POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2005, Vol. 14/55, No 3, pp. 243–247

RAPID CHROMATOGRAPHIC METHOD FOR SEPARATION OF GREEN TEA PROANTHOCYANIDINS

Magdalena Karamać[1], Agnieszka Kosińska[1], Uttam D. Chavan[2]

[1]Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland; [2]Biotechnology Centre, Mahatma Phule Agricultural University, Rahuri, India

Key words: green tea, proanthocyanidins, catechins, column chromatography

A rapid chromatographic method for the separation of proanthocyanidins from the phenolic extract of green tea was described. A crude catechin mixture from green tea was applied onto a Toyopearl HW-40S column (TosoHaas). Six fractions (I–VI) were eluted from the column using ethanol as a mobile phase. Fractions I–III were free of catechins. Catechins (EC, ECG, EGC, EGCG) were present in fractions IV–VI. Two proanthocyanidin fractions (VII–VIII) were eluted from the column with acetone-water (1:1; v/v). Proanthocyanidins present in these fractions exhibited a strong positive vanillin reaction. The absence of catechins in the proanthocyanidin fractions was confirmed by means of thin layer chromatography.

INTRODUCTION

Green tea is of great interest due to its beneficial medicinal properties such as antioxidant, antimicrobial, anticarcinogenic, and antimutagenic ones [Amarowicz & Shahidi, 1995a; Amarowicz *et al.*, 2000, 2005; Kondo *et al.*, 1999; Sartippour *et al.*, 2001]. The main polyphenols in green tea are catechins, namely (-) epicatechin (EC), (-) epigallocatechin (EGC), (-) epicatechin-3-gallate (ECG), and (-) epigallocatechin-3-gallate (EGCG). According to Hashimoto *et al.* [1992], the content of proanthocyanidins in green tea is at least 10 times lower than that of catechins. Four dimeric proanthocyanidin gallates (prodelphidin B-2 3'-*O*-gallate, procyanidins B-2 3,3'-di-*O*-gallate, B-2 3'-*O*-gallate, and B-4 3'-*O*-gallate) and two dimeric flavan-3-ol gallates in which two flavanol units are linked at the B-ring have been reported by Nonaka *et al*. [1983]. At least 12 proanthocyanidins were identified in green tea samples by means of HPLC-MS and HPLC-DAD [Kiehne *et al.*, 1997]. Among them EGC-EGCG, EC-EC-EC, EC-EGC, EGC-EGC, EGCG-EGCG, EC-ECG, EGCG-ECG, ECG-EGCG, ECG-ECG were present.

Proanthocyanidins of green tea were found to be the antimicrobial compounds [Amarowicz *et al.*, 2000]. Their antioxidant activity was confirmed using a meat model system [Amarowicz *et al.*, 2005]. The antitumor promoting compound of green tea was found as the complex of tannins with carbohydrates [Nakamura *et al.*, 1998].

Investigations of the biological activity of green tea proanthocyanidins must be preceded by their separation from catechins. The column chromatography with macroporous gel method which enables obtaining a procyanthocyanidin fraction free of catechins from green tea was described in this study.

MATERIAL AND METHODS

Chemicals. All solvents used were pure for analysis or HPLC grade. Methanol, chloroform, acetone, ethanol, *n*-butanol, acetic acid, and hydrochloric acid were acquired from the P.O.CH. S.A. (Gliwice, Poland), vanillin and Folin-Ciocalteau's reagent were from Sigma Chemical Co. (Poznań, Poland), and standards of catechins – from Extrasynthese S.A. (Genay Cedex, France).

Material. Chinese green tea leaves were obtained from Anhui Province (People Republic of China).

Extraction of crude catechins. A crude mixture of catechins was extracted from 50 g green tea leaves using 500 mL of hot water (80°C) over 1 h period [Price & Spitzer, 1993].

Column chromatography. Four grams of crude catechins were dissolved in 15 mL of ethanol and applied onto a silica gel (Merck, Darmstad, Germany) chromatographic column (2.5×30 cm) packed with Toyopearl HW-40S (TosoHaas) equilibrated with ethanol. The same solvent was used for elution. Fractions (7 mL) were collected using a fraction collector. The absorbance of eluates from each tube was measured at 280 nm. In addition, the absorbance at 500 nm was read after color development reaction for catechins [Price *et al.*, 1978]. When eluate obtained was free of phenolics, a new mobile phase acetone-water (1:1; v/v) was applied. This part of the column chromatography was mon-

Author's address for correspondence: Magdalena Karamać, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, ul. Tuwima 10, P.O. Box 55, 10-747 Olsztyn, Poland; tel.: (48 89) 523 46 27; fax: (48 89) 524 01 24; e-mail: magda@pan.olsztyn.pl

itored by absorbance at 500 nm and 725 nm after color development reaction for catechins [Price *et al.*, 1978] and phenolics [Naczk & Shahidi, 1989]. According to the absorbance values eluates were pooled into 8 fractions: I–VI eluted with ethanol and VII–VIII eluted with acetone-water. Organic solvents were evaporated, water removed by lyophylisation, and dried residues weighed. UV spectra of individual fractions, dissolved in methanol, were measured using a Beckman DY-7500 diode array spectrophotometer.

Total phenolics. The content of total phenolics in the extract and in each fraction was estimated using the Folin-Ciocalteau's reagent [Naczk & Shahidi, 1989]. (+)-Catechin was used as a standard.

Vanillin assay for proanthocyanidins. Tannin content of the fractions was determined using the modified vanillin assay [Price *et al.,* 1978] and expressed as absorbance (A_{500}) units per 1 mg fraction (A_{500}/mg).

Acid butanol assay for proanthocyanidins (according to Porter *et al.* [1986]). To a 10 mL screw cap tube 6 mL of the acid butanol reagent (950 mL of *n*-butanol with 50 mL concentrated HCl), 1.0 mg aliquot of the fraction, and 0.2 mL of the iron reagent (2% ferric ammonium sulfate in 2 mol/L HCl) were added and vortexed. The tube was capped loosely, and put in a boiling water bath for 50 min. Then the tube was cooled and the solution was transferred to a volumetric flask and adjusted to 25 mL with acid butanol. The absorbance was read at 550 nm. Results were expressed as absorbance (A_{550}) units per 1 mg (A_{550}/mg).

UV spectra. UV spectra of individual fractions were recorded with a Beckman DU 7500 diode array spectrophotometer.

Thin layer chromatography. Fractions separated were also examined on silica gel TLC plates (Sigma) with mobile phase chloroform-methanol-water (65:35:10, v/v/v, lower phase) [Amarowicz & Shahidi, 1995b]. Vanillin-hydrochloric acid reagent [Karchesy *et al.*, 1989] was used for a color development reaction of catechins.

RESULTS AND DISCISSION

Six fractions (I–VI) were eluted from the Toyopearl HW-40S column using ethanol as a mobile phase (Figure 1). Fractions I–III were free of catechins. Relative contents of these fractions were 0.8%, 1.9%, and 4.5% (Table 1). They exhibited the maximum UV absorbance at 274 nm (I), 272 nm (II), 270 nm and 353 nm (III), respectively. Shoulders of fraction I and II were observed at 310 nm and 306 nm, respectively (Figure 2, Table 2). The absorption band at 353 nm originated from flavonols [Mabry *et al.* 1970]. The presence of quercetin in green tea was reported by Wang *et al.* [2004].

Catechins (EC, ECG, EGC, EGCG) were present in fractions IV-VI. The results of TLC demonstrated the presence of all catechins in each fraction. The prevailing catechin was epigallocatechin gallate (EGCG). The relative

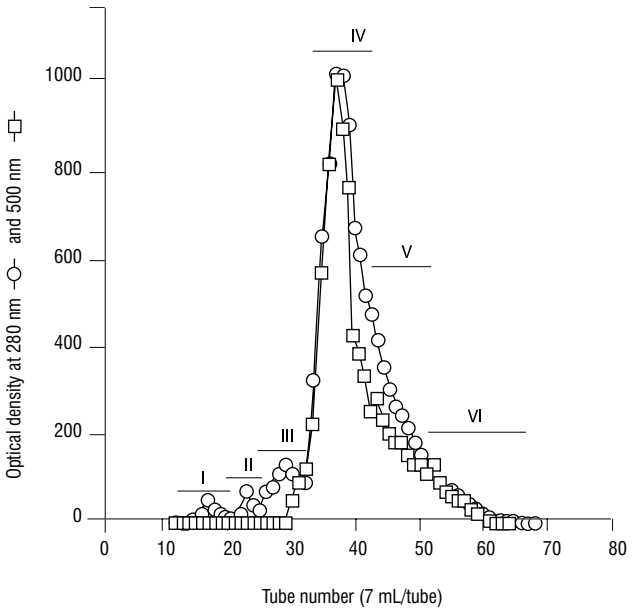

FIGURE 1. Separation of crude catechins of green tea using Toyopearl HW-40S column chromatography with ethanol as mobile phase.

TABLE 1. Relative content of separated fractions (%).

Mobile phase	Fraction	Relative content (%)*
Ethanol	I	0.8
	II	1.9
	III	4.5
	IV	72.6
	V	12.2
	VI	5.2
Acetone-water (1:1, v/v)	VII	1.6
	VIII	1.2

* the percent in the relation to the sum of the mass of the individual fractions

proportion of fraction IV in the extract was the greatest at 72.6% (Table 1). The content of total phenolics in catechin fractions was higher than in the non-catechin ones (Table 2). UV spectra of fractions IV-VI were characterised with absorption bands at 277 nm (IV), 271 nm (V), and 273 (VI). Similar spectral data were reported for a catechin fraction separated from green tea using Sephadex LH-20 [Amarowicz & Shahidi, 1995b] and silica gel column chromatography [Amarowicz *et al.*, 2003].

Two not base-line separated proanthocyanidin fractions (VII-VIII) were eluted from the column by acetone-water (1:1; v/v) (Figure 3). The relative content of these fractions was only 1.6%, and 1.2% (Table 1) but they exhibited a very high content of total phenolics: 612 and 684 mg/g, respectively (Table 2). Vanillin and *n*-butanol/HCl assays applied for proanthocyanidins fractions gave much higher absorbance readings than in the case of the fractions obtained by column chromatography with ethanol as a mobile phase (Table 3). The same UV spectra with maxima at 279 nm were observed for both proanthocyanidin fractions (Figure 4, Table 2). Similar spectral data were measured for proanthocyanidin fractions of leguminous seeds [Amarowicz & Troszyńska, 2003; 2004]. Thin layer chromatography confirmed the absence of catechins in the proanthocyanidin fractions.

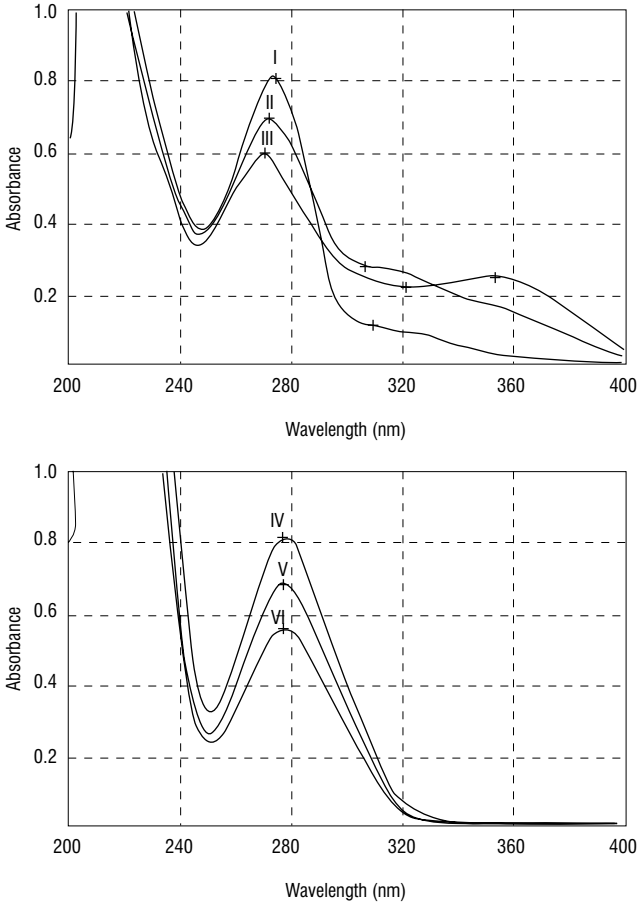

FIGURE 2. UV spectra of fractions separated on a Toyopearl HW-40S column with ethanol as mobile phase.

TABLE 2. Total phenolic content (mg/g) and UV spectra data of separated fractions.

Mobile phase	Fraction	Total phenolics (mg/g)	λ_{max} (nm)	λ_{sh} (nm)
Ethanol	I	75	274	310
	II	104	272	306
	III	184	270; 353	
	IV	342	277	
	V	434	271	
	VI	646	273	
Acetone-water	VII	612	279	
(1:1, v/v)	VIII	684	279	

TABLE 3. Proanthocyanidin content of separated fractions (absorbance/mg fraction).

Mobile phase	Fraction	A_{500}/mg	A_{550}/mg
Ethanol	I	-	-
	II	-	-
	III	-	-
	IV	0.447	0.245
	V	0.357	0.161
	VI	0.630	0.331
Acetone-water (1:1, v/v)	VII	0.794	0.473
	VIII	0.815	0.602

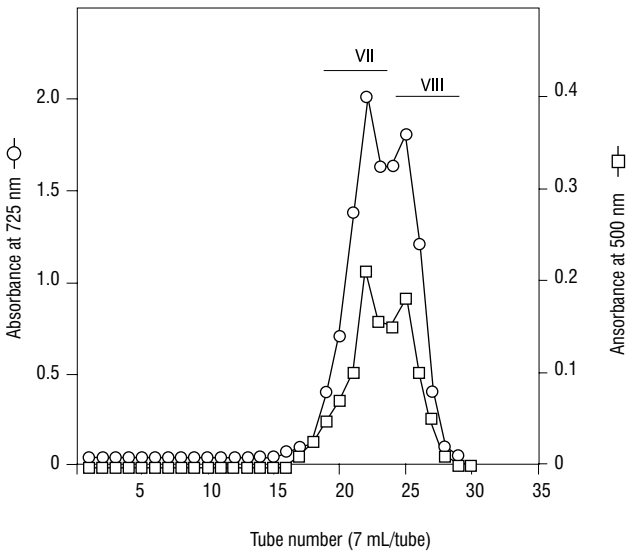

FIGURE 3. Separation of proanthocyanidin of green tea using Toyopearl HW-40S column chromatography with acetone-water (1:1) as mobile phase.

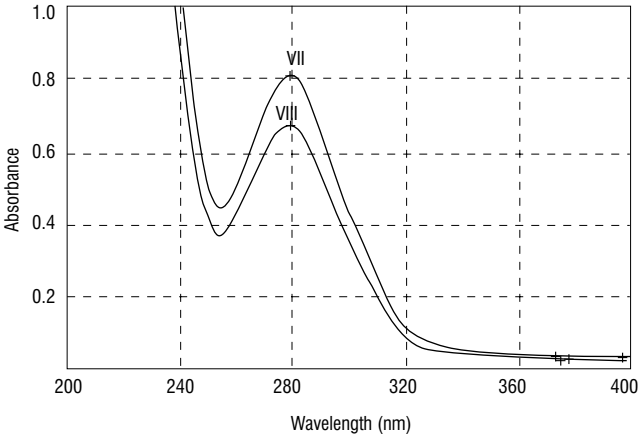

FIGURE 4. UV spectra of fractions separated on a Toyopearl HW-40S column with acetone-water (1:1) as mobile phase.

The method used for the separation of proanthocyanidin fractions was fast and needed one type of gel with two mobile phases. To the same end, other authors applied more advanced chromatographic methods. Kiehne *et al.* [1997] used a polyamide column chromatography for a clean-up procedure for separation of proanthocyanidins of green tea. Elution was achieved with methanol (2×100 mL), 2% acetic acid in methanol (100 mL), and N,N-dimethyl formamid (100 mL). Proanthocyanidins were present in the fraction eluted with the last two solvents. Using this system EGGC was accompanied by proanthocyanidins. In a study of Nonaka *et al.* [1983], the isolation of tannins from the extract of green tea leaves was successfully achieved by a combination of Sephadex LH-20 and high porosity polystyrene gel (Diaion HP-20) chromatography. Two hydrolysable tannins were isolated from green tea by Nonaka *et al.* [1984] who used Sephadex LH-20 with H_2O and that with H_2O containing an increasing amount of methanol.

CONCLUSIONS

The application of the column chromatography on Toyopearl HW-40S macropours gel with mobile phases of ethanol and acetone-water (1:1, v/v) is reliable for fast and easy separation of a proanthocyanidin fraction from catechin crude extract of green tea. The method can be used as the first step of separation of individual procyanidins from green tea before semi-preparative HPLC.

REFERENCES

1. Amarowicz R., Shahidi F., Antioxidant activity of green tea catechins in a β-carotene-linoleate model system. J. Food Lipids, 1995a, 2, 47–56.
2. Amarowicz R., Shahidi F., A rapid chromatographic method for separation of individual catechins from green tea. Food Res. Intern., 1995b, 29, 71–76.
3. Amarowicz R., Pegg R.B., Bautista D.A., Antibacterial activity of green tea polyphenols against *Escherichia coli* K12. Nahrung, 2000, 44, 60–62.
4. Amarowicz R., Shahidi F., Wiczkowski W., Separation of individual catechins from green tea using silica gel column chromatography and HPLC. J. Food Lipids, 2003, 10, 165–177.
5. Amarowicz R., Troszyńska A., Antioxidant activity of extract of pea and its fractions of low molecular phenolics and tannins. Pol. J. Food Nutr. Sci., 2003, 12/53 S1, 10–15.
6. Amarowicz R., Troszyńska A., Antioxidant and antiradical activity of extracts of phenolic compounds from red bean. Czech J. Food Sci., 2004, 22, 206–208.
7. Amarowicz R., Pegg R.B., Troszyńska A., Shahidi F., Assessment of the antioxidant activity of green tea extracts using a meat model system. ACS Symposium Series, 2005, in press.
8. Hashimoto F., Nonaka G., Nishioka I., Tannins and related compounds. CXIV. Structures of novel fermentation products, theogallinin, theaflavonin and desgalloyl theaflavonin from black tea, and changes of tea leaf polyphenols during fermentation. Chem. Pharm. Bull., 1992, 40, 1383–1389.
9. Karchesy J.J, Bae Y., Chalker-Scott L., Helm R.F., Foo L.Y., Chromatography of proanthocyanidins. 1989, *in*: Chemistry and Significance of Condensed Tannins (eds. R.W. Hemingway, J.J. Karchesy). Plenum Press, New York and London, pp. 139–151.
10. Kiehne A., Lakenbrink C., Engelhardt U.H., Analysis of proanthocyanidins in tea samples. Z. Lebensm. Unter. Forsch. A, 1997, 205, 153–157.
11. Kondo K., Masaaki K., Miyata N., Suzuki T., Toyoda M., Mechanistic studies of catechins as antioxidants against radical oxidation. Archiv. Biochem. Biophys., 1999, 363, 79–86.
12. Mabry T.J., Markham K.R., Thomas M.B., The Systematic Identification of Flavonoids. 1970, Springer-Verlag, New York-Heidelberg-Berlin, pp. 57–164.
13. Naczk M., Shahidi F., The effect of methanol – ammonia – water treatment on the content of phenolic acids of canola. Food Chem., 1989, 31, 159–164.
14. Nakamura Y., Matsuda M., Honma T., Tomita I., Shibata N., Warashina T., Noro T., Hara Y., Chemical constituents of mainly active components fractionated from the tea aqueous non-dialysates, an antitumor promoter. Third Tannin Conference, 20–25 July 1998, Bend, Oregon (USA).
15. Nonaka G., Kawahara O., Nishioka I., Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf. (1). Chem. Pharm. Bull., 1983, 31, 3906–3914.
16. Nonaka G., Sakai R., Nishioka I., Hydrolysable tannins and proanthocyanidins from green tea. Phytochem., 1984, 23, 1753–1755.
17. Porter L.J, Hirstich L.N., Chan B.G., The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochem., 1986, 25, 223–230.
18. Price M.L, Van Scoyoc S., Butler L.G., A critical evaluation of the vanillic reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem., 1978, 26, 1214–1218.
19. Price W.E., Spitzer J.C., Variation in the amounts of individual flavanols in a range of green tea. Food Chem., 1993, 47, 271–276.
20. Sartippour M.R., Heber D., Ma J., Lu Q., Go V.L., Nguyen M., Green tea and its catechins inhibit breast cancer xenografts. Nutr. Cancer, 2001, 40, 149–156.
21. Wang L.F., Park S.C., Chung J.O., Baik L.H., Park S.K., The compounds contributing to the greenness of green tea. J. Food Sci., 2004, 69, 301–305.

Received May 2005. Revision received and accepted June 2005.

SZYBKA CHROMATOGRAFICZNA METODA WYODRĘBNIANIA PROANTOCYJANIDYN Z ZIELONEJ HERBATY

Magdalena Karamać[1], Agnieszka Kosińska[1], Uttam D. Chavan[2]

[1]Oddział Nauki o Żywności, Instytut Rozrodu Zwierząt i Badań Żywności Polskiej Akademii Nauk, Olsztyn;
[2]Biotechnology Centre, Mahatma Phule Agricultural University, Rahuri, India

Ekstrakt polifenoli z zielonej herbaty rozdzielano na kolumnie wypełnionej żelem Toyopearl HW-40S (TosoHaas). Stosując etanol jako fazę ruchomą otrzymano 6 frakcji związków fenolowych (I–VI). W frakcjach I-III nie stwierdzono obecności katechin. Frakcje IV–VI zawierały epikatechinę (EC), epigalokatechinę (EGC), galusan epikatechiny (ECG) i galusan epigalokatechiny (EGCG). Następnie fazą ruchomą aceton-woda (1:1, v/v) wymyto z kolumny dwie kolejne frakcje proantocyjanidyn. Frakcje te dawały intensywną reakcję barwną z waniliną. Nieobecność katechin we frakcjach proantocyjanidyn potwierdzono metodą chromatografii cienkowarstwowej na żelu krzemionkowym.