Ars Separatoria Acta 5 (2007) 27-33

ARS SEPARATORIA ACTA

www.ars_separatoria.chem.uni.torun.pl

EVALUATION OF ANTIOXIDACTIVITY OF GREEN AND BLACK TEA (*Camellia sinensis)* AND ROOIBOS (*Aspalathus linearis)* TEA EXTRACTS BY MEANS OF HPLC WITH REACTION DETECTOR

Rasa MILAŠIENĖ[1)], Katarzyna SAWICKA[1),2)], Olga KORNYŠOVA[1)], Magdalena LIGOR[2)], Audrius MARUŠKA[1)], Bogusław BUSZEWSKI[2*)]

[1)] Department of Chemistry, Vytautas Magnus University, Vileikos 8, Kaunas LT 44404, Lithuania
e-mail: a.maruska@gmf.vdu.lt
[2)] Department of Environmental Chemistry and Bioanalytics, Nicolaus Copernicus University, Gagarina 7, 87-100 Toruń, Poland

ABSTRACT

Antioxidants are chemical compounds that scavenge free radicals in organisms and protect most important biomolecules such as DNA, proteins, lipids from their damaging activity. Antioxidants reduce a risk of cancer, inflammations, arthritis, cardiovascular diseases and others. The objective of this study was to prepare extracts of different tea species (*Camellia sinensis, Aspalathus linearis*) in order to compare their antioxidant and radical scavenging activities using reaction detection. Methanolic black, green and red tea extracts were prepared. Reaction detection was used for on-line determination of free radicals of 2,2-diphenyl-1-picrylhydrazil (DPPH) binding by high performance liquid chromatography.

Keywords: Antioxidant activity, HPLC, Reaction detector, DPPH, Tea

INTRODUCTION

Oxidation reactions are chemical reactions that involve the transfer of electrons from one substance to an oxidizing agent. While the vast majority of complex life requires oxygen for its existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species. The reactive oxygen species produced in cells include hydrogen

* Corresponding author

peroxide, hypochlorous acid and free radicals such as hydroxyl radical and the superoxide anion. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Oxidative stress is thought to contribute to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease, the pathologies caused by diabetes, rheumatoid arthritis and neurodegenaration in motor neurons diseases [1].

Antioxidants can slow down the mentioned reactions either by reacting with intermediates and suppressing the oxidation reaction or by reacting with the oxidizing agent and preventing the oxidation reaction from occurring [2-4]. Antioxidants neutralize free radicals in organisms. Even small amounts of antioxidants can suppress the formation of these radicals, eliminate them or induce their scavenging. All these actions can protect tissues of an organism from harmful affect [5].

Antioxidant activity can be evaluated by means of the β-carotene bleaching test [6, 7], reducing the activity of compounds over Fe^{3+} and Cu^{2+}, Fe^{3+} [8], UV, IR spectrophotometric analysis [9, 10], $DPPH^{\bullet}$, $ABTS^{\bullet+}$ radical scavenging methods [5, 6, 9, 11].

On-line reaction detection was widely described by van Beek et al. [6, 11-13]. It is a very convenient method for revealing antioxidant activities of different components of complex mixtures such as plant extracts. During one analysis, compounds are separated, and later, after reaction with free radicals, the antioxidant activity is evaluated in the same HPLC eluent.

Compounds that have been reported to exhibit strong antioxidant activity are flavonoids. Tea is a rich source of these compounds [14]. Tea infusions, consumed by two thirds of the world's population, are obtained from the leaves of one kind of a plant called *Camellia sinensis* L. (family Theaceae) or rooibos tea *Aspalathus linearis* (family Fabaceae). It can be categorized into unfermented – green, partially fermented – oolong or fermented – black tea. Rooibos tea, the product of South Africa, is the fermented one. The majority of compounds contained in tea is a group of catechins and flavonoids, which are also categorized into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids [15, 16]. They have antioxidant, cardioprotective, and antithrombogenic, anti-inflammatory effects, as well as improve the coronary flow velocity [14].

EXPERIMENTAL

Materials and instrumentation

Black, green, and rooibos teas were purchased in one of Polish supermarkets. 99 % methanol (G.R., Lachema, Czech Republic) was used for the extraction of plant materials. Ammonium acetate (Merck, Germany), formic acid, analytical grade acetonitrile (MeCN), gallic acid, theobromine (Sigma-Aldrich, Germany), caffeine (Reag. Ph Eur, Merck, Germany) and catechin (Sigma-Aldrich, Germany) were used for HPLC analysis. The following reagents were used for the evaluation of radical scavenging and

antioxidant activity: Na_2HPO_4 and NaH_2PO_4 (Merck, Germany), synthetic free radical 2,2-diphenyl-1-picrylhydrazil (DPPH$^{\bullet}$) (Sigma-Aldrich, Germany), rutin hydrate, min 95 % HPLC (Sigma-Aldrich Chemie, Steinheim, Germany).

The determination of antioxidants in tea was performed by a high-performance liquid chromatography (HPLC) system (Fig. 1), which consisted of a model 1100 quaternary pump (Hewlett Packard, Waldbronn, Germany), a model 1100 vacuum degasser (Hewlett Packard, Waldbronn, Germany), an autosampler (Perkin Elmer, USA), a precolumn, an analytical column ODS C-18 d_p=5 μm reversed-phase 250 mm × 4.6 mm (Altech, Germany); the detection was carried out using a UV detector "Spectra 200" (Spectra Physics, USA); the buffered DPPH$^{\bullet}$ solution was introduced to the system by a syringe pump (Phoenix 20 CU, Carlo Erba Instruments, Milan, Italy); the radical scavenging reaction was performed in the 0.75 m reaction coil (0.3 mm I.D.) and the signal of an antioxidant action was detected by a visible light detector "Linear UVIS 200" (Linear Instruments, Reno, USA). Two channel signals were collected, calculated, and stored by ChromStar 3.20 software (Bruker, Bremen, Germany). One, at wavelength of 270 nm, is an image of tea compounds separation, and the other – the negative signal at wavelength of 517 nm, depicts the antioxidant activity.

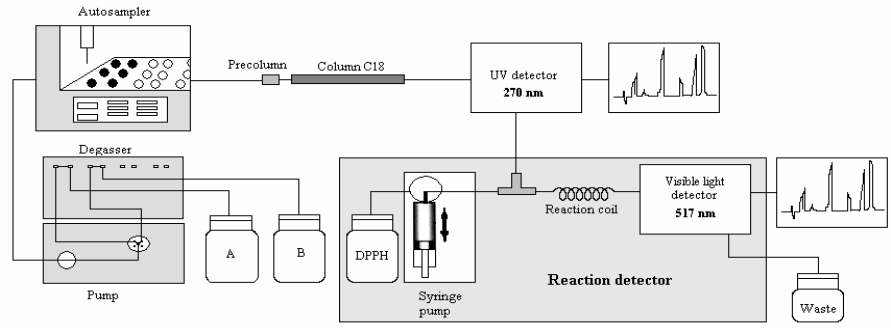

Fig.1. High-performance liquid chromatography (HPLC) system with reaction detector.

Before extraction, each tea sample was ground in a ceramic mortar; then 0.25g of the ground tea was extracted with 2.5ml of 100 % methanol. All the extracts were stirred for half an hour, and then filtered using a 0.2 μm membrane filter.

For HPLC, a separation gradient was used. Solvent A was 2.5 mM ammonium acetate in bidistilled water, which was acidified with formic acid (0.4 ml/l). HPLC grade MeCN mixed with solvent A in a volume ratio

of 1:1, was used as solvent B. The percentage of solvent B in the gradient started from 20 % and was as follows: 0 – 10 min from 20 to 30 %, then to 40 % in 10 min, hold for 4 min, then from 40 to 90 % in 9 min and to 100 % in 5 min. The flow rate was 0.4 ml/min. The DPPH reagent was dissolved in MeCN at the concentration of 10 mg/l. This solution was mixed with 50 mM phosphate buffer in a ratio of 1:2. The phosphate buffer was prepared of Na_2HPO_4, and pH = 7.6 was adjusted by adding NaH_2PO_4. The stream of the buffered DPPH$^{\bullet}$ solution was introduced to the system at the flow rate of 0.4 ml/min.

Evaluation of antioxidant activity

Method is based on reaction between DPPH$^{\bullet}$ and separated compounds from extracts of different sorts of tea. As a result – bleaching out of free radical from purple to yellow is obtained and measured. The results are expressed as rutin equivalents (RE) in mg per dry weight (g) of tea. Antioxidant activity of gallic acid, catechin, rutin and total antioxidant activity were evaluated. For total antioxidant activity determination all peaks of the mirror chromatogram were integrated and expressed in RE.

RESULTS AND DISCUSSION

Figure 2 shows UV and reaction detection chromatograms of black, green and rooibos tea. The identification of gallic acid, theobromine, catechin, caffeine, and rutin peaks was made using standard solutions of these compounds. Epigallocatechin was identified from literature data [17,18] according to its elution order. Obtained results were compared to the published data.

As the antioxidant activity was expressed in RE, calibration graph for rutin was constructed from data obtained using the DPPH* reaction detector [6]. The equation obtained was used to evaluate antioxidant activity of different compounds in RE (mg/g), where *Peak area* expressed in mV*min. (Equation 1)

$$\mathrm{RE}=\frac{Peak\_area}{3855.6} \qquad (1)$$

The quantification results for some important compounds (gallic acid, epigallocatechin, catechin, and rutin) are shown in Table 1, and their quantities, as obtained using UV detection, are expressed in mg per 1 g of dry tea. The comparison of tea species showed that the highest amount of rutin appears in black tea, whereas its content is lower in green tea. Moreover, it was not determined in rooibos tea. Epigallocatechin was identified in the green tea chromatogram using the epigallocatechin standard solution. However, from the literature sources it is known, that green tea contains more catechin group compounds comparing to black tea [16]. This is reflected in higher antioxidant activity of green tea. The highest amount

of gallic acid appears in black tea, although its content in green and black tea is comparable.

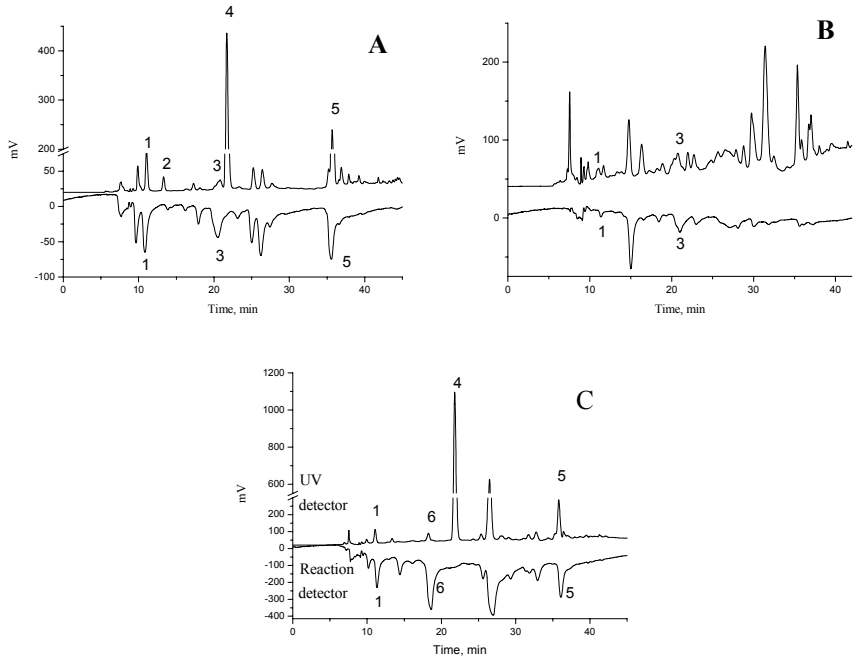

Fig. 2. HPLC-DPPH chromatograms of black (A), red (B) and green (C) teas. Peak identification:1- gallic acid; 2 – theobromine; 3 – catechin; 4 – caffeine; 5 – rutin; 6 – epigallocatechin.

Table 1. Quantity of some important compounds in tea expressed in mg per 1 g of dry tea obtained using UV detection

Sample	Gallic acid, mg/g	Catechin, mg/g	Epigalocatechin, mg/g	Rutin, mg/g
Black tea extract	1.907	4.624	-	10.12
Green tea extract	1.227	-	4.436	4.759
Rooibos tea extract	0.105	1.112	-	-

In Table 2 the antioxidant activity of main compounds i.e. gallic acid, catechins, and rutin in black, green and rooibos tea, determined using the reaction detection, is shown. The results indicate, that the amount of rutin, good correlation between quantitative analysis data (Table 1) and

antioxidant activity (Table 2) was obtained for rutin. Higher content and higher antioxidant activity of this compound was in black tea. The same correlation is observed between the amount of gallic acid and its radical scavenging activity, i.e. it is also higher in the case of black tea. The content of catechin in black tea was higher comparing to epigallocatechin in green tea. The antioxidant activity expressed in mg/g RE, however, was higher for epigallocatechin in green tea comparing to catechin in black tea.. The results obtained let us draw the conclusion, that epigallocatechin is a stronger antioxidant than catechin.

Table 2. Antioxidant activity of principle compounds gallic acid, catechins and rutin in black, green and rooibos tea determined using reaction detection

Sample	Antioxidant activity, mg/g RE			
	Gallic acid	Catechin compounds		Rutin
		Catechin	Epigallocatechin	
Black tea extract	3.12	3.16	-	3.29
Green tea extract	2.41	-	4.56	2.65
Rooibos tea extract	0.11	0.91	-	0

The total antioxidant activity was evaluated by summing up all the negative peak areas of the mirror chromatogram expressed in mg/g RE. The results obtained are shown in Table 3. The total antioxidant activity varied from 4.29 mg/g to 24.19 mg/g and was highest for green tea and lowest for rooibos tea. This can be explained by the fact that unfermented green tea has a higher content of catechin group compounds, which are stronger radical scavengers.

Table 3. Total radical scavenging activity for black, green and rooibos tea determined using reaction detection

Sample	Antioxidant activity, mg/g RE
Black tea extract	18.71
Green tea extract	24.19
Rooibos tea 100% methanol extract	4.29

Summarizing Tables 2 and 3, principal compounds, i.e. gallic acid, catechins, and rutin represent 51%, 43% and 24% of the total antioxidant activity in black, green, and rooibos tea, respectively.

The limit of detection (LOD) and the limit of quantification (LOQ) for rutin was evaluated. LOD was calculated when the peak height exceeded the baseline noise twice and was 0.751 μg/ml. LOQ was evaluated when the peak height exceeded the baseline noise four times and was 1.52 μg/ml.

CONCLUSIONS

For the antioxidant activity evaluation in methanolic extracts of black and green tea (*Camellia sinensis)* and rooibos tea *(Aspalathus linearis)* a novel convenient on-line ESC – $DPPH^{\bullet}$ reaction detection assay was used.

The main compounds such as: gallic acid, theobromine, epigallocatechin, catechin, caffeine and rutin, were identified in black, green and rooibos tea.

The antioxidant activity was evaluated. In general, green tea has been found superior to black tea in terms of its antioxidant activity owing to the higher catechins content the value of which is approximately 30 % higher when compared to black tea. Rooibos tea showed a lower antioxidant activity. Catechin group compounds (epigallocatechin etc.) provide a higher radical scavenging activity than gallic acid in it. The amount of rutin and gallic acid in black tea is approximately 20 % higher comparing to green tea.

ACKNOWLEDGEMENTS

Erasmus scholarship for Katarzyna Sawicka is acknowledged.
Financial support from the Lithuanian State Science and Studies Foundation ("Vaistabiotas" project N-07008) is acknowledged.

REFERENCES

[1] Access through internet 09 05 2007: http://lpi.oregonstate.edu/f-w00/flavonoid.html
[2] K.E. Heim, D.J. Bobilya, *J. of Nutr. Bioch.*, 2002, 13, 572 – 584.
[3] H.E. Seifrieda, J.A. Milnera, *J. of Nutr. Bioch.*, 2007, 18 (9), 567 –579.
[4] B.C. Behera, N. Verma, *Biotech. letters*, 2005, 27, 991 – 995.
[5] V. Briedis, V. Povilaitytė, *Medicina*, 2003, 39 (2), 104-112.
[6] A. Dapkevičius, T.A. van Beek, *J. of Chr. A,* 2001, 912, 73-82.
[7] F. Poli, M. Muzzoli, *Ph. Biol. (Form. Int. J. of Pharmac.)*, 2005, 41 (5), 379-383.
[8] J.A. Rufian-Henares, J. Morales, *Eur. Food Res. and Tech.*, 2006, 223, 225–231.
[9] O. Yesil-Celiktas, G. Girgin, *Eur. Food Res. and Tech.*, 2006, 224 (4), 443–451.
[10] G. Miliauskas, P.R. Venskutonis, *Food Ch.*, 2003, 85, 231-237.
[11] V. Exarchou, Y.C. Fiamegos, *J. of Chr. A*, 2006, 1112, 293 – 302.
[12] G. Miliauskas, T.A. van Beek, *J. Sc. Food and Agr*., 2004, 84 (15), 1997-2009.
[13] G. Miliauskas, T.A. van Beek, *Eur. Food Res. and Tech.*, 2004, 218 (3), 253–261.
[14] T.O. Cheng, *Int. J. of Card.*, 2006, 108, 301 – 308.
[15] *USDA Database for the Flavonoid Content of Selected Foods, USA,* 2003
[16] J. Petersona, J. Dwyera, *J. of Food Comp. and An.,* 2005, 18, 487–501.
[17] M. Bonoli, G. Lercker, *Food Ch.,* 2003, 81, 631 – 638.
[18] X. R. Yang, Y. M. Jiang, *Food Ch.,* 2007, 100 (3), 1132 – 1136.