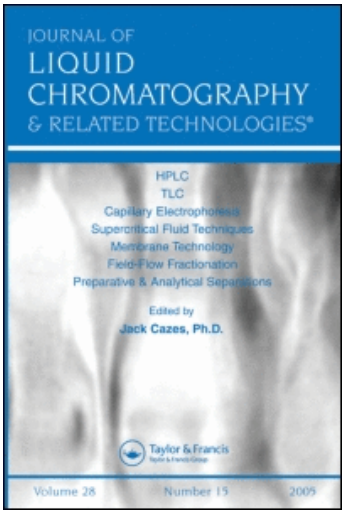

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

ISOLATION OF NATURAL PIGMENTS BY HIGH SPEED CCC

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Online publication date: 30 June 2001

To cite this Article Degenhardt, Andreas and Winterhalter, Peter(2001) 'ISOLATION OF NATURAL PIGMENTS BY HIGH SPEED CCC', Journal of Liquid Chromatography & Related Technologies, 24: 11, 1745 — 1764
To link to this Article: DOI: 10.1081/JLC-100104376
URL: http://dx.doi.org/10.1081/JLC-100104376

J. LIQ. CHROM. & REL. TECHNOL., 24(11&12), 1745–1764 (2001)

EXTRACTIONS AND PURIFICATIONS

ISOLATION OF NATURAL PIGMENTS BY HIGH SPEED CCC

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ABSTRACT

Many natural food colorants are highly water soluble, and their separation on a preparative scale still remains a challenge. The use of high-speed countercurrent chromatography (HSCCC) for the separation of anthocyanins from red wine, betalains from red beet, carotenoids from *Gardenia jasminoides,* and theaflavic acids from black tea is presented in this paper. Moreover, the purification of carminic acid using the pH-zone refining technique and the isolation of hydrophilic thearubigins from black tea by HSCCC is demonstrated. Isolated compounds can be used for quantification of colorants in foods, for testing of physiological activities, as well, as for monitoring changes that occur during the processing of foods.

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INTRODUCTION

Color plays an important role in our perception of freshness, taste, and quality of all foods. The food industry is, therefore, interested in gaining a better understanding of color generation and color stabilization during the various steps of food processing. Nowadays, a trend towards "naturalness" represents a challenge for food manufacturers, since many natural pigments, are to some extent, unstable on storage or susceptible to pH changes. Natural food colorants can be originally present in the foodstuff, or may be added as an extract to enhance the natural color. Physical aspects like colorant location, light-scattering ability, and crystal size must be taken into consideration when assessing the color quality of a product.[1] Apart from colorimetric measurements of food systems, there is a need to understand color on the molecular level, i.e., the structures of compounds containing the chromophores and their changes during processing of foods which often involves high temperatures, pressure, and strong frictional forces. HPLC methods are often used to monitor the changes seen in the composition of colorants. However, due to the lack of commercially available standards, exact quantification is often hampered. The potential of CCC with regard to the rapid, preparative isolation of standard compounds was tested in the present study.

EXPERIMENTAL

Apparatus

A high speed Model CCC-1000 manufactured by Pharma-Tech Research Corporation (Baltimore, Maryland, USA) was equipped with 3 preparative coils, connected in series (total volume: 850 mL). The separations were run at a revolution speed of 1000 rpm. The flow was delivered by a Biotronik HPLC pump BT 3020. Freeze-dried XAD-7 extracts and plant extracts 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 1 g to 3 g. Stationary phase retention was in the range of 40-80%. 10 mL fractions were collected with a Pharmacia LKB Super Frac fraction collector. Elution was monitored with a Knauer UV-Vis detector and chromatograms were recorded on a Knauer L 250 E plotter. The HSCCC from Pharma-Tech Research was used for subsequent separations, unless otherwise stated. The second CCC instrument used was a Multi-Layer Coil Countercurrent Chromatograph by P.C. Inc. (Potomac, Maryland, USA) equipped with a single coil (volume: 350 mL). Revolution speed was set at 800 rpm for the single coil instrument. The sample size was typically in the range of 500 mg to 1 g for the single coil instrument.

Separation of Anthocyanins by HSCCC

Isolation of anthocyanins was carried out according to a procedure described by Degenhardt et al.[2] XAD-7 isolates of red grape sources were separated by HSCCC using MTBE-*n*-butanol-acetonitrile-water (2/2/1/5), acidified with 0.1% TFA, less dense layer as stationary phase, flow rate: 5.0 mL/min and anthocyanins **1**-**6** were obtained (for structures cf. Fig. 2).

HPLC Analysis of Anthocyanins from Red Wine

A Jasco ternary gradient unit LG-980-02, with degasser and UV-975 detector, was used. The column was a RP18 5 μm LUNA 150 × 4.6 mm (Phenomenex, Aschaffenburg, Germany), flow rate was 0.8 mL/min 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, 40% B in 15 min; to 40% A, 60% B in 5 min; to 30% A, 70% B in 6 min; back to initial conditions.

Isolation and Analysis of Water Soluble Carotenoids from *Gardenia jasminoides*

A methanolic extract from fruits of *Gardenia jasminoides* was donated by Professor N. Watanabe, Shizuoka University, Japan. A LUNA RP18 column 5 μm (150 × 4.6 mm) from Phenomenex (Aschaffenburg, Germany) was used for the separation by HPLC, solvents were: water (solvent A), acetonitrile (solvent B). Conditions: initial conditions: 95% A, 5% B; linear gradient over 30 min to 50% A, 50% B; detection at 450 nm, flow rate: 0.5 mL/min. Crocetin derivatives **7**-**9** (for structures cf. Fig. 4) were separated by HSCCC using MTBE-*n*-butanol-acetonitrile-water (2/2/1/5, less dense layer as stationary phase, flow rate: 5.0 mL/min).

Spectroscopic data of *trans*-crocetin-mono-gentiobiosyl-ester (**9**) (cf. Fig. 4): λ_{max} determined by HPLC-DAD: 439 and 467 nm. ESI-MS: 652 daltons (pseudomolecular ion at m/z 675: $[M(652)+Na]^+$. ^{1}H-NMR (360 MHz, CD_3OD, ppm): δ 1.97 (6 H, d, J = 1.0 Hz, H_3C-C 2/H_3C-C 15); 2.01 (6 H, d, J = 1.0 Hz, H_3C-C 6/H_3C-C 11); 3.22 (1 H, dd, J = 10.0, 8.0 Hz, H 2"); 3.31 (H 5"/H 3"/H 4", m, partially obscured by solvent peak); 3.46 (3 H, m, H 4'/H 2'/H 5'); 3.54 (1 H, dd, J = 9.5, 9.5 Hz, H 3'); 3.67 (1 H, dd, J = 11.5, 5.0 Hz, H 6a"); 3.78 (1H, dd, J = 11.5, 5.0 Hz, H 6a'); 3.84 (1 H, dd, J = 11.5, 2.0 Hz, H 6b"); 4.16 (1 H, dd, J = 11.5, 2.0 Hz, H 6b'); 4.33 (1 H, d, J = 8.0 Hz, H 1"); 5.53 (1 H, d, J = 8.0

Hz, H 1'); 6.49 (2 H, ddq, *J* = 10.0, 1.0, 1.0 Hz, H 7/10); 6.68 (2 H, dd, *J* = 15.0, 11.5 Hz, H 4/13); 6.70 (1 H, d, *J* = 15.0 Hz, H 5); 6.75 (1 H, d, *J* = 15.0 Hz, H 12); 6.82 (2 H, m, H 8/9); 7.31 (1 H, dq, *J* = 11.0, 1.0 Hz, H 3); 7.43 (1 H, dq, *J* = 11.0, 1.0 Hz, H 14). ^{13}C-NMR (90 MHz, CD_3OD, ppm): δ 12.7 (CH_3-C 2/CH_3-C 15); 12.9 (CH_3-C 6/CH_3-C 11), 62.7 (C 6"), 69.5 (C 6'), 70.9 (C 4'), 71.5 (C 4"), 73.9 (C 2'), 75.0 (C 2"), 77.7 (C 5"), 77.9 (C 3'/C 3"), 78.0 (C 5'), 96.0 (C 1'), 104.5 (C 1"), 124.6 (C 4), 125.0 (C 13), 126.3 (C 2), 127.9 (C 15), 132.6 (C 8), 133.1 (C 9), 136.6 (C 7), 137.4 (C 10), 137.8 (C 6), 138.2 (C 11), 140.2 (C 3), 141.9 (C 14), 145.0 (C 5), 146.2 (C 12), 168.5 (C 1), 172.0 (C 16).

Analysis and Separation of Carminic Acid

Crude carminic acid (**10**) was obtained from Sigma (St. Louis, MO, USA). HPLC conditions: A Hypersil RP18 column 5 µm (250 × 4.6 mm) from Phenomenex (Aschaffenburg, Germany) was used, solvents were: 2% acetic acid (v/v; solvent A), acetonitrile (solvent B). Conditions: initial conditions: 80% A, 20% B; linear gradient over 30 min to 55% A, 45% B; detection at 500 nm, flow rate: 1.0 mL/min. ESI-MS (negative mode) of **10**: 491 daltons $[M-H]^-$. HSCCC (single coil instrument) separation of **10** (for structure cf. Fig. 6) was carried out using MTBE-*n*-butanol-acetonitrile-water (1/3/1/5, lower phase mobile), 15 mM TFA was added to the upper phase; 15 mM of NH_3 was added to the more dense phase; flow rate: 1.9 mL/min.

Isolation and Analysis of Theaflavic Acids

A commercially available black tea (20 g) was extracted for 10 minutes with 1000 mL of boiling water. After cooling to about 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 lyophilisate 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.[3] The elution of theaflavins (TF) and theaflavic acids was carried out with 10% acetone in ethanol (v/v) and all colored fractions were collected, pooled, evaporated to dryness. and freeze-dried. The HPLC separation was carried out as follows: a Nucleosil RP18 column (5 µm, 150 mm × 4.6 mm) from Phenomenex (Aschaffenburg, Germany) was used. 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. ESI-MS (negative mode) of **11**: 579 daltons $[M-H]^-$; **12**: 428 daltons $[M-H]^-$ (for structures cf. Fig. 8). HSCCC separation: hexane-ethyl acetate-methanol-water (2/5/2/5, less dense layer as stationary phase, flow rate: 2.8 mL/min) was used as solvent system.

Analysis of Thearubigins (TR) and Betalains

Isolation and clean-up are described in detail by Degenhardt et al.[4] and depicted schematically in Fig. 9. HPLC conditions were the same as for theaflavic acids. A commercial red beet juice concentrate was donated by Plantextrakt (Vestenbergsgreuth, Germany). The concentrate was separated by HSCCC (sample load up to 3 grams on the single coil system) without further work-up of the sample. The resulting fractions were evaporated *in vacuo* and desalted on a short Polyamide column. The pigments were absorbed on the column. After rinsing with water, the elution of betalains was carried out with 0.05% NH_3 in methanol (v/v). The eluate was evaporated *in vacuo* and freeze-dried. HPLC analysis: Column was a Hypersil RP18 5 μm (250 × 4.6 mm) from Techlab (Erkerode, Germany). Solvents were: methanol/0.05 m KH_2PO_4 (18/82, v/v, solvent A) and methanol/water (40/60, v/v, solvent B). Conditions: initial 100% A, 0% B; linear gradient over 45 min to 10% A, 90% B; detection at 538 nm, flow rate: 0.6 mL/min.[5] ESI-MS (positive mode) of **13** (for structure cf. Fig. 12): 551 daltons $[M+H]^+$, MS/MS of 551: m/z 389 $[M\text{-glucosyl}+H]^+$. HSCCC conditions (single coil instrument): ethanol-acetonitrile-ammonium sulfate solution (saturated)-water (1/0.5/1.2/1); flow rate: 1.8 mL/min.

RESULTS AND DISCUSSION

Isolation of Anthocyanins from Red Wine by HSCCC

Color of red wine is an important quality marker. Simple, sensorial assessments of red wines are often complemented by photometry or tristimulus measurements.[6-8] In young red wines, the color is generated by monomeric anthocyanins with malvidin-3-glucoside being the major component in many varieties.[9] The anthocyanin composition is considered to be characteristic for a grape variety. Also, wine making may have an influence on the anthocyanin composition.[10] Wines made from Pinot Noir grapes contain only simple monoglucosylated anthocyanins of the malvidin, peonidin, petunidin, delphinidin, and cyanidin type, whereas, the composition of other grape varieties can be far more complex and a large number of acylated forms of anthocyanins are present. The anthocyanin profile is further useful in the detection of hybrid grapes or the adul-

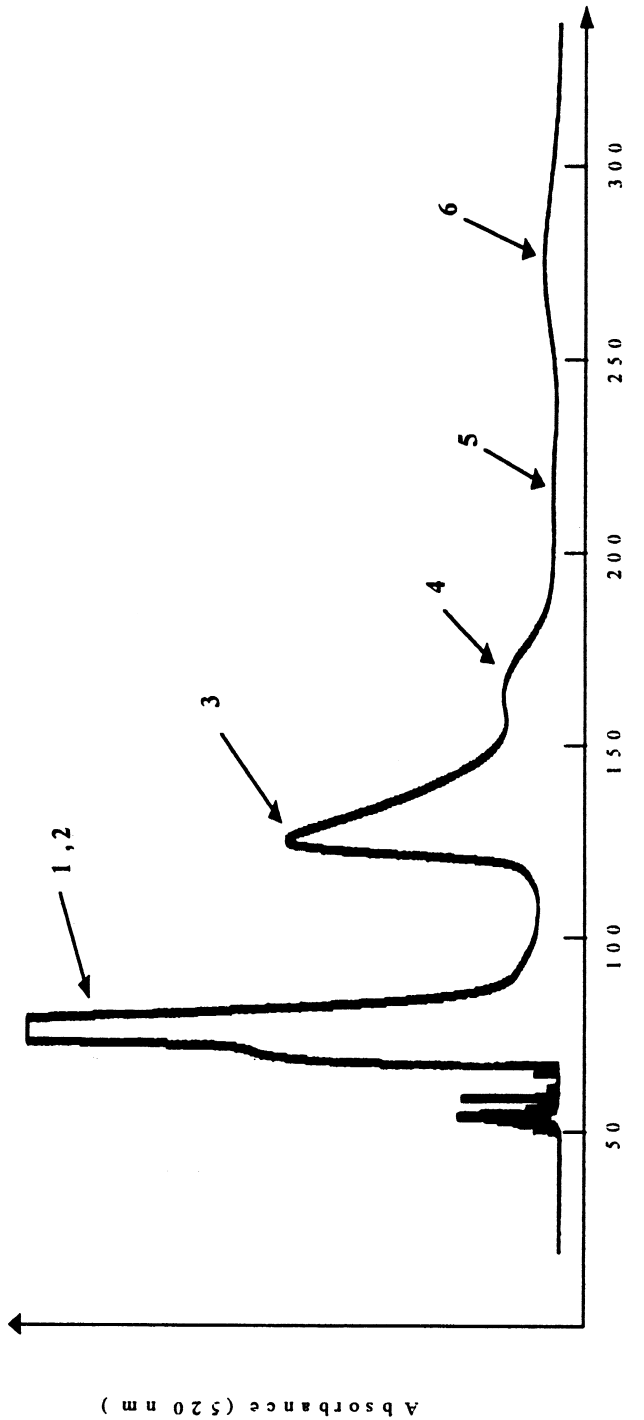

Figure 1. Separation of anthocyanins from a Californian red wine, for experimental conditions see text. Peonidin-3,5-diglucoside (**1**); malvidin-3,5-diglucoside (**2**); malvidin-3-glucoside (**3**); malvidin-3-(6”-coumaroylglucoside)-5-glucoside (**4**); peonidin-3-(6”-coumaroylglucoside)-5-glucoside (**5**); malvidin-3-(6”-acetylglucoside) (**6**).

teration of red wines.[11] With the aging of red wine, a shift in the anthocyanin profile takes place and leads to the formation of not well-characterized polymeric pigments. Moreover, aged wines show an increase in yellow color at $\lambda \sim 420$ nm and a shift from the brightness and purple tints of young wines to a more brownish color.[12]

A clean-up on Amberlite XAD-7 resin was carried out prior to HSCCC separation in order to remove sugars and organic acids present. The separation of a Californian red wine is shown in Fig. 1 (for structures cf. Fig. 2). The solvent system was MTBE-*n*-butanol-acetonitrile-water (2/2/1/5, acidified with 0.1% TFA, less dense layer as stationary phase, flow rate: 5.0 mL/min). Since anthocyanins **1** and **2** coeluted under the conditions applied, a slightly more hydrophilic solvent system was used for the separation (cf. Fig. 3). The solvent system employed was ethyl acetate-*n*-butanol-water (2/3/5), acidified with 0.1% TFA (less dense layer as stationary phase, flow rate: 2.8 mL/min). Identification of isolated fractions and purity control was done by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), electrospray ionization mass spectrometry (ESI-MS/MS), and HPLC with diode array detection (HPLC-DAD).[13] The isolated fractions were pure enough to serve as standards for HPLC determinations.

We managed to obtain up to 500 mg of pure malvidin-3-glucoside (**3**) from a bottle of a German 'Dornfelder' wine. Sample loads on the HSCCC with 850 mL of volume were typically in the range of 1 to 2 grams of the XAD-7 isolate. A solvent system for the isolation of coumaroyl and caffeoyl derivatives of anthocyanidins is ethyl acetate-water (1/1; acidified with 0.1% TFA). Suitable for the isolation of acetylated anthocyanins, is ethyl acetate-*n*-butanol-water (4/1/5; acidified with 0.1% TFA). The substitution of malvidin-3-glucoside with different groups necessitates solvent systems with a different polarity. The isolation of further pigments from red wine and studies on the color of red wines using the color activity concept, is described in detail by Degenhardt et al.[14]

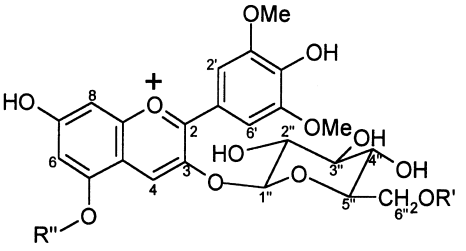

Figure 2. Structure of malvidin-based anthocyanins from red wine. Malvidin-3,5-diglucoside (**2**): R' = H, R" = glucose; malvidin-3-glucoside (**3**): R' = H, R" = H; malvidin-3-(6"-coumaroylglucoside)-5-glucoside (**4**): R' = coumaric acid, R" = glucose; malvidin-3-(6"-acetylglucoside) (**6**): R' = acetyl, R" = H.

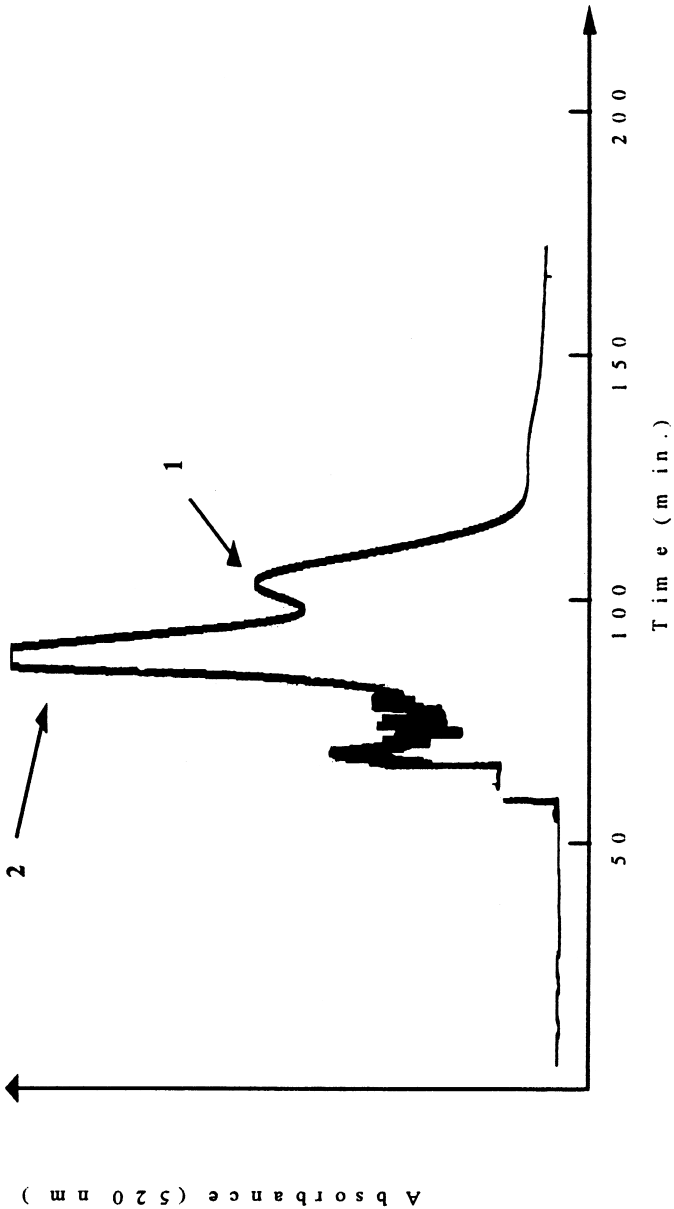

Figure 3. Separation of coeluting compounds from Fig. 1. For experimental conditions see text.

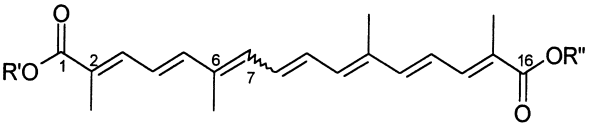

Figure 4. Structure of crocins. *all-trans*-crocin (**7**): R', R" = gentiobiosyl; 6-*cis*-crocin (**8**): R', R" = gentiobiosyl; *trans*-crocetin-mono-gentiobiosyl ester (**9**): R' = gentiobiosyl, R" = H.

Isolation of Water Soluble Carotenoids from Saffron (*Crocus sativus* L.) and *Gardenia jasminoides* by HSCCC

Saffron is the dried, dark-red stigmata of *Crocus sativus*. Saffron is used as spice for flavoring, as well as, for its coloring properties.[15] Saffron and *Gardenia jasminoides* contain a range of water soluble carotenoids, the so-called crocins which are glucosyl esters of crocetin. Due to the high price of saffron (150 000 flowers are needed to obtain 1 kg of the precious spice), the fruits of *Gardenia jasminoides* may serve as a substitute in terms of coloring properties. A separation of crocins (cf. Fig. 4) by HSCCC using ethyl acetate-*n*-butanol-water (2/3/5) as solvent mixture was published by Oka et al.[16] Reversed elution order of the solvent system MTBE-*n*-butanol-acetonitrile-water (2/2/1/5, flow rate: 5mL/min) at the end of the HSCCC separation, led to the elution of the more hydrophobic crocetin-mono-gentiobiosyl ester (**9**) (cf. Fig. 5). **9** was detected by HPLC-MS in foods colored with gardenia yellow.[17]

Spectroscopic data (^{13}C-NMR and two-dimensional NMR experiments HMBC and HMQC) showed the presence of 32 carbon atoms (i.e. C20 of crocin plus two hexoses), and HMBC experiments showed cross peaks, which proved that the two glucose moieties had a (1→6)-linkage (i.e. forming the disaccharide gentiobiose) and unambiguously identified the site of attachment of the gentiobiose to the crocin molecule. The structure of **9** was, therefore, deduced as depicted in Fig. 4. However, it should be noted that an unknown compound is coeluting with **8** under the HSCCC conditions applied (cf. Fig. 5), whereas **7** and **9** were of good purity as shown by HPLC and MS analysis of the fractions.

Purification of Carminic Acid by pH-Zone Refining

Carminic acid is a colorant that is obtained from the bodies of female insects which live on cactus and the roots of some trees. Carmine is the word used to describe the aluminium chelate of carminic acid. The word cochineal is often encountered and describes the dried insects themselves, as well as, the color

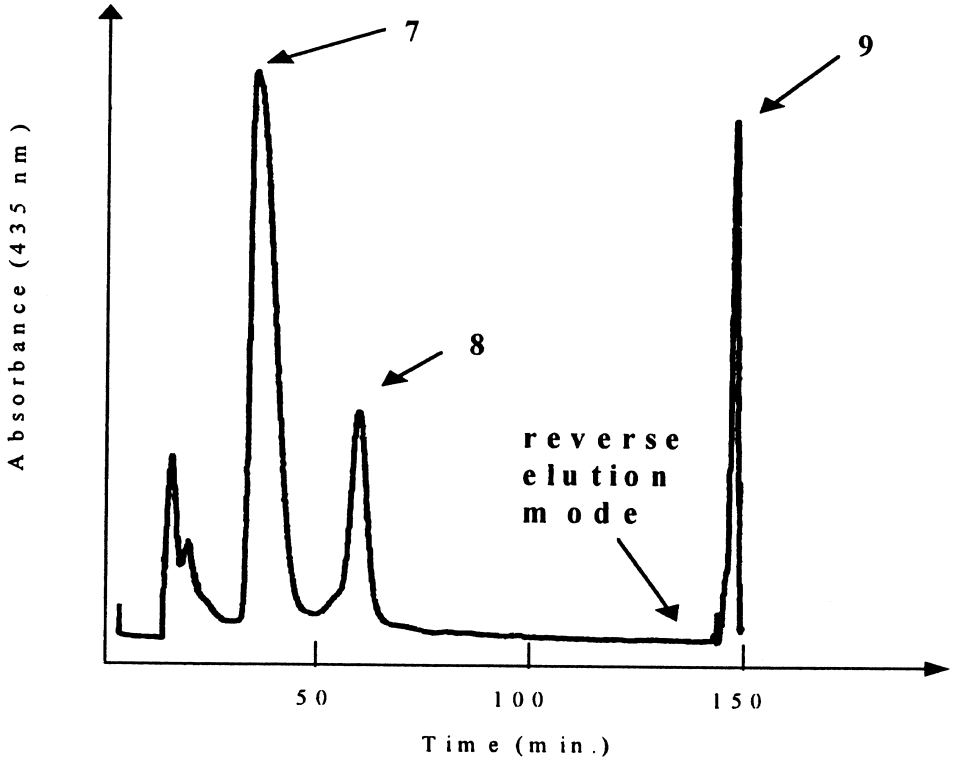

Figure 5. HSCCC separation of a methanolic extract from fruits of *Gardenia jasminoides*, solvent system was MTBE-*n*-butanol-acetonitrile-water (2/2/1/5, less dense layer acting as stationary phase), flow rate 5 mL/min. The arrow indicates when the elution order was reversed. For structures cf. Fig. 4.

derived from them. Cochineal insects have been used for thousands of years as a source of red color. The current principal source of cochineal insects is Peru.[18] Carminic acid (cf. Fig. 6 for structure) is water soluble and its shade in solution is pH dependent. It is orange in acidic solution and violet when alkaline. Cochineal and carmine are widely permitted for use as a food colorant. Recently, allergic reactions due to ingestion of carmine colored foods were reported.[19, 20] HSCCC separation of a commercial carminic acid sample by pH-zone refining is shown in Fig. 6. 700 mg of pure **10** were obtained in a single run on the single coil instrument with 350 mL of volume. HPLC chromatograms (detection at 500 nm) of the crude sample, and after purification, are shown in Fig. 7. ^{1}H-NMR spectra also demonstrated that the purity of **10** is good (cf. Fig. 7 bottom).

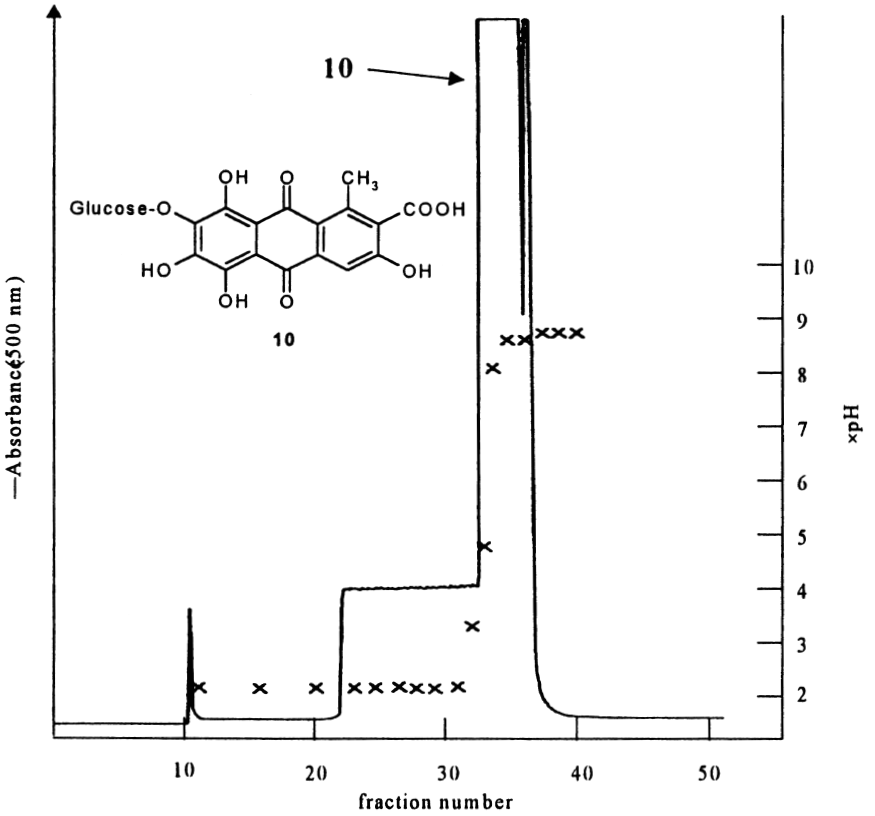

Figure 6. pH-Zone refining of carminic acid (**10**). Solvent system: MTBE – *n* – butanol –acetonitrile - water (1 /3 /1 /5, lower phase mobile), 15 mM TFA was added to the upper phase; 15 mM of NH_3 was added to the more dense phase; flow rate: 1.9 mL/min; single coil HSCCC with 350 mL of volume.

Purification and Isolation of Theaflavic Acids from Black Tea by HSCCC

While green tea is heat-treated to prevent enzymatic oxidation of flavanols (catechins), black tea has undergone a fermentation process. Colorless catechins form a variety of pigments during this process. Theaflavins (TF), compounds, which exhibit a bright orange-red color in solution are formed. TF are believed to make important contributions to the "brightness" of tea infusions, an important marker for the evaluation of tea quality.[21] Also, theaflavic acids (cf. Fig. 8 for

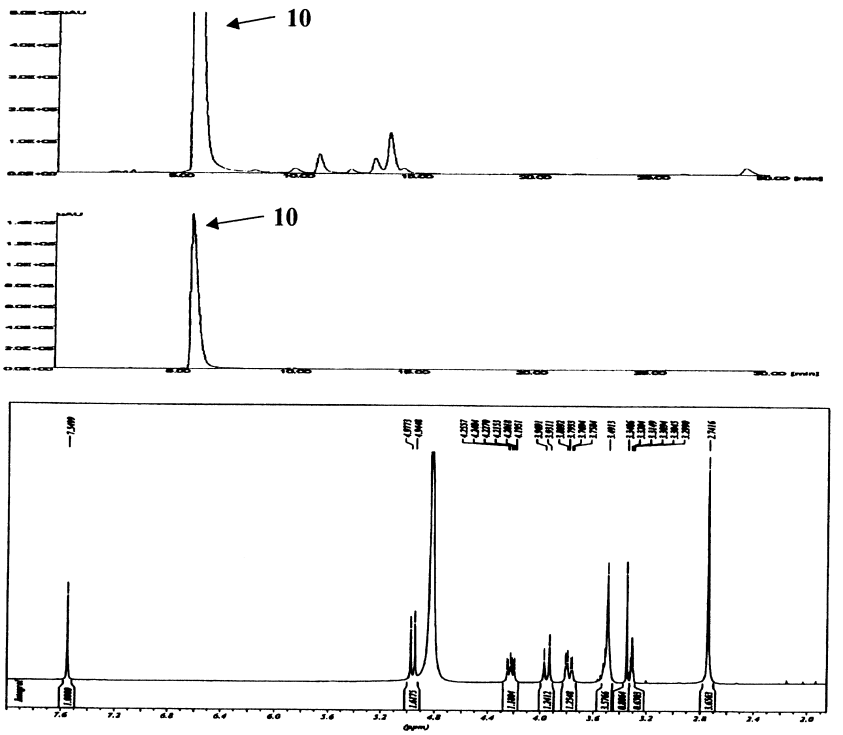

Figure 7. HPLC analysis of crude carminic acid (top), detection at 500 nm; HPLC analysis of **10** purified by pH-zone refining (middle); ^{1}H-NMR spectrum (in CD_3OD) of purified **10** (bottom).

structures) are formed from catechins and gallic acid.[3] Both groups of compounds contain a benzotropolone functionality in the molecule. Isolation of these compounds can be carried out by a combination of chromatography on Sephadex LH20 and HPLC.[3] HSCCC represents another method for rapid isolation of theaflavic acids from black tea. A clean-up of a black tea extract was done on Sephadex LH20.[3] This clean-up step allows removal of catechins, compounds which potentially coelute under the HSCCC conditions applied for theaflavic acid isolation. Hexane-ethyl acetate-methanol-water (2/5/2/5, less dense layer as stationary phase, flow rate: 2.8 mL/min) was found to be a suitable solvent system for the separation of theaflavic acids (cf. Fig. 8). ^{1}H-NMR, HPLC, and ESI-MS analysis showed that the fractions were of good purity. Spectral data was found to be in line with literature data.[3,22]

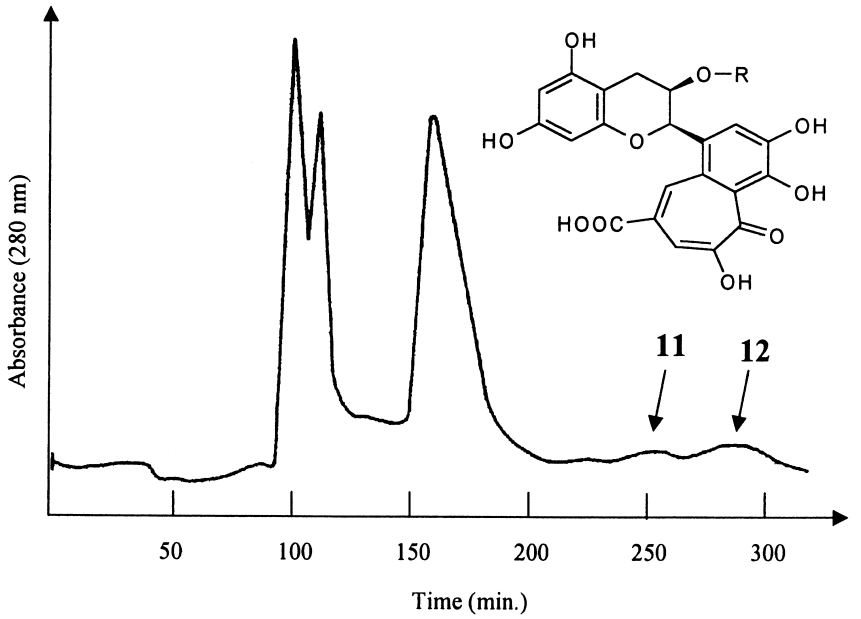

Figure 8. Isolation of theaflavic acids from a black tea extract. For experimental conditions see text. Epitheaflavic acid-3'-monogallate (**11**): R = gallate; epitheaflavic acid (**12**): R = H.

Isolation of Hydrophilic Thearubigins (TR) from Black Tea by HSCCC

Whereas TF and theaflavic acids are well-characterized classes of compounds, the structure of thearubigins, which also provide color to the black tea beverage, still remains unknown. TR represent a heterogeneous group of compounds which originate from the polyphenol oxidase mediated oxidation of catechins. The name TR goes back to Roberts (1958) who assigned the name TR to all acidic brown pigments of black tea.[23] The author classified this heterogeneous class of compounds into SII, SI, and SIa thearubigins. SI TR are extractable into ethyl acetate, while those remaining in the aqueous phase are named the SIa and SII TR, respectively, with the SIa group being more soluble in diethyl ether. Chromatographed on reversed-phase packings by HPLC, TR elute in the form of a "hump", upon which more or less resolved polyphenols can be seen.[24,25] TR represent a challenge for the preparative isolation, because it is widely known that this polymeric class of polyphenols exhibits a strong affinity to many solid sup-

ports used in column chromatography. Therefore, we attempted isolation of TR by HSCCC and found that the solid support free technique is ideally suited for the preparative TR isolation and separation from coeluting monomeric polyphenols.

A clean-up of a black tea extract was performed on Amberlite XAD-7 resin according to a procedure described in Fig. 9. HSCCC separation of the methanolic XAD-7 isolate from black tea using ethyl acetate-*n*-butanol-water (2/3/5, more dense layer as mobile phase, flow rate: 2.8 mL/min) resulted in separation of TR from monomeric phenolics (cf. Fig. 10). HPLC analysis of the isolated TR fraction showed a convex "hump" which was free of coeluting monomers (cf. Fig. 11, right side). Typical sample loads were in the range of 1 to 2 grams of the XAD-7 isolate on the 850 mL triple coil HSCCC. Further studies on the isolated TR are described in more detail by Degenhardt et al.[4]

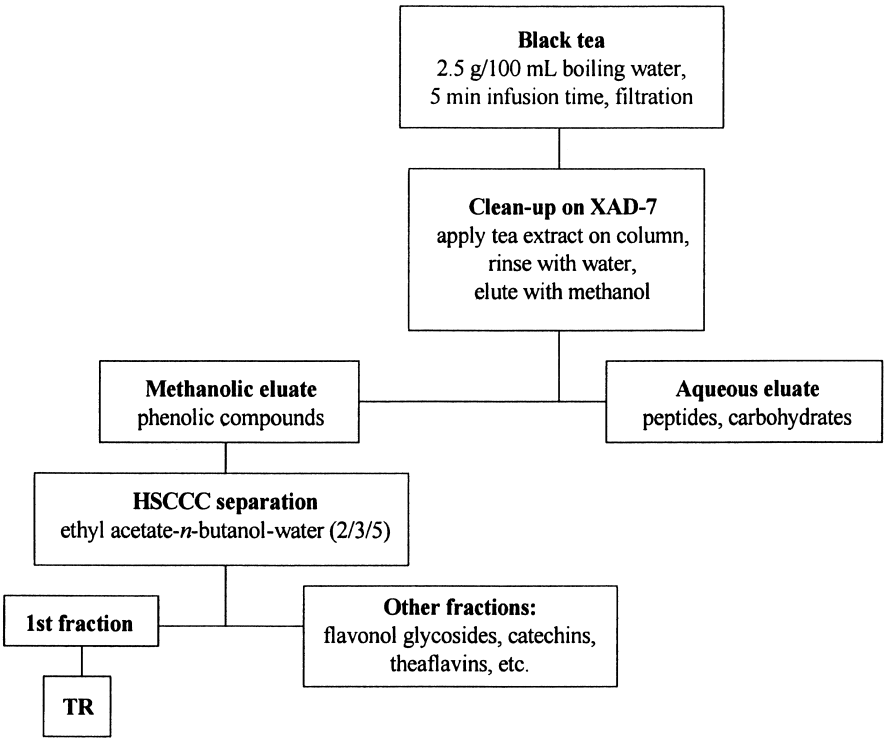

Figure 9. Clean-up of black tea extract.

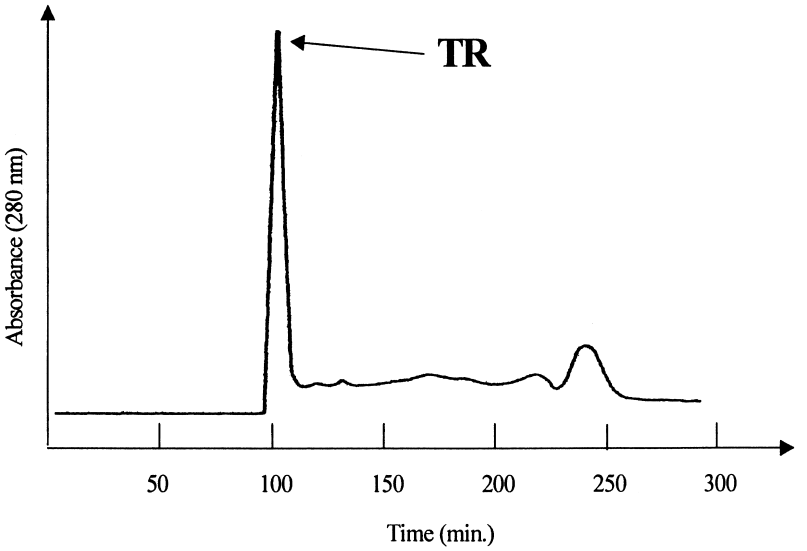

Figure 10. HSCCC separation of XAD-7 isolate from black tea. For experimental conditions see text.

Isolation of Betalains from Red Beet by HSCCC

The pigments in red beet root are collectively known as betalains and can be divided into two classes, the red betacyanins and the yellow betaxanthins. The most prominent betacyanin is the red-violet betanin, which represents up to 95% of the total betacyanins.[18] Vulgaxanthin I and II are the principal yellow colorants (betaxanthins). Although betanin has a very intense color and may be stronger than many synthetic colorants, its application is often limited, since the pigment is susceptible to heat degradation.[18] Another factor affecting betanin stability is oxygen.[26] The stability of betanin in red beet juice is better than in isolated form. It is known that a partial re-synthesis from the products of hydrolysis can take place.[27] Betaxanthins were, furthermore, recently identified as potent antioxidants and exhibit various other physiological effects.[28,29]

Due to the very hydrophilic character of betalains, the search for a suitable solvent system for HSCCC separation resulted in discovery of ethanol-acetonitrile-ammonium sulfate solution (saturated)-water (1 /0.5 /1.2 /1), a solvent system that exceeds the polarity of butanol-acetic acid-water systems. It was found that equal volumes of upper and lower phase were produced from the mixture and that the settling time of the system was shorter than 30 sec. Furthermore, the retention of stationary phase was excellent (often > 70%). The polarity of this

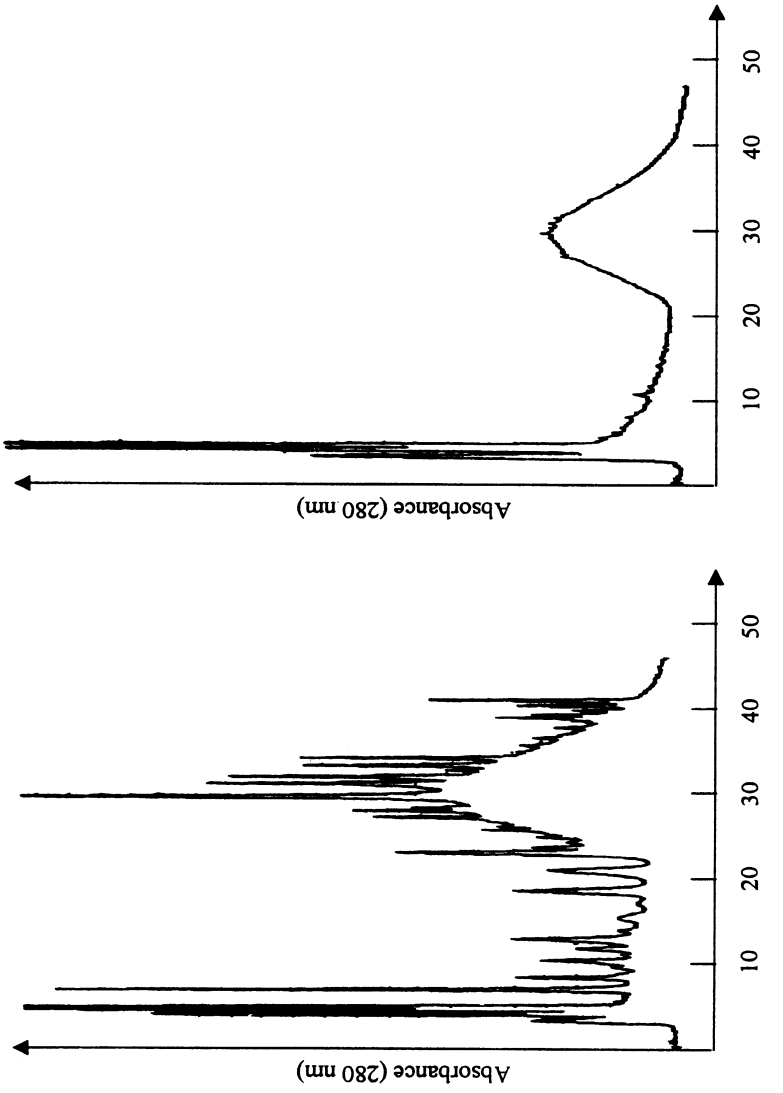

Figure 11. HPLC chromatogram of black tea extract (left side), detection at 280 nm; the whole range of flavonoids can be detected. HPLC chromatogram of the isolated TR fraction (right side); TR are free of coeluting monomeric phenolics.

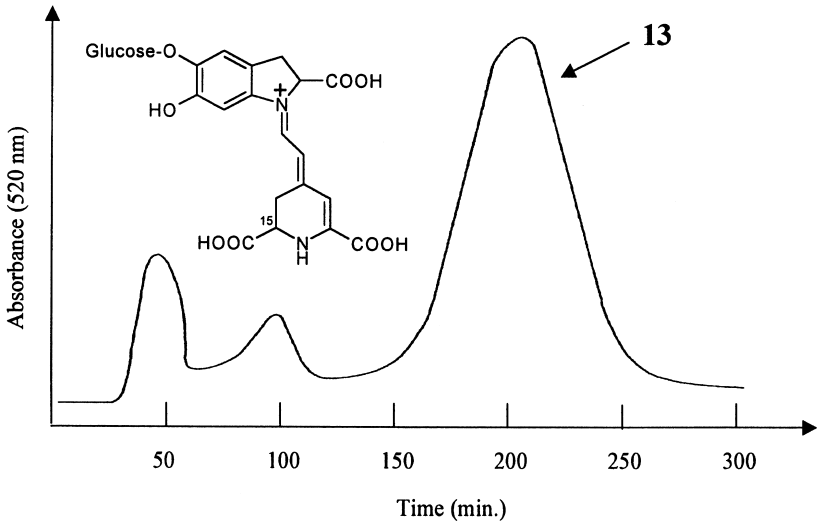

Figure 12. HSCCC separation of a concentrated red beet juice. For experimental conditions see text. Betanin (**13**) (5-*O*-glucoside of betanidin) and its epimer at C-15.

solvent system can be adjusted within certain limits by changing the concentration of the ammonium sulfate solution (below 45% ammonium sulfate (w/v) the system does not generate two phases any more).

The separation of a concentrated red beet juice is shown in Fig. 12. The more dense layer acted as stationary phase in the Tail→Head mode, the flow rate was set at 1.8 mL/min. The single coil HSCCC with 350 mL of volume was used for the separation. Peak identity and purity control after desalting on a Polyamide column (pigments are absorbed, salt is rinsed with water, elution of pigments is done with 0.05% NH_3 in methanol (v/v)), was carried out by HPLC-DAD, ESI-MS/MS, and ^{1}H-NMR (recorded in MeOD + trace of TFA (d_6); data was in accordance with reference (30)). The peak indicated in Fig. 12 represented a mixture of betanin and its C-15 epimer isobetanin.

CONCLUSION

HSCCC is ideally suited for the preparative isolation of hydrophilic pigments, which was demonstrated for a range of water soluble compounds from a variety of foods.

ACKNOWLEDGMENTS

The authors wish to thank Dr. H. Knapp for MS analysis, Mrs. C. Stingl and Mrs. M. Messerer for recording NMR spectra, as well as, Mrs. C. Balcke for help in preparing the manuscript. Prof. Watanabe's donation of a *Gardenia jasminoides* extract is gratefully acknowledged.

REFERENCES

1. Joshi, P. Physical Aspects of Color in Foods. Chem. Innov. **2000**, *30* (2), 19-24.
2. Degenhardt, A.; Knapp, H.; Winterhalter, P. Rapid Isolation of Malvidin 3-Glucoside from Red Wine by High-Speed Countercurrent Chromatography. Vitis **2000**, *39*, 43-44.
3. Davis, L.; Cai, Y.; Davies, A.P. H and ^{13}C NMR Assignment of Theaflavin, Theaflavin Monogallate and Theaflavin Digallate. Magn. Reson. Chem. **1995**, *33*, 549-552.
4. Degenhardt, A.; Engelhardt, U.H.; Wendt, A.-H.; 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**, in press.
5. Pourrat, X.; Lejeune, B.; Grand, A.; Pourrat, H. Betalains Assay of Fermented Red Beet Root Extract by High Performance Liquid Chromatography. J. Food Sci. **1988**, *53,* 294-295.
6. Kreuz, S.; Flak, W.; Kernbauer, E.; Pitschmann, M.; Schaber, R. Qualitative Assessment of Wines by Tristimulus Measurements and Related Techniques. Mitt. Klosterneuburg **1998**, *48*, 25-33.
7. Heredia, J.; Guzmán-Chozas, M. The Color of Wine: A Historical Perspective I. Spectral Evaluations. J. Food Qual. **1993**, *16*, 429-437.
8. Heredia, F.J.; Guzmán-Chozas, M. The Color of Wine: A Historical Perspective. II. Trichromatic Methods. *J. Food Qual.* **1993**, *16*, 439-449.
9. Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*, CRC Press: Boca Raton, FL, 1993.
10. Fischer, U.; Strasser, M.; Gutzler, K. Impact of Fermentation Technology on the Phenolic and Volatile Composition of German Red Wines. Int. J. Food Sci. Technol. **2000**, *35*, 81-94.
11. Holbach, X.; Marx, R.; Ackermann, M. Determination of Anthocyanin Composition of Red Wines using High Pressure Liquid Chromatography (HPLC). *Lebensmittelchemie* **1997**, *51*, 78-80.
12. Heredia, F.J.; Francia-Aricha, E.M.; Rivas-Gonzalo, J.C.; Vicario, I.M.; Santos-Buelga, C. Chromatic Characterization of Anthocyanins from Red Grapes - I. pH Effect. Food Chem. **1998**, *63*, 491-498.

13. 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.
14. 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**, Submitted.
15. Straubinger, M.; Winterhalter, P. Saffron – Renewed Interest in an Ancient Spice. Food Rev. Int. **2000**, *16*, 39-59.
16. Oka, H.; Ikai, Y.; Yamada, S.; Hayakawa, J.; Harada, K.-I.; Suzuki, M.; Nakazawa, H.; Ito, Y. Separation of Gardenia Yellow Components by High-Speed Countercurrent Chromatography. In *Modern Countercurrent Chromatography*; Conway, W.D.; Petroski, R.J., Eds.; ACS Symposium Series 593; American Chemical Society: Washington, DC, 1995; 92-106.
17. Ichi, T.; Higashimura, Y.; Katayama, T.; Koda, T.; Tada, M. Determination of a Food Colorant, Gardenia Yellow Pigment, in Processed Foods with a High Performance Liquid Chromatograph/Photodiode-Array Detector/ Mass Spectrometer System. *J. Food Hyg. Soc. Japan* **1995**, *36*, 482-489.
18. *Natural Food Colorants*, Hendry, G.A.F., Houghton, J.D., Eds.; Blackie Academic & Professional: Bishopbriggs, Glasgow, UK, 1996.
19. Acero, X.; Tahar, A.I.; Alvarez, M.J.; Garcia, B.E.; Olaguibel, J.M.; Moneo, I. Occupational Asthma and Food Allergy Due to Carmine. Allergy **1998**, *53*, 897-901.
20. DiCello, M.C.; Myc, A.; Baker, J.R.; Baldwin, J.L. Anaphylaxis After Ingestion of Carmine Colored Foods: Two Case Reports and a Review of the Literature. Allergy and Asthma Proceedings **1999**, *20*, 377-382.
21. Balentine, X.; Wiseman, S.A.; Bouwens, L.C.M. The Chemistry of Tea Flavonoids. Crit. Rev. Food Sci. Nutr. **1997**, *37*, 693-704.
22. Collier, P.D.; Bryce, T.; Mallows, R.; Thomas, P.E. The Theaflavins of Black Tea. Tetrahedron Lett. **1973**, *29*, 125-142.
23. Roberts, H. The Phenolic Substances of Manufactured Tea. II. – Their Origin as Enzymic Oxidation Products in Fermentation. J. Sci. Food Agric. **1958**, *9*, 212-216.
24. 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.
25. 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.
26. Attoe, L.; Von Elbe, J.H. Oxygen Involvement in Betanine Degradation: Effect of Antioxidants. J. Food Sci. **1985**, *50*, 106-110.

27. Czapski, J. Heat Stability of Betacyanins in Red Beet Juice and in Betanin Solutions. Z. Lebensm. Unters. Forsch. **1990**, *191*, 275-278.
28. Zakharova, N.S.; Petrova, T.A. Relationships Between the Structure and Antioxidant Activity of Certain Betalains. Prikl. Biokhim. Mikrobiol. **1998**, *34*, 199-202.
29. Pourrat, X.; Lejeune, B.; Grand, A.; Bastide, P.; Bastide, J. Propriétés du jus et des Colorants de la Betterave Rouge. Méd. et Nut. **1987**, *23*, 166-172.
30. Heuer, S.; Wray, V.; Metzger, J.W.; Strack, D. Betacyanins from Flowers of *Gomphrena Globosa.* Phytochemistry **1992**, *31*, 1801-1807.

Received August 19, 2000 Author's Revision October 10, 2000
Accepted October 11, 2000 Manuscript 5485