Bulgarian Chemical Communications, Volume 50, Special Issue C (pp. 138 – 143) 2018

Antioxidant potential of high molecular weight polyphenol fraction from green tea

M. A. Janiak*, R. Amarowicz

Department of Chemical and Physical Properties of Food, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10 Tuwima Str., 10-748 Olsztyn, Poland

Received September 30, 2017; Accepted November 9, 2017

Green tea is one of the most popular beverages. Due to the high content of bioactive compounds it exhibits many health-promoting properties. Our study focused on analyzing the high molecular weight (HMW) fraction obtained from 13 green tea samples for their antioxidant capacity and chelating ability and comparing the results with those recorded for green tea extract (Ex) and its low molecular weight (LMW) fraction. HMW and LMW were obtained using Sephadex LH-20 column chromatography. Obtained fractions were characterized by total phenolic content, size-exclusion high performance liquid chromatography (SE-HPLC), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging capacity, and ability to chelate Fe(II) ions. HMW exhibited the highest total phenolic content and scavenging activity. The results obtained for the chelating capacity showed that changes in UV-spectra after addition of ferric (II) chloride observed for HMW solutions were the most significant.

Keywords: Proanthocyanidins, Green tea, SE-HPLC, DPPH, Chelation, Ferric ions, Sephadex LH-20, UV spectrum analysis

INTRODUCTION

Green tea is a widely consumed beverage across the whole world. There are many health-beneficial aspects of regular drinking infusion prepared from dry leafs of *Camellia sinensis* [1-3]. For example, drinking green tea can help patients with obesity in reducing body mass [4]. Green tea is an abundant source of polyphenols [6] and is well known for its antioxidant properties [7, 8]. It was proven that antioxidants present in green tea leafs are responsible for many health-beneficial attributes, in particular EGCG which can be detected as over 50% of the total sum of polyphenols [9-11]. Mandel *et al.* [12] claimed that the ability of EGCG to chelate ferric ions might have protective role against neurodegenerative diseases connected to abnormal iron metabolism. Although proanthocyanidins obtained from green tea exhibit anti-inflammatory properties [13] it seems that the high molecular fraction of green tea, which is composed by proanthocyanidins [14] is still not well described. However, there are reports suggesting protective role of proanthocyanidins from other sources against neurodegenerative diseases when tested on cell lines or mice [15, 16], proanthocyanidins have limited absorption through the gut barrier. Therefore, one of the aims of our study was to test the ability of green tea extract and of its low (LMW) and high molecular weight (HMW) fractions to chelate ferric (II) ions.

In our study we focused on highlighting the antioxidant potential of green tea's high molecular fraction, compared with the extract and the low molecular fraction. Research was focused on characterizing HMW separated from different commercially available green teas and comparing it with corresponding Ex and LMW using total phenolic content, DPPH radical scavenging activity test, size-exclusion high performance liquid chromatography (SE-HPLC) and changes in UV spectrum of extract/fractions inducted by ferric (II) chloride addition.

EXPERIMENTAL

Chemicals

Methanol, acetone, ethanol, sodium carbonate and ferric (II) chloride 4-hydrate were purchased from POCH S.A. (Polskie Odczynniki Chemiczne). Folin-Ciocalteu reagent, EGCG, gallic acid, tannic acid, DPPH, acetonitrile and trifluoroacetic acid were purchased from Sigma–Aldrich, USA. Procyanidin B2 was purchased from Extrasynthese (Genay, France).

Samples and extraction

13 samples of green tea were purchased from local shops in Olsztyn, Poland. Before extraction the samples were powdered in a coffee mill (Bosh, Ljubljana, Slovenia) and then phenolics were extracted from the green tea by mixing 20 g of powdered leafs with 80 % acetone (v/v) in a solid:liquid ratio 1:10 at 70°C for 15 min in a water shaking bath (SW 22, Julabo GmbH, Seelbach, Germany). After cooling, the obtained extract was filtered through Whatman filters. The extraction procedure was repeated two times. Filtrates were

* To whom all correspondence should be sent:
E-mail: m.janiak@pan.olsztyn.pl

combined, organic solvent was evaporated (Büchi, Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland) and the obtained aqueous concentrate was frozen and lyophilized for 72 h at -48°C (Freezone 6, model 77530, Labconco Co., Kansas City, MO, USA). Dry crude green tea extracts were stored at -20°C until further analysis.

Fractionation of crude extracts

Separation of two different molecular size fractions from green tea extract was conducted using the method of Naczk *et al*. [17]. Ex was dissolved in absolute ethanol and applied onto the column (5 × 40 cm) filled with Sephadex LH-20 gel in ethanol. Elution was performed with ethanol to obtain LMW fraction (approximately 1000 ml of eluate). Next, the mobile phase was changed for 50 % (v/v) acetone. HMW was eluted using 600 ml of the second eluent. Organic solvents were evaporated and the aqueous residue was lyophilized and stored under -4°C until further analysis.

Total phenolics assay

Total phenolics contetnt (TPC) was characterized using Folin-Ciocalteu's method adjusted for microplate readers and described by Horszwald and Andlauer [18]. Samples of Ex, LMW and HMW that were firstly dissolved in methanol (25 µl; 0.2 ml/ml) were placed in 96-well microplates. Next, 250 µl of Folin-Ciocalteu reagent was added (1:15; v/v) using built-in microplate reader's (TECAN Infinite M1000) injector. After 10 min of incubation 15 µl of 20% sodium carbonate was automatically added and incubated for 20 min. Process was conducted with plate shaking after each reagent addition. Absorbance was measured at λ=755 nm. Results were expressed as (-) epigallocatechingallate (EGCG) equivalent as mg per g of extract/fraction. For this purpose a calibration curve was prepared using a solution of EGCG (1 mg/ml) to prepare a series of dilutions. As reference sample pure methanol was used. Green tea extract and its fractions were analyzed in triplicate.

Antiradical scavenging activity against (2,2-diphenyl-1-picrylhydrazyl) radical (DPPH•)

For investigation of DPPH radical scavenging activity the microplate method was used [18]. 10 mg of DPPH powder was dissolved in 25 ml of methanol. DPPH• solution was diluted to obtain A~1.1 measured at λ=517 nm freshly before its injection. Each sample was dissolved in methanol (1 mg/ml), diluted (in the range 0.006-0.035 mg/ml) and placed inside the microplate (20 µl). Solution of DPPH• was automatically added (270 µl) and then the plate was shaken and incubated for 30 min in the dark with a cover on which was removed before absorbance measurement. Results are presented as the curve of absorbance (λ=517 nm) reduction, and % of radical reduction for final concentration of the sample calculated using the equation:

$$\%R = (A_0 - A_{fc}) \times 100\% / A_0$$

where %R is % of reduction of DPPH radical; A_0 is absorbance of blank; A_{fc} is absorbance of sample at final concentration.

Size exclusion-high performance liquid chromatography (SE-HPLC)

HMW fraction was dissolved in the mobile phase (0.5 mg/ml), filtered through Whatman 0.45 µm NYL w/GMF filters and injected (20 µl) into the HPLC system (LC-10AD pump, controller SCTL 10A and photodiode array detector SPD-M 10AQ). Samples were analyzed using TSK $G2000SW_{XL}$ column in isocratic mode where mobile phase was 45 % (v/v) acetonitrile with 0.1 % of trifluoroacetic acid and flow rate of 0.2 ml/min. Separation was monitored at λ=280 nm. Retention times of recorded peaks were compared with three standards: gallic acid, procyanidin B2 and tannic acid.

Iron (II) chelation

Method of Stookey [19] with modifications of Karamać and Pegg [20] was adjusted to microplate reader and UV spectrum analysis. Ex, LMW and HMW were dissolved in water (0.18 mg/ml). Samples (200 µl) were placed in quartz-bottom microplates and mixed with freshly prepared, automatically added ferric (II) chloride (0.4 mM $FeCl_2 \times 4H_2O$). Plates were incubated for 10 min and the UV spectrum was recorded in the wavelength range of 230-400 nm. The reference UV spectrum was recorded for Ex, LMW and HMW without ferric (II) chloride addition.

Statistical analysis

All analyses were conducted in triplicate. Statistical analysis was performed using STATISTICA 10, StatSoft. Differences between treatments were determined using ANOVA and Duncan's test.

RESULTS AND DISCUSSION

Results obtained for the ability of Ex, LMW and HMW to reduce Folin-Ciocalteu reagent are presented in Table 1.

Table 1. Total phenolics content (mg EGCG / g of extract or fraction) in 13 green teas.

Green tea	Ex				LMW				HMW			
1	599	±	35.9	fgh	494	±	10.30	h	588	±	40.5	cd
2	565	±	63.8	efg	466	±	9.31	gh	706	±	32.6	g
3	526	±	18.2	de	400	±	17.7	de	550	±	15.5	c
4	529	±	13.5	de	387	±	38.7	cd	421	±	7.38	b
5	615	±	23.8	gh	528	±	18.1	i	672	±	24.0	efg
6	406	±	22.1	b	423	±	17.2	ef	583	±	25.6	cd
7	312	±	4.38	a	286	±	27.5	a	341	±	8.64	a
8	463	±	42.1	c	367	±	7.16	bc	642	±	25.8	ef
9	548	±	17.0	ef	418	±	23.4	def	699	±	56.7	g
10	513	±	35.3	cde	416	±	7.42	def	626	±	23.8	de
11	473	±	16.0	c	355	±	14.1	b	689	±	38.1	fg
12	478	±	13.8	cd	447	±	9.57	fg	384	±	7.88	ab
13	643	±	4.70	h	480	±	4.94	h	718	±	37.3	g
mean	513	±	89.6		421	±	64.5		586	±	128	

Data are expressed as mean ± standard deviation (n=3); values in the same column having different letters differ significantly (P<0.05)

Total phenolics content was determined in the samples in a broad range. Mean value for HMW was 586 ± 128 mg/g and was the highest among all three groups (513 ± 89.6 mg/g for Ex and 421 ± 64.5 mg/g for LMW). The range for which results were obtained was 312 – 643 mg/g for Ex, 286 – 480 mg/g for LMW and 341 – 718 mg/g for HMW. Statistical analysis revealed that HMW was the most differentiated group, however all three groups showed moderate variability. What is interesting, the highest results were more than two times higher (for Ex and HMW) or nearly two times higher (in case of LMW) than the lowest ones. It presented the differences in ability to synthesize those compounds by different *Camellia sinensis* varieties and/or differences in the quality of final product. The range in which total phenolics content can be found in literature data is broad. There are findings that report TPC from 120-185 mg gallic acid equivalent (GAE) per g dry weight (DW) for infusions [7], 13.75-20.43% of DW as GAE [21] and up to 837.6 mg/g of extract in catechin equivalent [22]. Such variability of the results can be explained by different varieties, manufacturing processes and storage conditions. Other reports present that HMW from other sources that contain polymeric polyphenols exhibit higher values of absorbance [23, 24].

Typical results obtained after analyzing the scavenging activity of Ex, LMW fraction and HMW fraction from green tea against DPPH radical are depicted in Fig. 1.

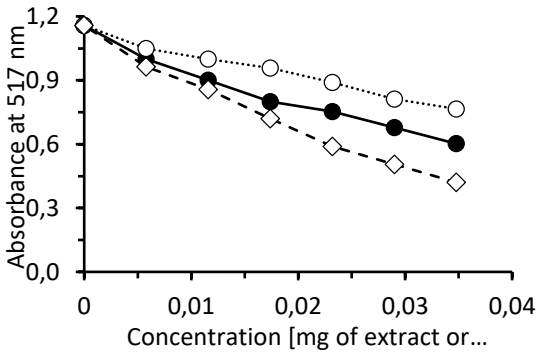

Figure 1. DPPH radical scavenging capacity for Ex, LMW and HMW (Black circles – Ex, white circles – LMW, white panes – HMW).

Curves for Ex, LMW and HMW are not linear. In case of each sample set (Ex, LMW and HMW) the most significant decrease of absorbance by solutions with different concentration of the samples was recorded for HMW. Ex with final concentration of 0.035 mg/ml was able to scavenge 48.00 % of DPPH•, LMW 33.88 % and HMW 63.72 %. DPPH assay presents results as the reduction of DPPH radicals measurable spectroscopically at 515nm. It can be observed as a loss of dark-purple color. Due to this method of expressing radical scavenging activity, the lowest results indicate that the sample which caused the strongest change in absorbance is the most active scavenger. Presented curves of decrease of absorbance in relation to concentration of the extract/ fractions clearly indicate that HMW was characterized by the highest free radical activity. Amarowicz *et al*. [23] reported that HMW fraction from red lentil exhibited few times higher activity against DPPH radical than Ex or LMW. In case of

LMW and HMW in our study, the difference was not as significant, because in both low and high molecular fractions compounds with high antioxidant potential could be detected. Nevertheless, differences in radical scavenging activity between Ex and LMW might suggest that compounds that can be separated to obtain HMW possess significant impact on antioxidant activity against DPPH$^{\bullet}$ of Ex.

Phenolic compounds extracted from green tea leafs were separated using SE-HPLC technique. Typical results are depicted in Fig. 2.

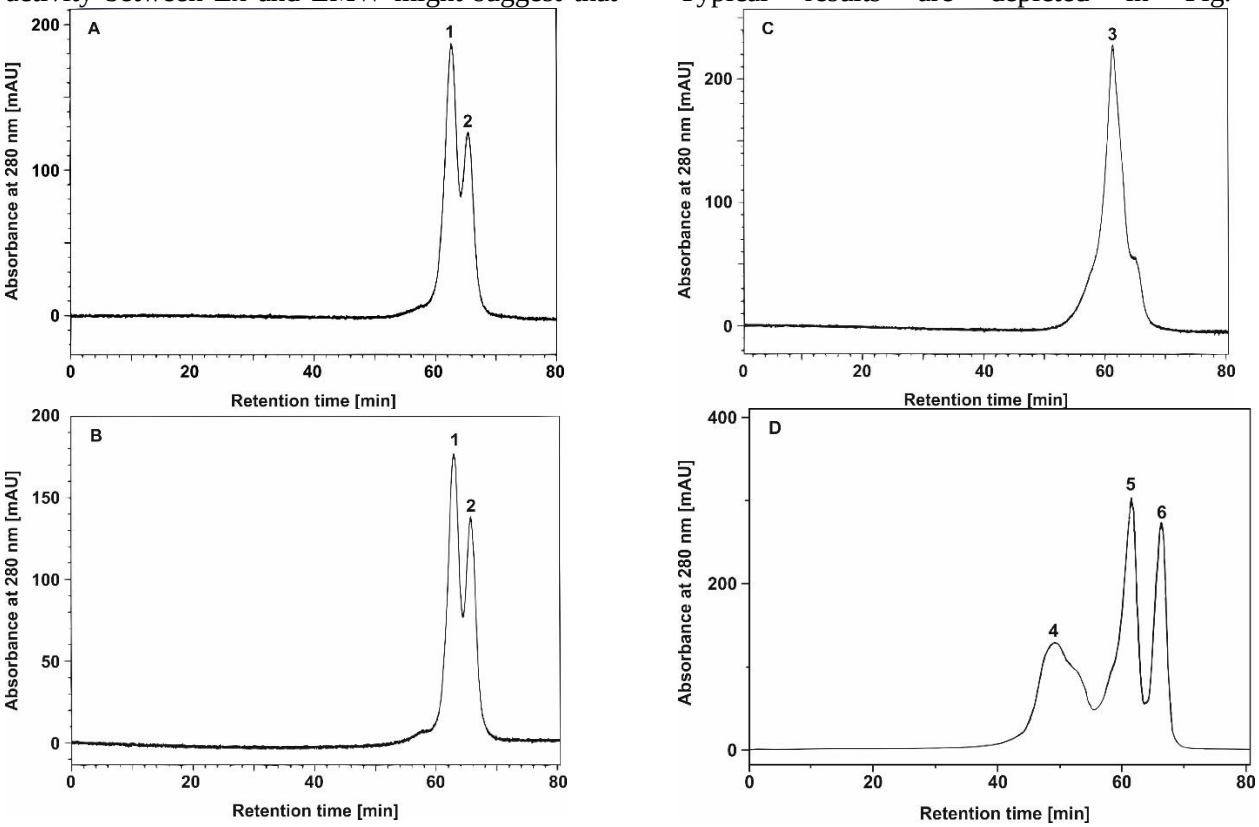

Figure 2. SE-HPLC chromatograms of Ex (A), LMW (B), HMW (C) obtained from green tea sample 11 and mix of standards (D): tannic acid (peak 4), procyanidin B2 (peak 5) and gallic acid (peak 6).

To compare the retention times of the separated compounds three standards with gradation of molecular weight were used. They were tannic acid (1701.19 g/mol), procyanidin B2 (578.52 g/mol) and gallic acid (170.12 g/mol). The results are presented in Fig. 2d. By using SE-HPLC technique some relatively small organic compounds like polyphenols extracted from different plant sources were investigated [24-27]. Using size exclusion column, compounds with higher molecular mass are eluted earlier than those with lower mass. As it can be noticed, peak 3 has similar retention time to peak 4, which corresponds to procyanidin B2. Peaks 1 and 2 were recorded for catechins so their retention times are shorter. Retention time of peak 2 is shorter than peak 6 recorded for gallic acid.

HMW fraction from green tea is dark, brownish and exhibit high values of absorbance around λ=562 nm. This wavelength was used in the colorimetric method with ferrozine [20]. There are also reports of other authors that assign changes of absorbance in the range of 546-600 nm to formation of complexes of polyphenol with metal ions [28, 29]. Due to those facts it was decided to present differences after ferric (II) chloride addition within the spectra in the UV wavelength range of Ex, LMW and HMW. Few other authors made successful attempts to compare UV spectra of pure polyphenolic compounds before and after metal ions addition [28, 31, 32]. Typical UV spectra obtained in this study before and after addition of Fe^{2+} are presented in Fig. 3.

It can be noticed that addition of ferric ions shifted areas around 250, 300 and 316 nm. It was previously reported that addition of metal ions to EGCG solution resulted in changes at similar areas, including shifting maximum of spectra to 312 nm [31]. Similar effect was noticed also after addition of Cu^{2+} to different flavonoids [30]. Addition of Al^{3+} to apigenin-7-O-glucopyranoside resulted in bathochromic shifting [32].

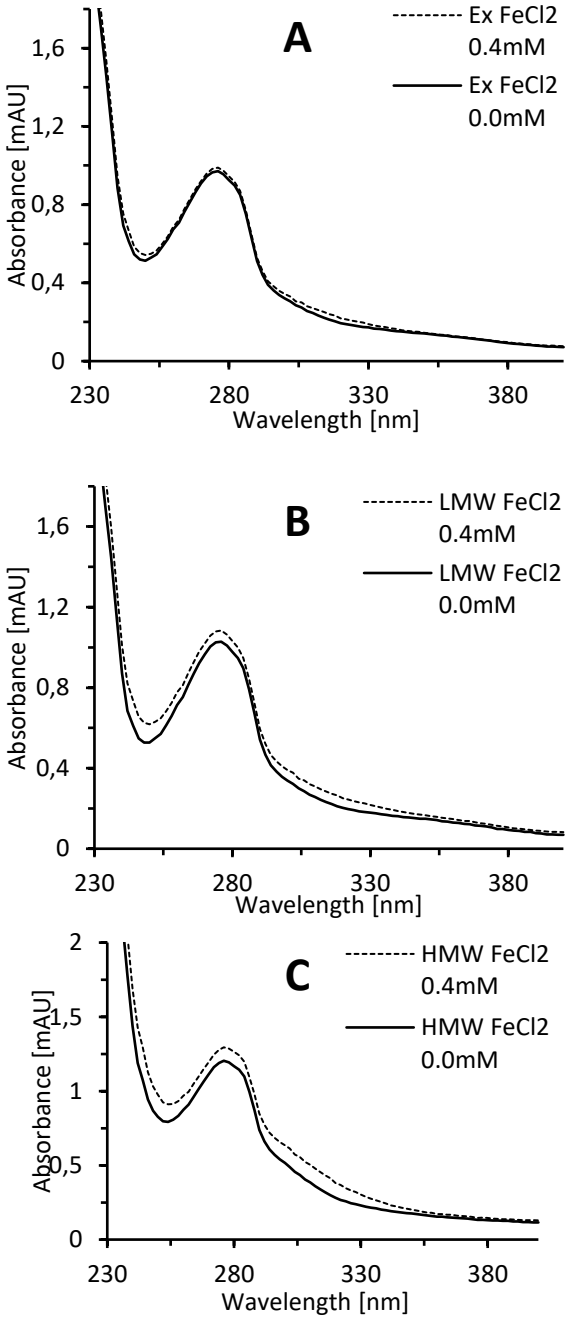

Figure 3. Chelation ability of Fe (II) by Ex (A), LMW (B) and HMW (C) obtained from green tea sample 11.

However, it was demonstrated that tannins from the same source bind Cu^{2+} in greater amounts than Fe^{2+} [20] which can lead to the conclusion that changes of spectrum after addition of Fe^{2+}might be less significant. In case of HMW, the strongest changes were within the area 316-320 nm of the UV spectrum. LMW exhibited weaker and Ex the weakest changes. This can be explained by the ability of tannins to bind metal ions at higher levels than monomeric polyphenols [20]. HMW as a stronger chelating agent than LMW might mitigate changes in spectrum of Ex in which condensed tannins are in minority, but can greatly participate in metal ions binding properties. Condensed tannins possess a significant number of hydroxyl groups which are potential places where metal ions can be bound to. UV spectrum of Ex is mostly a result of overlapping spectra of monomeric catechins which are in majority in Ex. Thus bigger changes of the spectrum of tannins that are also present in Ex are covered by the spectrum of monomers. Catechins in Ex bind less Fe^{2+} than catechins in LMW, because some amounts of ions were bonded to condensed tannins. Moreover [28], it was observed that after addition of a stronger chelator (EDTA) to a solution of a weaker chelator (phenolic acids), the spectra and λ_{max} of the polyphenol-metal ion complexes were changed. This suggests that a similar phenomenon can be found in case of natural mixtures of phenolics. It appears that priority in binding ferric ions can have stronger chelators, in case of Ex – proanthocyanidins. However, Ex, LMW and HMW are mixtures of many compounds what causes difficulties in presenting simple conclusions.

CONCLUSION

HMW fraction exhibits significant impact on green tea's antioxidant capacity. Especially, HMW was also the fraction which showed the highest values obtained for total phenolic content. The method of analyzing chelating ability of the colored plant extracts by observing changes in the UV-spectrum after addition of ferric (II) chloride seems to be a promising way that will help to analyze complexes between polyphenols and metal ions.

REFERENCES

1. H. Mukhtar, N. Ahmad, *Am. J. Clin. Nutr*. **71,** suppl**.,** 1698 (2000).
2. D.L. McKay, J.B. Blumberg, *J. Am. Coll. Nutr*., 1, 1 (2002).
3. D. Richard, K. Kefi, U. Barbe, A. Poli, P. Bausero, F. Visioli, *Pharmacol. Res*., **59**, 351 (2009).
4. W. Tang, S. Li, Y. Liu, M.-T. Huang, C.-T. Ho, *J. Funct. Foods*, **5**, 1784 (2013).
5. C. Snoussi, R. Ducroc, M.H. Hamadaoui, K. Dhaoudai, H. Abaidi, F. Cluzeaud, C. Nazaret, M. Le Gall, A. Bado, *J. Nutr. Biochem*., **25**, 557 (2014).
6. C.-T. Ho, C.W. Chen, U.N. Wanasundara, F. Shahidi, Natural antioxidants from tea. in: *Natural Antioxidants. Chemistry. Health Effects and Application* (Shahidi F., ed.). AOCS Press Champaign, Illinois. 213, 1997.
7. A. Rusaczonek, F. Świderski, B. Waszkiewicz-Robak, *Pol. J. Food. Nutr. Sci.*, **60**, 33 (2010).
8. A.K. Dutta, M.A. Siddiquee, S. Hossain, Y. Kabir, *Malay. J. Pharm. Sci*., **11**, 11 (2013).
9. H. Wang, G.J. Provan, K. Helliwell, *Trends Food. Sci. Technol*., **11**, 152 (2000).

10. G. Williamson, C. Manach, *Am. J. Clin. Nutr*. **81,** suppl., 243 (2005).
11. D.G. Nagle, D. Ferreira, Y-D. Zhou, *Phytochemistry*, **67**, 1849 (2006).
12. S. Mandel, G. Maor, M.B. Youdim , *J. Mol. Neurosci*. **24**, 401 (2004).
13. D.-X. Hou, S. Masuzaki, F. Hashimoto, T. Uto, S. Tanigawa, M. Fuji, Y. Sakata ,*Arch. Biochem. Biophys.*, **460**, 67 (2007).
14. A. Kiehne, C. Lakenbrink, U.H. Engelhardt, *Eur. Food Res. Technol*., **205**, 153 (1997).
15. K.E. Strathearn, G.G. Yousef, M.H. Grace, S.L. Roy, M.A. Lila, J.-C. Rochet, *Brain Res*., **1555**, 60 (2014).
16. Y.-S. Gong, J. Guo, K. Hu, Y.-Q. Gao, B.-J. Xie, Z.-D. Sun, E.-N. Yang, F.-L. Hou, *Exp. Gerontol*. , **74**, 21 (2016).
17. M. Naczk, R. Amarowicz, R. Zadernowski, F. Shahidi, *Food Chem*. **73**, 467 (2001).
18. Horszwald A., Andlauer W.*J. Berry Res*. **1**, 189 (2011).
19. L.L. Stookey, *Anal. Chem*., **42**, 779 (1970).
20. M. Karamać, R.B. Pegg, *J. Agric. Food Chem*., **57**, 6425 (2009).
21. C. Astill, M.R. Birch, C. Dacombe, P.G. Humphrey, P.T. Martin, *J. Agric. Food Chem*., **49**, 5340 (2001).
22. A. Gramza, K. Pawlak-Lemańska, J. Korczak, E. Wąsowicz, M. Rudzińska, *Pol. J. Environ. Stud*., **14**, 861 (2005).
23. R. Amarowicz, I. Estrella, T. Hernández, M. Dueńas, A. Troszyńska, A. Kosińska, R.B. Pegg, *Int. J. Mol. Sci*. **10**, 5513 (2009).
24. E. Pelvan Pelitli, M.A. Janiak, R. Amarowicz, C. Alasalvar , *Food Chem*., **218**, 584 (2017).
25. A. Yanagida, T. Shoji, T. Kanada, *Biosci. Biotechnol. Biochem*., **66**, 1972 (2002).
26. M. Karamać, A. Kosińska, A. Rybarczyk, R.B. Pegg, *Pol. J. Food Nutr. Sci.*, **57**, 87 (2007).
27. A Kosińska., A. Urbalewicz, K. Penkacik, M. Karamać, R. Amarowicz, *Pol. J. Food Nutr. Sci*., **61**, 263 (2011).
28. M. Andjelkocić, J. Van Camp, B. De Meulenaer, G. Depaemelaere, C. Socaciu, M. Verloo, R. Verhe , *Food Chem*., **98**, 23 (2009).
29. N.R. Perron, H.C. Wang, S.N. DeGuire, M. Jenkins, M. Lawson, J.L. Brumaghim, *Dalton Trans*. ,**39**, 9982 (2010)
30. J.E. Brown, H. Khodr, R.C. Hider, C.A. Rice-Evans, *Biochem. J*., **330**, 1173 (1998).
31. M. Kumamoto, T. Sonda, K. Nagayama, M. Tabata, *Biosci. Biotechnol. Biochem*., **65**, 126 (2001).
32. T.H. Costa Marques, C.H. Santos De Melo, R.B., Fonseca De Carvalho, L.M. Costa, A.A. De Souza, J.M. David, J. David, P. De Lima, R.M. De Freitas, *Biol. Res*., **46**, 231 (2013).

АНТИОКСИДАНТЕН ПОТЕНЦИАЛ НА ВИСОКОМОЛЕКУЛНА ПОЛИФЕНОЛНА ФРАКЦИЯ ОТ ЗЕЛЕН ЧАЙ

М. А. Яниак*, Р. Амарович

Департамент по химични и физични свойства на храните, Институт по възпроизводство на животните и изследвания върху храните, Полска академия на науките, ул. Тувима 10, 10-748 Олщин, Полша

Постъпила на 30 септември, 2017 г.; приета на 9 ноември, 2017 г.

(Резюме)

Зеленият чай е една от най-популярните напитки. Поради високото си съдържание на биоактивни съединения той проявява много стимулиращи здравето свойства. В настоящото изследване е анализирана високомолекулната част (HMW) от 13 проби зелен чай по отношение на антиоксидантния капацитет и хелатообразуващата способност, като резултатите са сравнени с тези, получени за екстракт от зелен чай и нискомолекулната му фракция (LMW). HMW и LMW фракции са получени чрез колонна хроматография с използване на Sephadex LH-20. Получените фракции са характеризирани чрез определяне на общото фенолно съдържание, високоефективна течна хроматография с изключване на размера (SE-HPLC), капацитет за улавяне на 2,2-дифенил-1-пикрилхидразил радикал (DPPH$^{\bullet}$) и способност за образуване на хелат с Fe(II) йони. HMW има най-високо фенолно съдържание и проявява най-висока радикал-улавяща активност. Резултатите за хелатообразуващата активност показват, че най-значителни промени в UV-спектрите след добавяне на железен (II) хлорид се наблюдават за HMW разтвори.