see text.

burg, Germany), and solvents were water−formic acid−acetonitrile (87/10/3, v/v/v, solvent A), water−formic acid−acetonitrile (40/10/50, v/v/v, solvent B). Linear gradient from 94% A and 6% B to 80% A and 20% B in 20 min; to 60% A and 40% B in 15 min; to 40% A and 60% B in 5 min; to 30% A and 70% B in 6 min; back to initial conditions. The flow rate was set at 0.8 mL/min. Peak detection was carried out at 280 and 520 nm. Spectra were also visualized as a contour plot in the wavelength region 200−600 nm.

HPLC Coupled with Electrospray-Ionization Mass Spectrometry (ESI−MS/MS) for Red Wine Pigments. A Bruker Esquire HPLC−MS with UV−vis detector in series (set at 280 nm) was used. MS parameters were: negative mode; capillary, 2500 V; end plate, 2000 V; capillary exit, −120 V; skim 1, −40 V; skim 2, −8 V; dry gas, 325 °C; gas flow, 9 L/min; nebulizer, 40 psi; fragmentation amplitude, 1.0 V. Hypersil RP-18 column, 5-μm (250 mm × 2.0 mm), from Phenomenex (Aschaffenburg, Germany). Solvents: 2% aqueous acetic acid (v/v, solvent A), acetonitrile (solvent B); linear gradient from 95% A and 5% B (initial), to 85% A and 15% B in 35 min; to 20% A and 80% B in 50 min; to 0% A and 100% B in 5 min; isocratic at 100% B for 10 min; flow rate, 0.35 mL/min.

HPLC−DAD for Black Tea Pigments. A Beckman System Gold programmable solvent module 126, equipped with Beckman autosampler 502 and 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 RP-18 column (5 μm, 150 mm × 4.6 mm) from Phenomenex (Aschaffenburg, Germany). 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 were initial 100% A and 0% B; isocratic at 100% A and 0% B for 15 min; in 20 min to 68% A and 32% B; isocratic at 68% A and 32% B for 5 min; back to initial conditions; flow rate, 0.8 mL/min.

RESULTS AND DISCUSSION

Preliminary Studies. In a simple test tube experiment it was found that when dissolved in ethanol, addition of methyl *tert*-butyl ether (MTBE) resulted in a precipitation of the red wine extract, whereas dissolved catechin (a flavan-3-ol) and rutin (quercetin-3-*O*-rutinoside) were hardly precipitated. The black tea extract could be precipitated with hexane. This fact made us think about exploring the different solubility of polymeric fractions in hydrophobic organic solvents for a chromatographic separation. In a recent study, Labarbe et al. (*6*) reported the quantitative fractionation of grape seed proanthocyanidins by precipitation on glass powder, and fractionation according to the degree of polymerization was found. However, the polymeric grape seed material separated is chemically different from the red wine polymers used in the present investigation (*3*).

Centrifugal Precipitation Chromatography (CPC). CPC is a novel chromatographic technique based on the

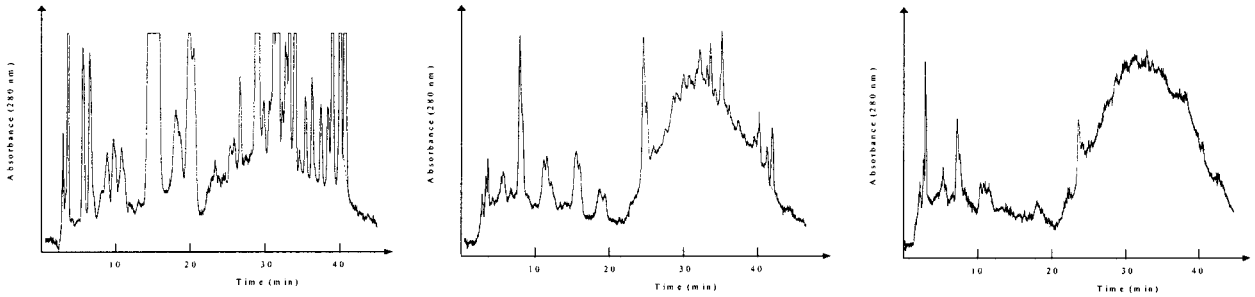

Figure 6. HPLC chromatograms of CPC fractions from black tea extract (cf. Figure 5). Left side, fraction at 100 min; center, fraction at 340 min; right side, fraction at 535 min.

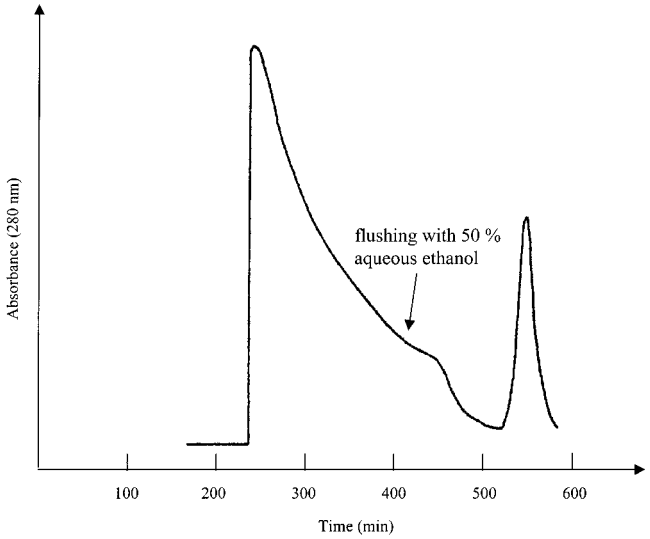

Figure 7. CPC fractionation of TR obtained by HSCCC with ethyl acetate−*n*-butanol−water (2/3/5, less dense layer as stationary phase, reference *16*); CPC equipped with Teflon disk.

separation of compounds between two miscible liquid phases. In contrast to conventional high-speed countercurrent chromatography (HSCCC) in which two immiscible liquid phases are employed, CPC uses a dialysis membrane to separate the two miscible liquids. Hexane or MTBE is eluted through one channel and ethanol through the other channel in the opposite direction at a lower flow rate (Figure 4). This countercurrent process causes a migration of the organic solvent in the ethanol channel, similar to the process shown for ammonium sulfate and water (*17*). An exponential gradient of organic solvent is formed through the ethanol channel. Under a centrifugal force field, black tea or red wine polymers introduced into the ethanol channel are exposed to gradually increasing concentrations of the hydrophobic organic solvent. This causes precipitation and deposition of polymers at various locations along the ethanol channel according to their solubility in the hydrophobic organic solvent. The hydrophobic organic solvent concentration is then gradually decreased (by starting the gradient from 100% hexane or MTBE to 100% ethanol). As a result, chromatographic elution of tea and wine compounds is achieved. Once-deposited compounds dissolve and reprecipitate at an advanced location in the channel. Because the organic solvent concentration in the ethanol channel falls at a rate much lower than the flow rate of the ethanol phase, the compounds undergo a repetitive process of precipitation and dissolution and elute in decreasing order of their solubility in the organic solvent (i.e., hexane or MTBE).

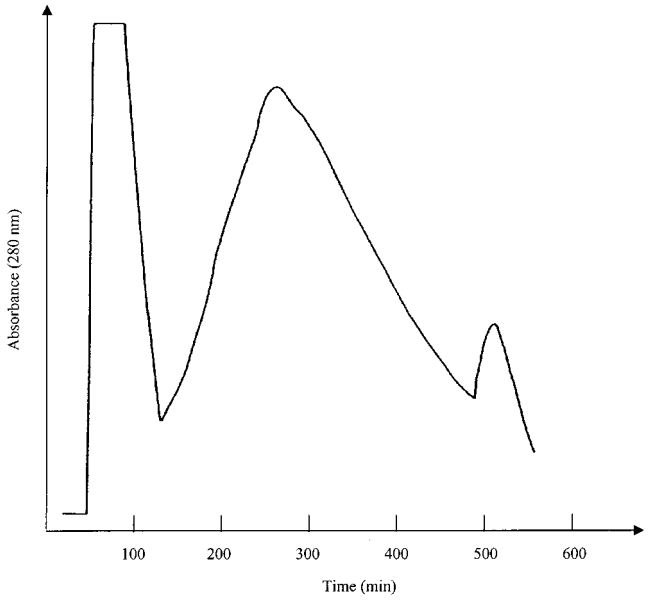

Figure 8. CPC fractionation of red wine XAD-7 extract, Teflon disk sandwiched between the two layers; for experimental conditions see text.

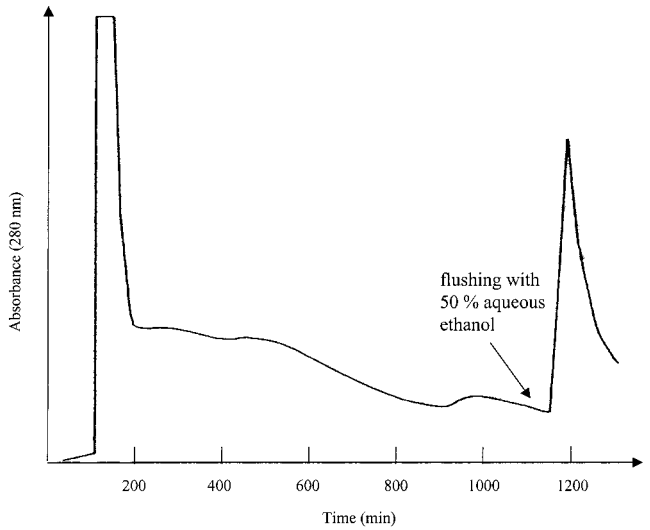

Figure 9. CPC fractionation of red wine XAD-7 extract, dialysis membrane sandwiched between the two layers; for experimental conditions see text.

Fractionation of a Black Tea Extract by CPC. A series of basic experiments was performed to evaluate the feasibility of fractionating biopolymers by CPC. For these experiments the membrane was replaced by a Teflon disk. In this way, diffusion processes through the membrane are stopped, and the CPC apparatus was

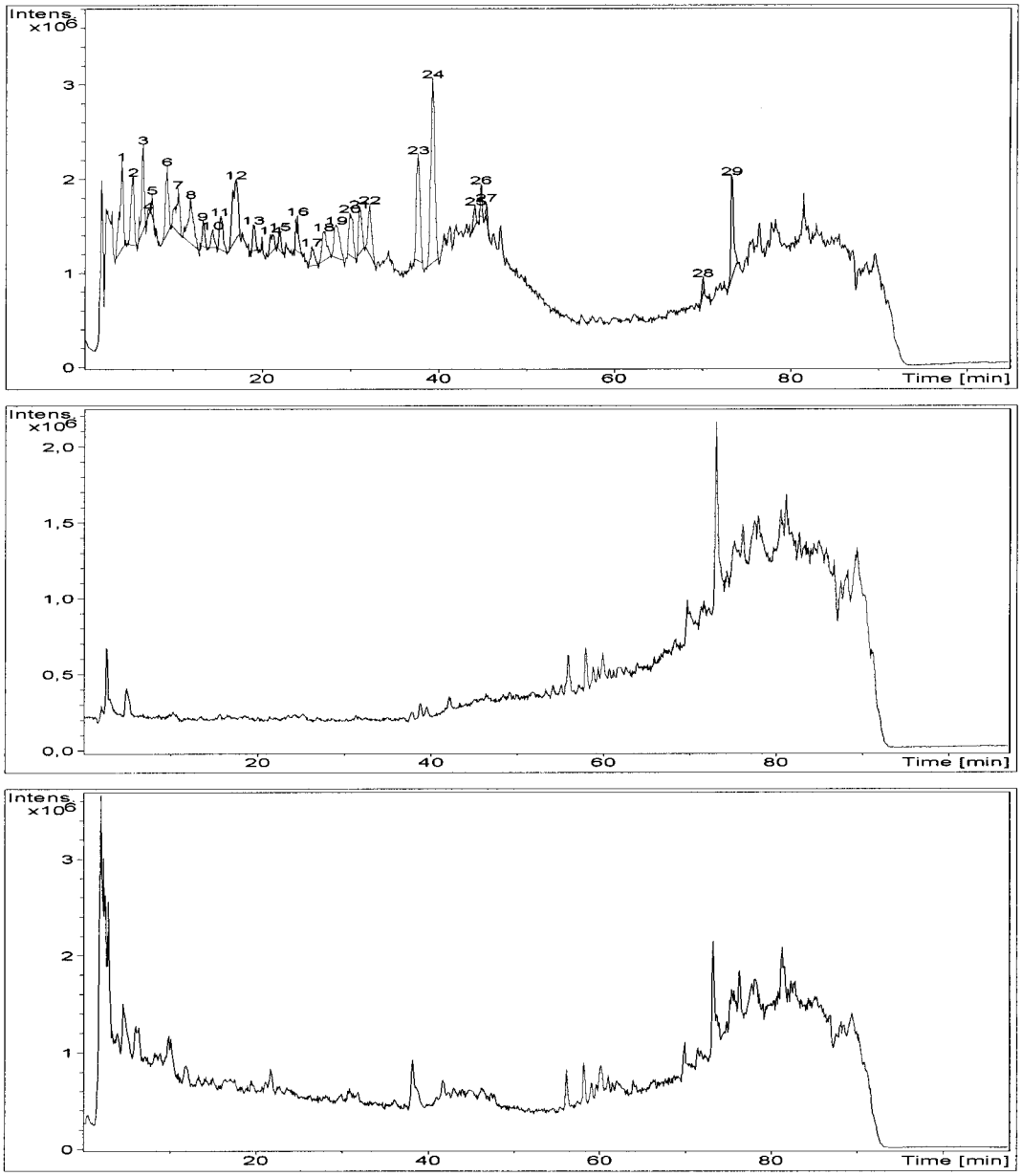

Figure 10. HPLC−ESI-MS chromatograms of red wine fractions from Figure 9. Top, early fraction at 100 min; middle, fraction at 550 min; bottom, fraction at 1200 min.

operated with only one long separation channel to represent the simplest possible instrumental setup. One channel was entirely filled with hexane. The black tea extract was dissolved in ethanol and injected into the system by loop injection. The sample was first precipitated by the organic solvent (i.e., hexane) and then gradually dissolved by increasing ethanol concentrations. After 400 min of separation time, the column was flushed with 50% aqueous ethanol (v/v). The separation of the black tea extract, which was monitored at 280 nm, is shown in Figure 5. HPLC−DAD analyses revealed fractionation of the extract (cf. HPLC traces at 280 nm in Figure 6). The left side shows an early fraction (at 100 min) containing the whole range of low-molecular-weight flavonoids. In the center of Figure 6, HPLC analysis of a fraction at 340 min demonstrates a shift from monomeric compounds toward more polymerized material. It can be seen that the number of resolved peaks decreases and the "hump-like" material increases. A fraction at 535 min (right side of Figure 6) is nearly free of known chromatographically resolved flavonoids and contains thearubigins which elute as a "hump" from RP-18 HPLC columns (*15*). Consequently, CPC allows partial separation of black tea constituents according to their degree of polymerization. To further investigate the capabilities of CPC, a polymeric fraction from black tea was isolated by HSCCC with a solvent system consisting of ethyl acetate-*n*-butanol−water (2/3/5, more dense layer as mobile phase) according to a procedure described in reference *16*. TRs elute under these conditions with, or shortly after, the solvent front. Such a HSCCC fraction was shown to be free of flavonoid monomers and elutes from reversed-phase HPLC packings as a convex hump. Furthermore, the fraction is free of carbohydrates and proteins and exhibits a high phenolic content. This fraction was separated by CPC using conditions identical to those used before. As can be seen in Figure 7, the early eluting part is missing, thus confirming that CPC separates the early-eluting monomers from polymeric material and also leads to a fractionation of polymers. Interestingly, one part of the polymeric tea compounds was not soluble

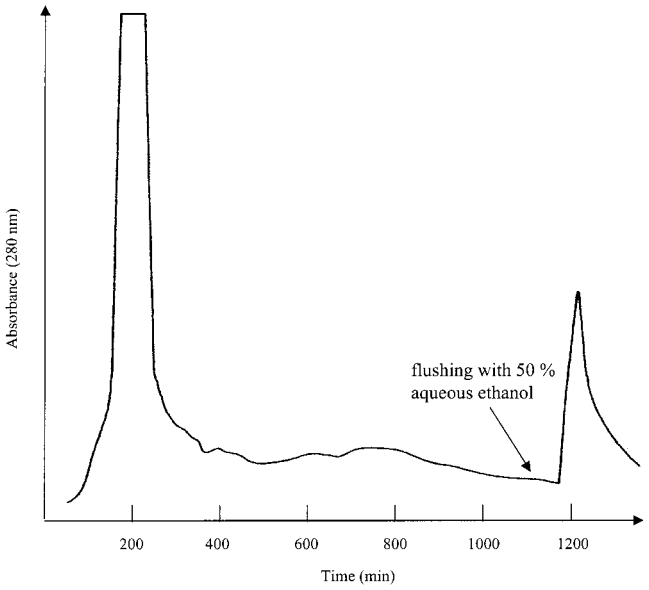

Figure 11. CPC fractionation of black tea extract, dialysis membrane sandwiched between the two layers; for experimental conditions see text.

in pure ethanol and only eluted from the column with 50% aqueous ethanol. On the basis of solubility differences, separation of this more hydrophilic portion of total TRs from the other flavonoids was possible.

Fractionation of a Red Wine Extract by CPC. Prior to CPC, the red wine used in this study was investigated by HPLC using detection in the visible wavelength region. The content of monomeric anthocyanins was found to be very low (<10 mg/L, assayed by an HPLC method using reference compounds isolated from grape sources by HSCCC, reference *20*). The majority of colored compounds eluted as a "hump" together with some resolved condensed pigments of unknown structure. The red wine extract used for CPC was cleaned-up on an Amberlite XAD-7 resin which allows removal of organic acids and sugars (*21*). Moreover, extraction with ethyl acetate was carried out in order to remove less polar compounds such as catechins and flavonol-*O*-glycosides. MTBE proved to be more efficient in precipitating red wine polymers. Therefore, a MTBE−ethanol gradient was generated on the CPC (equipped with Teflon disk to prevent migration). After isocratic elution for 30 min at 100% MTBE a linear gradient to 50% ethanol was performed in 370 min. After 400 min of separation time, a linear gradient to 100% ethanol in 50 min led to the elution of more hydrophilic material (Figure 8). It was found that all fractions were colored. HPLC analysis (detection at 520 nm to visualize colored material and at 280 nm to detect phenolics) showed that resolved peaks predominated in the early fractions. With increasing separation time monomeric compounds disappeared and polymeric material predominated.

The Teflon disk was subsequently replaced by the dialysis membrane in order to increase separation efficiency. Migration through the membrane was expected to create exponential gradients, thus initiating the repetitive precipitation and dissolution process. In addition, separation time was extended and separations were run overnight to improve fractionation. A CPC separation of the red wine XAD-7 extract is shown in Figure 9. After 1170 min, the column was flushed with 50% aqueous ethanol which resulted in elution of more

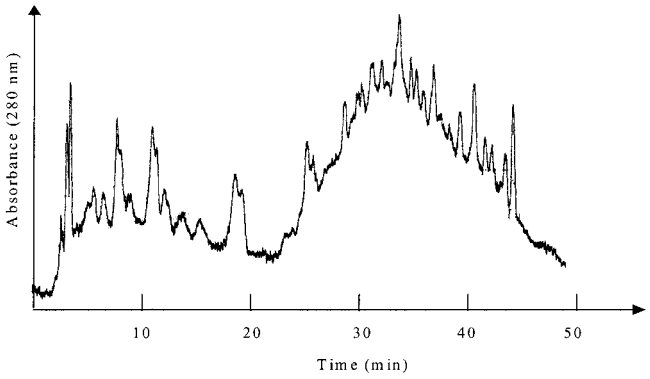

Figure 12. HPLC analysis of a fraction eluting at 1240 min from Figure 11.

hydrophilic material. HPLC analysis with a system optimized for monomeric anthocyanins showed that in fractions up to 400 min traces of monomeric anthocyanins could be detected (i.e., malvidin-3-glucoside), whereas later eluting fractions contained no discrete peaks at 520 nm, although the fractions were deeply colored. HPLC−ESI−MS analysis of an early fraction of the CPC fractionation (according to a procedure used in our lab for the analysis of proanthocyanidins) showed the presence of flavonol-*O*-glycosides (FOG), proanthocyanidin dimers, trimers, and some unidentified peaks (*22*−*25*) which eluted from the HPLC column up to 50 min (Figure 10, top, peaks numbered 1−27). Figure 10, middle, shows HPLC−MS analysis of a fraction at 550 min (cf. Figure 9). Under the conditions applied, the later eluting material (70−90 min) from the HPLC column becomes more prominent and the early eluting FOG, dimers, and trimers were missing. Mass spectra obtained in the range from 70 to 90 min showed signals in the range from *m*/*z* 500 up to *m*/*z* 1752. The fractions obtained after flushing with 50% aqueous ethanol (cf. Figure 9, 1200 min) were also analyzed by HPLC−MS. Peaks were present which eluted shortly after the dead volume of the HPLC column (cf. Figure 10 bottom). MS spectra contained signals up to *m*/*z* 1671. These compounds may represent polymeric material that is excluded from the HPLC column, similar to group I pigments proposed by Bailey et al. (*26*) for black tea thearubigins. As polycharged ions are likely to be formed, molecular weights could be in the range of up to several thousand Daltons.

Identification of pigments is a difficult task, because, at the present stage, the sample capacity of CPC is very limited and therefore, the actual amount of pigment present in each fraction is tiny. Preparative-scale CPC instruments currently being designed in our laboratory may provide sufficient amount of separated material for NMR analysis and hydrolysis experiments, respectively.

CPC fractionation of the black tea extract is demonstrated in Figure 11. A gradient from 100% hexane to 100% ethanol was performed in 1100 min. HPLC analysis proved the fractionation of the polyphenolic mixture. Figure 12 shows the RP−HPLC chromatogram of a late-eluting fraction. A partial resolution of the hump could be achieved after fractionation of the complex TR mixture by CPC.

The use of the two miscible solvents ethanol and water as a solvent system in CPC is also feasible. The hydrophilic TR (last peak in Figure 11) was fractionated by CPC using a gradient from 100% ethanol to 40%

ethanol and 60% water after elution of the more hydrophobic portion of total TR with a hexanes−ethanol gradient (CPC profile not shown). Consequently, it is also possible to apply ethanol−water gradients to fractionate highly hydrophilic polymeric pigments.

Summary. The potential of CPC to carry out fractionation of polymeric pigments is shown in this paper. In CPC, precipitation of the analyte in the more hydrophobic solvent is required to achieve chromatographic separation. CPC is a versatile technique because many miscible solvents are available. At present, CPC is still limited to analytical-scale separations. As soon as the construction of the preparative CPC apparatus is completed, determination of the degree of polymerization using hydrolysis/thiolysis experiments and elucidation of the monomeric units and chromophores present in the polymers by NMR techniques will be carried out.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) Liao, H.; Cai Y.; Haslam E. Polyphenol interactions. Anthocyanins: co-pigmentation and colour changes in red wines. *J. Sci. Food Agric.* **1992**, *59*, 299−305.

(2) Brouillard, R.; Dangles, O. Anthocyanin molecular interactions: The first step in the formation of new pigments during wine aging? *Food Chem.* **1994**, *51*, 365−371.

(3) Remy, S.; Fulcrand, H.; Labarbe, B.; Cheynier, V.; Moutounet, M. First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions. *J. Sci. Food Agric.* **2000**, *80*, 745−751.

(4) Johnston, T. V.; Morris, J. R. Circular dichroism and spectroscopic studies of *Vitis vinifera* cv. Cabernet Sauvignon and *Vitis rotundifolia* cv. Noble red wine liquid chromatographic fractions. *Am. J. Enol. Vitic.* **1996**, *47*, 323−328.

(5) Shoji, T.; Yanagida, A.; Kanda, T. Gel permeation chromatography of anthocyanin pigments from rosé cider and red wine. *J. Agric. Food Chem.* **1999**, *47*, 2885−2890.

(6) Labarbe, B.; Cheynier, V.; Brossaud, F.; Souquet, J.-M.; Moutounet, M. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1999**, *47*, 2719−2723.

(7) Krueger, C. G.; Carter Dopke, N.; Treichel, P. M.; Folts, J.; Reed, J. D. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem.* **2000**, *48*, 1663−1667.

(8) Sun, B.; Leandro, C.; Ricardo da Silva, J. M.; Spranger, I. Separation of grape and wine proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1998**, *46*, 1390−1396.

(9) Roberts, E. A. H. The phenolic substances of manufactured tea. II. − Their origin as enzymic oxidation products in fermentation. *J. Sci. Food Agric.* **1958**, *9*, 212−216.

(10) Balentine, D. A.; Wiseman, S. A.; Bouwens, L. C. M. The chemistry of tea flavonoids. *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 693−704.

(11) Roberts, E. A. H.; Cartwright, R. A.; Oldschool, M. The phenolic substances of manufactured tea. I. − Fractionation and paper chromatography of water-soluble substances. *J. Sci. Food Agric.* **1957**, *8*, 72−80.

(12) Cattell, D. J.; Nursten, H. E. Separation of thearubigins on Sephadex LH-20. *Phytochemistry* **1977**, *16*, 1269−1272.

(13) Clifford, M. N.; Powell, C. A partial resolution of black tea tannins by HPLC. In *Polyphenols Communications 96. Vol. 1. XVIIIe Journées Internationales Groupes Polyphenols: Paris INRA Editions*, Vercauteren, J., Chèze, C., Dumon, M. C., Weber, J. F., Eds.; INRA: Paris, 1996; pp 217−218.

(14) Wedzicha, B. L.; Donovan, T. J. Separation of derivatized black tea thearubigins by high-performance liquid chromatography. *J. Chromatogr.* **1989**, *478*, 217−224.

(15) Bruschi, L.; Copeland, E. L.; Clifford, M. N. Unexpected hyperchromic interactions during the chromatography of theafulvins and simple flavonoids. *Food Chem.* **1999**, *67*, 143−146.

(16) Degenhardt, A.; Engelhardt, U. H.; Wendt, A.-S.; Winterhalter, P. Isolation of black tea pigments using high-speed countercurrent chromatography and studies on properties of black tea polymers. *J. Agric. Food Chem.* **2000**, *48*, 5200−5205.

(17) Ito, Y. Centrifugal Precipitation Chromatography: Principle, apparatus, and optimization of key parameters for protein fractionation by ammonium sulfate precipitation. *Anal. Biochem.* **2000**, *277*, 143−153.

(18) Ito, Y.; Suaudeau, J.; Bowman, R. L. New flow-through centrifuge without rotating seals applied to plasmapheresis. *Science* **1975**, *189*, 999−1000.

(19) Ito, Y. Sealless Continuous Flow Centrifuge. In *Apheresis: Principle and Practice*; McLeod, B., Price, T. H., Drew, M. J., Eds.; AABB Press: Bethesda, MD, 1997; 9−13.

(20) Degenhardt, A.; Hofmann, S.; Knapp, H.; Winterhalter, P. Preparative isolation of anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to red wine. *J. Agric. Food Chem.* **2000**, *48*, 5812−5818.

(21) Degenhardt, A.; Knapp, H.; Winterhalter, P. Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity. *J. Agric. Food Chem.* **2000**, *48*, 338−343.

(22) Lakenbrink, C. PhD Thesis. Technical University of Braunschweig, Braunschweig, Germany, 2000.

(23) Degenhardt, A.; Engelhardt, U. H.; Lakenbrink, C.; Winterhalter, P. Preparative separation of polyphenols from tea by high-speed countercurrent chromatography. *J. Agric. Food Chem.* **2000**, *48*, 3425−3430.

(24) De Pascual-Teresa, S.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. Prodelphinidins and related flavanols in wine. *Int. J. Food Sci. Technol.* **2000**, *35*, 33−40.

(25) De Freitas, V. A. P.; Glories, Y.; Laguerre, M. Incidence of molecular structure in oxidation of grape seed procyanidins. *J. Agric. Food Chem.* **1998**, *46*, 376−382.

(26) Bailey, R. G.; Nursten, H. E.; McDowell, I. Comparative study of the reversed-phase high-performance liquid chromatography of black tea liquors with special reference to the thearubigins. *J. Chromatogr.* **1991**, *542*, 115−128.

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