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The composition of polyphenols and methylxanthines in teas and herbal infusions

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ABSTRACT

The popularity of tea is increasing on the global aspect because of its role as a significant source of phenolic compounds in human diet. The purpose of this study was to determine and compare the phenolic and methylxanthine composition as well as the antioxidant capacity of white, green, Oolong and black teas, and chamomile and linden infusions depending on the extraction conditions (water temperature and multiple extractions). The content of total phenols and total flavonoids in teas and herbal infusions was determined by using UV/vis spectrophotometric methods, whilst individual polyphenols (phenolic acids and flavan-3-ols) and methylxanthines were identified and quantified by using high performance liquid chromatography coupled with photodiode array detection. In order to determine the antioxidant capacity of teas the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assays were applied. The highest content of phenolic compounds was determined in green tea, which also demonstrated the highest antioxidant capacity, whilst herbal infusions were characterised with the lowest content of phenolic compounds, as well as the lowest antioxidant capacity. The highest content of caffeine, as the most abundant methylxanthine, was determined in black tea. Extraction at 100 °C is the most effective to extract the highest content of polyphenols and methylxanthines in all studied teas.

1. Introduction

In recent years, researchers have paid particular attention to the biologically active ingredients, especially alkaloids and polyphenols in food and beverages due to their positive effects on human health. Tea is one of the most commonly consumed beverages throughout the world. Beside the attractive aroma and specific taste, its popularity is also a result of its potentially health-promoting properties. Numerous epidemiological studies link tea consumption to a reduction of the risk of cardiovascular diseases (Hertog, Hollman, Katan, & Kromhout, 1993; Young, Hotovec, & Romero, 1967), high cholesterol levels (Maron et al., 2003; Vinson, Teufel, & Wu, 2004), diabetes (Vinson & Zhang, 2005), arthritis (Haggi et al., 1999), ostheoporosis (Hegarty, May, & Khaw, 2000) and dental caries (Otake, Makimura, Kuroki, Nishihara, & Hirasawa, 1991). These beneficial effects of tea have been attributed to the antioxidant properties of polyphenolic compounds, particularly of the catechin derivatives: (−)-epicatechin, (−)-epigallocatechin gallate, (−)-epigallocatechin, (−)-epicatechin gallate, (+)-gallocatechin, and (+)-catechin (Roberts & Wood, 1953). In addition to the polyphenols, tea leaves are an important source of methylxanthines (caffeine, theobromine and theophylline) as well as amino acids (theanine), minerals and trace elements such as potassium, magnesium, calcium, nickel and zinc, which are essential to human health (Fernandez, Pablos, Martin, & Gonzalez, 2002). Methylxanthines have physiological and pharmacological effects on some body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory and renal systems (Nehlig, Daval, & Debry, 1992; Spiller, 1998).

Four types of tea are produced from the leaves of *Camellia sinensis*: white tea, green tea (both unfermented), Oolong tea (semi-fermented) and black tea (fermented). The fermentation of tea leaves induces enzymatic oxidation of catechins and leads to formation of two major pigments in black tea, theaflavins and thearubigins, which contribute to characteristic bright orange–red colour of black tea (Coggon, Moss, Graham, & Sanderson, 1973). In contrast with some Asian countries such as China and India, where tea drinking is a ritual and a life style, in many European countries tea consumption is infrequent and people still prefer various types of fruit teas or traditional herbal infusions (e.g. chamomile and linden). Tea consumption also differs, depending on the type of tea consumed and tea preparation. Habitually, in some parts of the world, tea is infused several times (repeated extractions) or prepared with water at different temperature. Due to the potential beneficial health effects related to tea drinking, it is interesting to closely determine and compare the chemical composition of different teas and herbal infusions. Moreover, there is no data available that shows bioactive composition of different teas and herbal infusions depending on various extraction conditions,

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especially multiple extractions. Therefore, the aim of this study is to compare the content of polyphenols and methylxanthines in teas and herbal infusions, as well as their antioxidant capacity, and to determine the effect of different extraction conditions (water temperature and multiple extractions) on the content of these biologically active compounds, in order to define the optimal extraction conditions for each type of tea.

2. Experimental

2.1. Chemicals

Folin–Ciocalteu, formic acid, ammonium peroxodisulphate, sodium carbonate, formaldehyde and hydrochloric acid were of analytical grade and supplied from Kemika (Zagreb, Croatia). DPPH (2,2-diphenyl-1-picrylhydrazyl) was supplied from Fluka (Buchs, Switzerland) and methanol (HPLC grade) was purchased from J.T. Baker (Deventer, Netherlands). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)diammonium salt) as well as caffeine (CF), theobromine (TB), theophylline (TP), (−)-epicatechin (EC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (+)-gallocatechin (GC), (−)-gallocatechin gallate (GCG), (+)-catechin (C), gallic acid (GA), chlorogenic acid (CHLA), ferulic acid (FA), caffeic acid (CA), *p*-coumaric acid (p-coumA) and vanillic acid (VA) were obtained from Aldrich (Sigma–Aldrich Chemie, Steinheim, Germany).

2.2. Sample preparation

Six types of tea in loose leaf form: Pai Mu Tan-superior (Chinese white tea, WT), Gyokuro (Japanese green tea, GT), Formosa Fine Oolong (Taiwanese Oolong tea, OT), Lingia (Indian black tea, BT), Linden (Croatian herbal tea, LT) and Chamomile (Croatian herbal tea, CT) were purchased from a local market. In order to simulate household brewing conditions for a cup of tea, teas were prepared using an aqueous extraction procedure. Tea samples (2.0 g) were poured with 200 ml of deionised water heated to 60, 80, and 100 °C and stirred with a glass rod for 3 min. Both tea extracts and herbal infusions were then filtered through a tea strainer. In order to study the effect of multiple extractions on the polyphenol and methylxanthine content of tea samples, each sample was extracted three times at the same conditions (80 °C/3 min). To determine the effect of milk or lemon juice on the antioxidant capacity of tea samples, 5 ml of milk or freshly squeeze lemon juice was added to 100 ml of tea extract.

2.3. Determination of total phenol and total flavonoid content

Total phenol content (TPC) in teas and herbal infusions was determined spectrophotometrically according to the modified Folin–Ciocalteu method described by Lachman, Hosnedl, Pivec, and Orsák (1998). The method is based on the reduction of phosphotungstic acid ($H_3P[W_3O_{10}]_4$) in alkaline solution to phosphotungstic blue. The absorbance of formed phosphotungstic blue is proportional to the number of aromatic phenolic groups and is used for their quantification, with gallic acid as the standard. Briefly, to a volume of 0.5 ml of a sample, 2.5 ml Folin–Ciocalteu's reagent, 30 ml distilled water and 7.5 ml of 20% Na_2CO_3 was added and diluted to 50 ml with distilled water. After 2 h, the absorbance was measured at 765 nm against blank.

To determine total flavonoid content (TFC), formaldehyde was added to tea extracts. Flavonoids containing characteristic 5,7-dihydroxy moiety react with formaldehyde forming methyl derivates at positions C-6 or C-8. These methyl derivatives are still reactive and undergo further reaction with other flavonoid molecules, leading to the formation of condensed products and their precipitation from the solution. Precipitated flavonoids were separated from the solution by filtration and non-flavonoid phenols remaining in the filtrate were determined by Folin–Ciocalteu method, as described previously. TFC was calculated as the difference between TPC and non-flavonoid phenol content. The results were expressed as mg/l of gallic acid equivalents (GAEs) (Kramling & Singleton, 1969). All measurements were performed in triplicate.

2.4. HPLC analysis of phenolic compounds and methylxanthines

Varian HPLC system (Varian, Walnut Creek, USA) consisting of Pro Star Solvent Delivery System 230 and Pro Star 330 photodiode array detector (PDA) and controlled by Star Chromatography Workstation Version 5 software was used for HPLC analysis of samples. Separation was performed using a reversed-phase Pinnacle II C-18 column (Restek, USA) (250 mm × 4.6 mm × 5 μm). The samples were filtered through a 0.45 μm membrane filter (nylon membranes, Supelco, USA) and 20 μl of each sample was injected for HPLC analysis. The solvent compositions used were 3% formic acid (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 ml/min. The elution was performed with a gradient starting at 2% B to reach 32% B at 20 min, 40% B at 30 min and 95% B at 40 min, and becoming isocratic for 5 min. Chromatograms were recorded at 278 nm. PDA detection was performed by recording the absorbance of the eluate between 200 and 400 nm, with a resolution of 1.2 nm. Phenolic compounds were identified by comparing the retention times and spectral data with those of authentic standards. All analyses were repeated three times.

2.5. Determination of antioxidant capacity

2.5.1. Free radical scavenging ability by the use of DPPH radical

This method is based on the reduction of stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical by antioxidants in a methanolic solution. In the presence of antioxidants the purple colour of the DPPH radical solution changes to a bright yellow and the intensity of this change can be monitored spectrophotometrically (Blois, 1958). The samples were analysed according to the method reported by Brand-Williams, Cuvelier, and Berset (1995). In brief, a volume of 3.8 ml of methanolic DPPH solution, c(DPPH) = 0.094 mmol/l, was added to 200 μl of diluted sample and the free radical scavenging capacity of the sample was evaluated by measuring the absorbance at 517 nm immediately after the addition of DPPH ($t = 0$) and at 1 min intervals, until the radical scavenging reaction reached to steady state. The results were expressed as the percentage of reduction (inhibition) of the DPPH (Q), which is defined by following expression: $Q = 100 \cdot (A_0 - A_s)/A_0$ where A_0 is the initial absorbance and A_s is the value of absorbance after the reaction reached to steady state (Yen & Duh, 1994). All determinations were performed in triplicate.

2.5.2. Free radical scavenging ability by the use of ABTS radical cation

Determination of antioxidant capacity of teas and herbal infusions was performed by Trolox Equivalent Antioxidant Capacity (TEAC) method adapted for flow injection analysis (FIA) (Iveković, Milardović, Roboz, & Grabarić, 2005). TEAC method is based on the scavenging of stable blue–green ABTS radical cation ($ABTS^{\bullet+}$), which is formed either by chemical (Re et al., 1999; Van der Berg, Haenen, Van der Berg, & Bast, 1999) or enzymatic (Arnao, Cano, & Acosta, 2001; Lemanska et al., 2001) oxidation of ABTS several hours prior to the analysis. The amount of ABTS radical cation scavenged by antioxidants is measured by monitoring the decrease of absorbance of ABTS radical cation, and compared with the decrease of absorbance produced by the addition of a known amount of Trolox, a water soluble vitamin E analogue. In order to avoid the

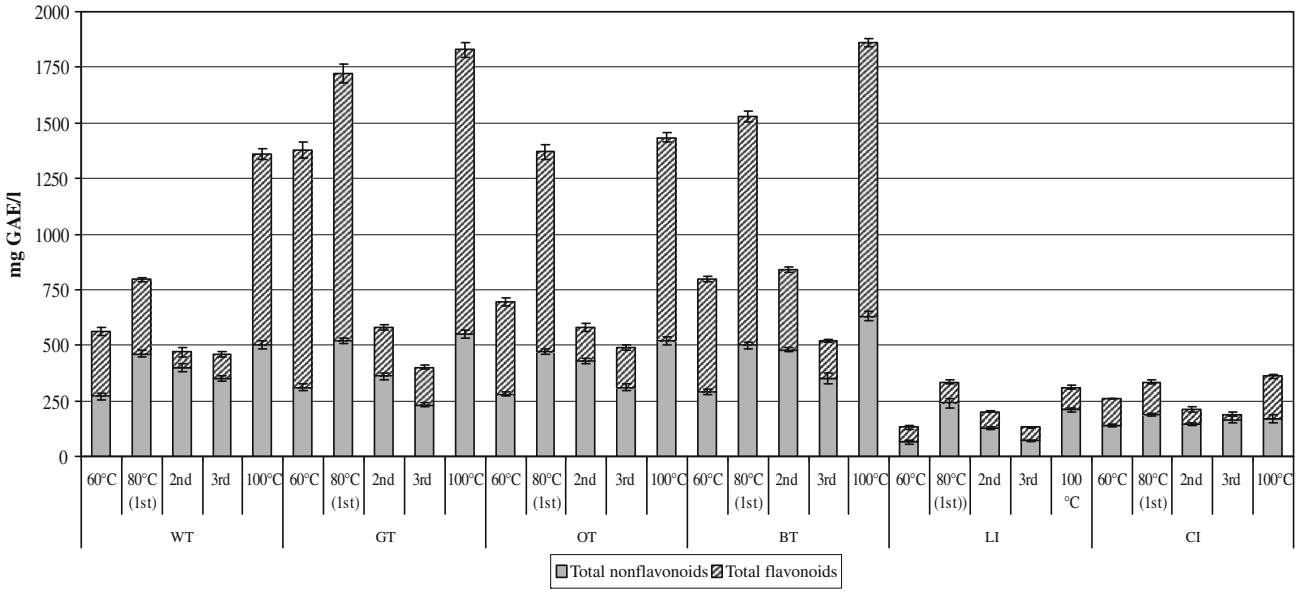

Fig. 1. Total nonflavonoid and flavonoid content in teas and herbal infusions affected by water temperatures and multiple extractions at 80 °C (1st extraction, 2nd extraction and 3rd extraction of the same sample – WT-white tea, GT-green tea, OT-oolong tea, BT-black tea, LI-linden infusion and CI-chamomile infusion). Results are expressed as mg GAE/l ± SD.

time consuming step of ABTS radical cation preparation, in this work we employed the FIA modification of TEAC method, based on the electrochemical production of ABTS radical cation in the electrolysis flow-cell forming the part of FIA system (Iveković et al., 2005). A 0.1 mol/l phosphate buffer solution (pH = 7.40) was used as a carrier stream, and the solution of ABTS radical cation generated electrochemically was employed as a second (reagent) stream. Both streams were pumped at a flow rate of 0.5 ml/min. The carrier and reagent streams were mixed by passing through a mixing coil and the absorbance at 734 nm was monitored by a detector placed just after the mixing coil. For the analysis an aliquot of 20 μl of diluted sample was injected into the carrier stream. The height of the FIA peak obtained by the sample injection (ΔA_{sample}) was compared with the height of the peak produced by the injection of 0.6 mmol/l Trolox solution (ΔA_{Trolox}). The TEAC values were then determined according to the formula: $\text{TEAC} = \Delta A_{\text{sample}} \cdot c(\text{Trolox}) \Delta A_{\text{Trolox}}$, and expressed as equivalent concentration of Trolox.

2.6. Statistical analysis

The results were statistically analysed using the Statistica 6.0 software to determine the mean value and standard error. The effect of water temperature and multiple extractions on the content of extracted polyphenols and methylxanthines were analysed by ANOVA and Duncan's multiple range test.

3. Results and discussion

Total phenol content (TPC) and total flavonoid content (TFC) of white, green, Oolong and black teas, linden and chamomile infusions, affected by water temperatures (60, 80, and 100 °C) and multiple extractions (1st, 2nd and 3rd), are presented in Fig. 1. As can be seen, TPC of teas decreases in the following order: GT > BT > OT > WT at water temperatures of 60 and 80 °C, and BT > GT > OT > WT at water temperature of 100 °C. In case of infusions, it decreases in following orders: CI > LI, CI = LI and CI > LI at water temperatures of 60, 80 and 100 °C, respectively. The obtained results confirmed previously published data (Perva-Uzunalić et al., 2006) that water at higher temperature extracted higher TPC and TFC, which reached to their maximum values at 100 °C. As shown in Fig. 1 green tea was recognised as the richest source of both total phenols (1380 mg/l GAE at 60 °C and 1830 mg/l GAE at 100 °C) and flavonoids (1070 mg/l GAE at 60 °C and 1280 mg/l GAE at 100 °C), whilst herbal infusions, especially linden, contained the lowest content of TPC and TFC. At 80 °C TPC and TFC in linden infusion were 230 and 65 mg/l GAE, respectively.

Multiple extractions of individual teas and herbal infusions at 80 °C yielded significantly ($p < 0.05$) lower TPC and TFC: 1st extraction > 2nd extraction ⩾ 3rd extraction. The 2nd extraction still yields a certain TPC and TFC, exhausting the original plant material of these compounds. As a consequence, TPC and TFC in the 3rd extracts were the lowest and in herbal infusions almost negligible, making the 3rd extraction fairly inefficient. Black tea extracts resulting from 2nd and 3rd extraction contained the highest TPC as well as TFC of all repeated extracts. Analysis of variance and Duncan's multiple range test point out a significant influence of multiple extractions ($p < 0.05$) on TPC and TFC.

Table 1 summarises the contents of identified flavan-3-ols in tested teas affected by water temperature. From the group of flavan-3-ols (−)-epicatechin (EC), (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (+)-gallocatechin (GC), (−)-gallocatechin-3-gallate (GCG), (+)-catechin (C) and (−)-epigallocatechin (EGC) were identified. These molecules with high nutraceutical potential are colourless and water-soluble compounds which impart bitterness and astringency to tea extracts (Wang, Provan, & Helliwell, 2000). The most abundant phenolic compound in all tested teas was EGCG, with the highest content determined in green tea, prepared at 100 °C (370 mg/l). In comparison with other teas, green tea contained the highest content of almost all identified flavan-3-ols, except GC and ECG (most abundant in black tea prepared at 100 °C). Lower content of the flavan-3-ol monomers is expected for the fully fermented black tea, whilst intermediate content is anticipated for the partially fermented Oolong tea (Kilmartin & Hsu, 2003). In chamomile infusion only EGCG and ECG were extracted at all water temperatures and in all repeated extracts, whilst EGC and EC were extracted only in infusion prepared at 80 °C. The water temperature showed significant influence

Table 1
Contents of flavan-3-ols [(−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin (EC), (+)-catechin (C), (−)-gallocatechin gallate (GCG), (−)-epicatechin gallate (ECG)] in teas and herbal infusions affected by water temperature (WT-white tea, GT-green tea, OT-oolong tea, BT-black tea, LI-linden infusion and CI-chamomile infusion). Values are expressed as means in mg/l ± SD (n = 3).

		GC	EGC	EGCG	EC	C	GCG	ECG	Total
WT	60 °C	5.48 ± 0.32	36.84 ± 0.63	96.57 ± 0.09	12.67 ± 0.03	10.77 ± 0.04	8.62 ± 0.13	35.32 ± 0.14	206.27
	80 °C	9.27 ± 0.41	39.62 ± 0.34	121.54 ± 0.07	14.29 ± 0.05	14.91 ± 0.03	0.53 ± 0.02	43.67 ± 0.17	243.83
	100 °C	32.05 ± 0.93	50.48 ± 0.51	184.71 ± 0.13	20.54 ± 0.06	27.63 ± 0.07	0.71 ± 0.00	67.41 ± 0.08	383.53
GT	60 °C	46.96 ± 1.02	245.99 ± 2.08	212.76 ± 0.18	120.61 ± 0.11	27.26 ± 0.05	4.46 ± 0.03	72.12 ± 0.13	730.16
	80 °C	51.10 ± 1.13	279.87 ± 1.87	324.54 ± 0.17	123.43 ± 0.13	19.70 ± 0.10	3.90 ± 0.06	108.55 ± 0.11	911.09
	100 °C	53.67 ± 1.72	288.80 ± 0,98	369.90 ± 0.21	133.90 ± 0.13	21.87 ± 0.04	4.04 ± 0.04	126.60 ± 0.15	998.78
OT	60 °C	19.49 ± 0.94	63.50 ± 0.42	70.19 ± 0.08	36.10 ± 0.08	10.44 ± 0.03	2.73 ± 0.01	38.50 ± 0.05	240.95
	80 °C	46.12 ± 0.65	78.22 ± 0.36	103.41 ± 0.13	38.68 ± 0.06	16.03 ± 0.08	1.69 ± 0.02	53.75 ± 0.07	337.90
	100 °C	93.60 ± 1.04	154.33 ± 1.11	194.24 ± 0.11	78.58 ± 0.11	36.14 ± 0.11	9.46 ± 0.10	99.26 ± 0.21	665.61
BT	60 °C	125.28 ± 5.05	54.73 ± 0.97	89.52 ± 0.09	28.26 ± 0.06	22.73 ± 0.07	4.35 ± 0.07	61.81 ± 0.71	386.68
	80 °C	187.98 ± 4.28	64.81 ± 0.56	143.83 ± 0.15	41.32 ± 0.09	34.12 ± 0.04	4.86 ± 0.07	105.23 ± 0.81	582.15
	100 °C	193.19 ± 2.07	52.75 ± 0.47	207.13 ± 0.19	32.59 ± 0.04	35.17 ± 0.10	0.93 ± 0.02	150.62 ± 0.23	672.38
LI	60 °C	n.d.	24.50 ± 0.13	50.49 ± 0.97	73.48 ± 0.12	n.d.	n.d.	n.d.	148.47
	80 °C	88.63 ± 0.87	40.91 ± 0.75	47.87 ± 0.82	14.25 ± 0.11	n.d.	n.d.	n.d.	191.66
	100 °C	66.30 ± 0.92	34.50 ± 0.56	14.32 ± 0.09	11.92 ± 0.07	n.d.	n.d.	n.d.	127.04
CI	60 °C	n.d.	n.d.	10.83 ± 0.08	n.d.	n.d.	n.d.	10.03 ± 0.09	20.86
	80 °C	n.d.	6.80 ± 0.07	28.30 ± 0.13	8.81 ± 0.06	n.d.	n.d.	24.33 ± 0.17	68.24
	100 °C	n.d.	n.d.	11.37 ± 0.07	n.d.	n.d.	n.d.	10.24 ± 0.10	21.61

n.d. – not detected.

($p < 0.05$) on flavan-3-ol content. These results are in agreement with the study of Sharma, Gulati, Ravindranath, and Kumar (2005), which demonstrated that catechins, especially EGCG, EGC and EC show noticeable difference when extracted at different temperatures. Unlike green tea and herbal infusions, the content of flavan-3-ols in white, Oolong and black tea was also significantly ($p < 0.05$) affected by water temperature. Price and Spitzer (1994) explained the effect of temperature on catechin diffusion by a difference in their molecular weight. The content of 999 mg/l total catechins in green tea (Table 1) is in agreement with the results published by Khokhar, Venema, Hollman, Dekker, and Jongen (1997) who extracted 849 mg/l of catechins from 1 g of Japanese green tea with 100 ml of water at 100 °C, but catechin content in teas is known to vary depending on the tea variety, origin, season of harvest and sun exposure. Kumamoto and Sonda (1998) reported that more intensive sun exposure relates to the higher catechin content.

According to the TPC and TFC (Fig. 1), the content of flavan-3-ols (Fig. 2) also gradually decreased in subsequent infusions and the lowest content was obtained in 3rd extracts of all teas and herbal infusions. However, some exceptions were observed for white and Oolong teas, whereas the 2nd extract exhibited higher total flavan-3-ols content, in relation to the 1st extract. This might be due to some differences in extraction performance, such as small variations in extraction time or pH value and some parameters of the chromatographic conditions, which can influence the polyphenol content and composition of the tested teas, thus giving variable measured concentrations by this HPLC method (Baptista, Tavares, & Carvalho, 1998). Repeated extractions of green, Oolong, black and linden samples showed significant influence ($p < 0.05$) on the content of flavan-3-ols.

Beside flavan-3-ols, phenolic acids also contribute to the overall antioxidant capacity of tea extracts. The effects of water temperatures and multiple extractions on the phenolic acid contents of teas and herbal infusions are presented in Table 2. Gallic, chlorogenic and *p*-coumaric acid were detected in "true" teas, whilst vanillic and caffeic acid were determined in linden infusion and chlorogenic, *p*-coumaric, ferulic and caffeic acid in chamomile infusion. The highest content of phenolic acids was determined in black tea, and it may have been released during the fermentation process (Harbowy & Balentine, 1997). In chamomile infusion, the highest content of caffeic (14 mg/l) and chlorogenic (2 mg/l) acids was

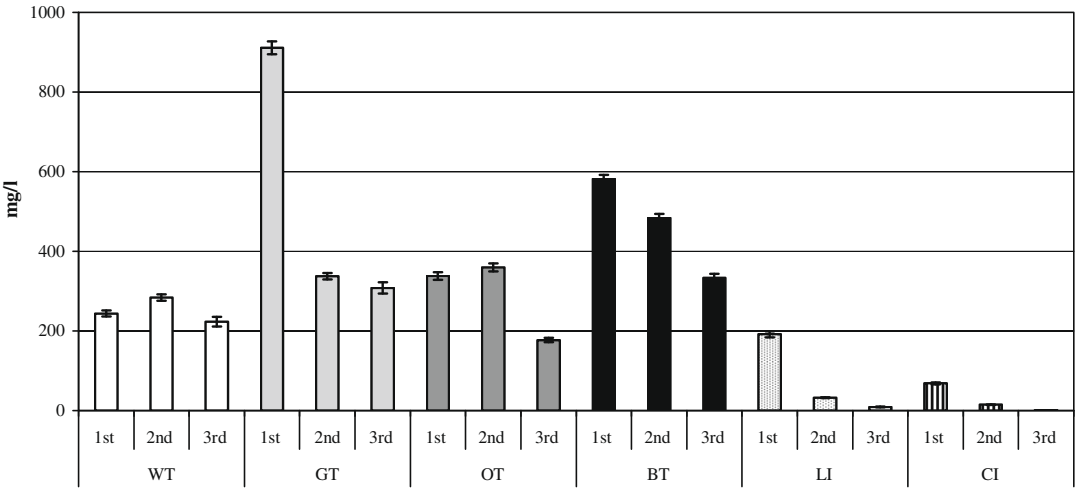

Fig. 2. Total flavan-3-ol content in teas and herbal infusions affected by multiple extractions at 80 °C (1st extraction, 2nd extraction and 3rd extraction of the same sample – WT-white tea, GT-green tea, OT-oolong tea, BT-black tea, LI-linden infusion and CI-chamomile infusion). Results are expressed as mg of total flavan-3-ol/l ± SD.

Table 2
Contents of phenolic acids [gallic (GA), chlorogenic (ChlA), *p*-coumaric acid (*p*-coumA), caffeic acid (CA), ferulic acid (FA) and vanillic acid (VA)] in teas affected by water temperature and multiple extractions (WT-white tea, GT-green tea, OT-oolong tea, BT-black tea, LI-linden infusion and CI-chamomile infusion). Values are expressed as means in mg/l ± SD (n = 3).

		GA	ChlA	*p*-coumA	CA	FA	VA	TOTAL
WT	60 °C	1.86 ± 0.12	0.65 ± 0.07	0.76 ± 0.09	n.d.	n.d.	n.d.	3.27
	80 °C (1st extract)	1.76 ± 0.15	1.13 ± 0.05	0.87 ± 0.05	n.d.	n.d.	n.d.	3.76
	2nd extract	2.03 ± 0.10	3.56 ± 0.06	2.68 ± 0.08	n.d.	n.d.	n.d.	8.27
	3rd extract	1.28 ± 0.09	2.59 ± 0.11	1.74 ± 0.10	n.d.	n.d.	n.d.	5.61
	100 °C	2.61 ± 0.11	1.34 ± 0.09	2.25 ± 0.07	n.d.	n.d.	n.d.	6.2
GT	60 °C	0.98 ± 0.11	2.44 ± 0.09	7.19 ± 0.13	n.d.	n.d.	n.d.	10.61
	80 °C (1st extract)	1.07 ± 0.07	2.92 ± 0.10	6.63 ± 0.23	n.d.	n.d.	n.d.	10.62
	2nd extract	1.25 ± 0.09	1.21 ± 0.05	1.88 ± 0.07	n.d.	n.d.	n.d.	4.34
	3rd extract	1.09 ± 0.04	0.59 ± 0.02	0.63 ± 0.03	n.d.	n.d.	n.d.	2.31
	100 °C	1.11 ± 0.08	1.81 ± 0.11	6.13 ± 0.18	n.d.	n.d.	n.d.	9.05
OT	60 °C	11.60 ± 0.19	1.59 ± 0.07	3.84 ± 0.12	n.d.	n.d.	n.d.	17.03
	80 °C (1st extract)	15.68 ± 0.31	3.32 ± 0.13	4.74 ± 0.08	n.d.	n.d.	n.d.	23.74
	2nd extract	8.33 ± 0.11	3.52 ± 0.11	4.58 ± 0.16	n.d.	n.d.	n.d.	16.43
	3rd extract	2.40 ± 0.07	1.45 ± 0.04	2.33 ± 0.07	n.d.	n.d.	n.d.	6.18
	100 °C	33.68 ± 0.51	8.89 ± 0.13	10.08 ± 0.25	n.d.	n.d.	n.d.	52.65
BT	60 °C	22.20 ± 0.13	2.91 ± 0.08	4.96 ± 0.09	n.d.	n.d.	n.d.	30.07
	80 °C (1st extract)	30.14 ± 0.21	3.22 ± 0.04	7.27 ± 0.11	n.d.	n.d.	n.d.	40.63
	2nd extract	3.23 ± 0.03	1.63 ± 0.02	4.96 ± 0.08	n.d.	n.d.	n.d.	9.82
	3rd extract	6.25 ± 0.15	0.80 ± 0.09	2.35 ± 0.17	n.d.	n.d.	n.d.	9.4
	100 °C	37.66 ± 0.79	1.56 ± 0.21	5.62 ± 0.16	n.d.	n.d.	n.d.	44.84
LI	60 °C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	80 °C (1st extract)	n.d.	n.d.	n.d.	2.53 ± 0.11	n.d.	11.39 ± 0.81	13.92
	2nd extract	n.d.	n.d.	n.d.	0.16 ± 0.02	n.d.	2.17 ± 0.09	2.33
	3rd extract	n.d.	n.d.	n.d.	n.d.	n.d.	0.09 ± 0.01	0.09
	100 °C	n.d.	n.d.	n.d.	0.38 ± 0.04	n.d.	14.86 ± 1.07	15.24
CI	60 °C	n.d.	n.d.	12.45 ± 0.76	9.91 ± 0.64	17.32 ± 1.03	n.d.	39.68
	80 °C (1st extract)	n.d.	2.03 ± 0.18	12.66 ± 0.91	14.27 ± 1.06	16.07 ± 0.96	n.d.	45.03
	2nd extract	n.d.	n.d.	12.08 ± 0.73	n.d.	5.07 ± 0.34	n.d.	17.15
	3rd extract	n.d.	n.d.	n.d.	n.d.	3.89 ± 0.18	n.d.	3.89
	100 °C	n.d.	1.22 ± 0.09	12.69 ± 1.03	n.d.	11.80 ± 0.89	n.d.	25.71

n.d. – not detected.

extracted with water at 80 °C, the highest content of *p*-coumaric acid (12.69 mg/l) was extracted at 100 °C, whilst the highest content of ferulic acid (17 mg/l) was extracted at 60 °C. First extraction of linden sample at 80 °C was the most effective in obtaining the highest content of caffeic acid (2.5 mg/l), whilst the highest content of vanillic acid was extracted at 100 °C (15 mg/l).

Compared to the content of flavan-3-ols, the content of detected phenolic acids was lower and it was also significantly ($p > 0.05$) affected by water temperature and by multiple extractions. This could be explained as a result of hydrophobic interactions of some phenolic compounds present in tea infusions, extracted at different temperatures, as well as synergistic or antagonistic effect of various compounds, including tea methylxanthines, on the content of extracted flavan-3-ols (Baptista et al., 1998). Variations in the obtained results confirm previously published data (Singleton & Rossi, 1965) that the presence of various phenolic compounds causes diversity in the sample behaviour.

Beside polyphenols, methylxanthines (Table 3) are also important biologically active constituents of tea. Amongst all determined biologically active compounds, caffeine was quantitatively the major constituent of tea extracts, except herbal infusions where it was not detected. The highest caffeine content were found in all teas extracted at 100 °C, and decreased in following order: WT > OT > GT > BT. Since the water temperature in household conditions usually ranges from 80 to 100 °C, it is interesting to mention that the lowest content of caffeine at 80 °C was determined in Oolong tea (156 mg/l), followed by black tea (184 mg/l), white tea (198 mg/l) and green tea (297 mg/l). Tea extracts contained relatively low content of theobromine and theophylline. The highest content of theobromine was determined in black tea extracted at 100 °C (46 mg/l), whilst the highest content of theophylline was detected in Oolong tea (17 mg/l) at the same temperature. Namely, theobro-

Table 3
Contents of methylxanthines [theobromine (TP), theophyline (TP) and caffeine (CF)] in teas and herbal infusions affected by water temperature and multiple extractions (WT-white tea, GT-green tea, OT-oolong tea, BT-black tea). Values are expressed as means in mg/l ± SD (n = 3).

		TB	TP	CF	TOTAL
WT	60 °C	23.19 ± 1.97	1.79 ± 0.18	106.54 ± 2.09	131.52
	80 °C (1st extract)	25.49 ± 1.66	2.30 ± 0.21	197.79 ± 2.71	225.58
	2nd extract	23.72 ± 1.03	2.68 ± 0.22	134.54 ± 2.12	160.94
	3rd extract	16.76 ± 0.78	2.63 ± 0.31	75.46 ± 1.54	94.85
	100 °C	38.61 ± 0.11	4.38 ± 0.08	335.05 ± 1.78	378.04
GT	60 °C	13.83 ± 0.16	5.33 ± 0.04	234.25 ± 1.89	253.41
	80 °C (1st extract)	15.41 ± 0.13	6.49 ± 0.06	296.86 ± 2.12	318.76
	2nd extract	7.32 ± 0.62	4.00 ± 0.47	124.29 ± 1.78	135.61
	3rd extract	4.46 ± 0.39	1.79 ± 0.19	42.54 ± 0.81	48.79
	100 °C	14.54 ± 0.05	6.34 ± 0.11	309.30 ± 2.32	330.18
OT	60 °C	22.52 ± 0.13	7.85 ± 0.07	110.69 ± 1.45	141.06
	80 °C (1st extract)	23.41 ± 0.19	7.04 ± 0.03	155.80 ± 0.79	186.25
	2nd extract	13.54 ±	6.01 ± 0.39	105.67 ± 1.33	125.22
	3rd extract	4.20 ±	2.27 ± 0.16	54.81 ± 0.66	61.28
	100 °C	34.93 ± 0.81	17.20 ± 1.78	316.18 ± 2.15	368.31
BT	60 °C	36.44 ± 0.79	5.65 ± 0.08	116.97 ± 1.07	159.06
	80 °C (1st extract)	45.08 ± 0.35	6.07 ± 0.07	183.97 ± 3.20	235.12
	2nd extract	23.28 ± 0.54	n.d.	117.54 ± 1.18	140.82
	3rd extract	9.03 ± 0.23	n.d.	62.48 ± 1.07	71.51
	100 °C	45.93 ± 0.29	9.27 ± 0.09	293.97 ± 3.03	349.17
LI	60 °C	n.d.	n.d.	n.d.	n.d.
	80 °C (1st extract)	3.83 ± 0.22	n.d.	n.d.	3.83
	2nd extract	0.33 ± 0.04	n.d.	n.d.	0.33
	3rd extract	n.d.	n.d.	n.d.	n.d.
	100 °C	2.94 ± 0.08	n.d.	n.d.	2.94
CI	60 °C	n.d.	n.d.	n.d.	n.d.
	80 °C (1st extract)	n.d.	0.55 ± 0.03	n.d.	0.55
	2nd extract	n.d.	n.d.	n.d.	n.d.
	3rd extract	n.d.	n.d.	n.d.	n.d.
	100 °C	n.d.	n.d.	n.d.	n.d.

n.d. – not detected.

Table 4
Antioxidant capacity of teas and herbal infusions evaluated by DPPH and ABTS assays, affected by water temperatures, multiple extractions and addition of milk and lemon (WT-white tea, GT-green tea, OT-oolong tea, BT-black tea, LI-linden infusion, CI-chamomile infusion). Values are expressed as% of inhibition ± SD, and mmol/l Trolox ± SD (n = 3).

	WT	GT	OT	BT	LI	CI
DPPH (% inhibition)						
60 °C	85.22 ± 5.8	86.13 ± 4.1	87.96 ± 8.2	86.86 ± 4.6	66.12 ± 3.9	65.13 ± 6.5
80 °C (1st extract)	85.77 ± 3.4	88.50 ± 3.6	88.87 ± 7.3	88.32 ± 5.2	68.23 ± 8.1	71.10 ± 4.7
2nd extract	72.78 ± 4.9	33.39 ± 0.7	76.24 ± 7.3	78.16 ± 8.2	38.63 ± 3.1	48.25 ± 3.6
3rd extract	51.08 ± 3.7	21.05 ± 2.0	35.42 ± 2.9	52.76 ± 4.6	36.41 ± 0.7	45.97 ± 2.9
100 °C	88.32 ± 9.9	89.23 ± 8.7	89.59 ± 4.9	89.42 ± 7.1	67.92 ± 2.8	69.57 ± 3.7
ABTS (mmol/l Trolox)						
60 °C	3.21 ± 0.19	8.12 ± 0.22	4.44 ± 0.16	7.95 ± 0.23	0.58 ± 0.02	0.27 ± 0.01
80 °C (1st extract)	5.21 ± 0.2	9.78 ± 0.31	6.22 ± 0.08	8.79 ± 0.25	0.96 ± 0.13	0.95 ± 0.14
2nd extract	3.74 ± 0.11	4.95 ± 0.27	4.13 ± 0.21	6.34 ± 0.29	0.54 ± 0.06	0.20 ± 0.02
3rd extract	2.35 ± 0.13	1.74 ± 0.11	2.27 ± 0.09	4.39 ± 0.17	0.17 ± 0.03	0.10 ± 0.01
milk	4.43 ± 0.09	6.34 ± 0.15	6.04 ± 0.11	6.82 ± 0.16	–	–
lemon	5.39 ± 0.08	6.21 ± 0.23	6.40 ± 0.11	8.82 ± 0.24	1.32 ± 0.17	1.12 ± 0.09
100 °C	6.6 ± 0.09	10.47 ± 0.04	7.73 ± 0.40	9.38 ± 0.18	0.80 ± 0.07	0.72 ± 0.07

mine is found in younger leaves, which are the choice leaves used in the production of black tea because of their high polyphenol content (Graham, 1992). Therefore, black teas should contain more theobromine than other teas. According to the obtained results, the content of extracted theobromine amounts to approximately 1/10 of the extracted caffeine content, which corresponds to the data found in earlier research (Friedman et al., 2005). Herbal infusions tested in this study contained limited content of methylxanthines, with theobromine detected in linden infusion and very low content of theophylline detected in chamomile infusion. The results obtained in this study suggested that multiple extractions of the analysed teas and herbal infusions also affected the total methylxanthines content (Table 3). Total methylxanthine content decreased in the following sequence: 1st extract > 2nd extract > 3rd extract, exhibiting an average 3.8-fold difference between the 1st and 3rd extracts of all teas, whilst the subsequent extracts of herbal infusions showed almost negligible content of total methylxanthines.

Obtained results (i.e. high TPC) suggest considerable antioxidant capacity of tested teas. The methods used to measure antioxidant capacity are extremely dependent on the reaction conditions and the substrates or products; all do not yield the same values for the activity. In order to obtain more reliable results, Fukumoto and Mazza (2000) suggested that antioxidant capacity should be measured using more than one method, by detecting the primary and secondary oxidation products. In this study two methods, DPPH assay and flow injection assay with electrochemically generated ABTS radical cation, were used. Measurement of the antioxidant capacity using DPPH radical scavenging assay has been widely spread due to its simplicity and reproducibility (Kitts, Wijewickreme, & Hu, 2000). Teas with higher inhibition percentage values (Table 4), react faster with the stable free DPPH radical, and reach their steady states before the others. Green tea and Oolong tea reached their plateau state after 10 min, followed by black tea (after 15 min), white tea (after 20 min) and herbal infusions (30 min). These results indicate that the DPPH radical scavenging ability of the tested samples can be predicted based on the previously determined total phenol content (Katsube et al., 2004). It should be emphasised that the antioxidant capacity of tea is not a property of a single phytochemical compound, but is widely distributed amongst the phenolic constituents (Simonetti, Pietta, & Testolin, 1997) and that the polyphenols identified in this paper represent a part of the total polyphenols found in tea.

Beside DPPH assay, an improved TEAC decolourisation assay-based flow injection analysis (FIA) method for evaluation of the antioxidant capacity was used. Antioxidant capacity of studied teas and herbal infusions, determined by scavenging the ABTS radical (Table 4), decreased in the following order: green tea > black tea > Oolong tea > white tea > chamomile infusion ≈ linden infusion. The percent inhibition determined by DPPH assay (Table 4), displays slightly different sequence of antioxidant capacities of teas and infusions: Oolong tea > black tea > green tea > white tea > chamomile infusion > linden infusion. According to these results, antioxidant capacity of tea extracts also increased with water temperature (60 °C < 80 °C < 100 °C) and decreased with the number of repeated extractions (1st extraction > 2nd extraction > 3rd extraction). The water temperature and repeated extractions showed significant influence ($p < 0.05$) on the antioxidant capacity.

The addition of milk as well as lemon juice to teas, particularly black teas, provides a means of lowering the astringency of the tea extracts. The results of some *in vitro* tests have produced diverse results, which indicate that phenolic compounds interact with the lipid fraction (Langley-Evans, 2000), or with casein proteins (Arts et al., 2002) in milk. These interactions could lower the antioxidant capacity (Langley-Evans, 2000) or produce no change at all (Richelle, Tavazzi, & Offord, 2001). Adding squeezed lemon juice to black tea clears the liquid, quickly transforming it from a dark, nearly opaque brown to transparent orangey yellow. Namely, the hydrogen ions derived from the citric acid in the lemon juice, suppress the ionisation of thearubigins (tannins), the polyphenols that impart brown colour to the tea. This reaction decolorizes thearubigins and eliminates their astringency, so strong black tea becomes more palatable by this way (Haslam, 2003). Lemon juice is also used as an addition to various herbal infusions. In this study the effect of milk and lemon juice addition (5%) on the antioxidant capacity of tea extracts was tested (Table 4). The addition of lemon juice to tea extracts did not show a significant effect on their antioxidant capacity ($p > 0.05$). In green tea the antioxidant capacity slightly decreased, whilst in other tested teas and herbal infusions it was the same, or its value was even slightly increasing. The addition of milk to the tea extracts caused a significant decrease ($p < 0.05$) in the antioxidant capacity from 3% (Oolong tea) to 35% (green tea).

The significant linear correlation was confirmed between TPC and antioxidant capacity of tea and herbal extracts prepared at all water temperature ($r_{ABTS} = 0.91$, $r_{DPPH} = 0.71$) as well as between TFC and their antioxidant capacity ($r_{ABTS} = 0.89$, $r_{DPPH} = 0.66$).

The relationship between the chemical structures (including sterical structures) and the free radical-scavenging activities of tea catechins (EGCG, EGC, EC) and their corresponding epimers (GCG, GC, (+)-C) is very complex. The antioxidant activity responds broadly to the tenet that the structures with the most hydroxyl groups exert the highest antioxidant activity, with the catechin isomers being more than twice as effective as vitamins E and C

(Rice-Evans, Miller, & Paganga, 1996). Also, the relationship between the steric hindrance and the accessibility of the radical centre of various radicals to each polyphenol could also influence the antioxidant capacity of tea (Yoshida et al., 1989).

According to previous findings by Rice-Evans et al., 1996 who studied the antioxidant activity of the polyphenolic constituents of green tea in relation to their relative compositions, the order of contribution to the antioxidant effectiveness in green tea was epigallocatechin ≈ epigallocatechin gallate ≫ epicatechin gallate = epicatechin > catechin. Sun and Ho (2001) established the order of antiradical capacities of green tea components obtained in a reaction with DPPH radical: EGCG > ECG > EGC > gallic acid > EC > BHT > 1,3,7-trimethyluric acid > caffeine. This order agrees partly with previous data on the antioxidant activity of tea catechins. According to these statements the scavenging abilities of EGCG and ECG are stronger even if the steric hindrance of EGC, GC, EC and (+)-C is smaller than that of EGCG and GCG, indicating that the presence of a gallate group at the 3 position plays the most important role in their abilities to scavenge free radicals (Guo et al., 1999).

The results of this study indicated that the antioxidant capacity of teas and infusions determined by both ABTS and DPPH assays, corresponds to their content of EGCG. EGCG was the most abundant polyphenol in all tested teas and in chamomile infusion. In linden infusion GC was the most abundant compound.

The content of EGCG was the highest in green tea prepared at 100 °C, it was 369.90 mg/L, which accounts for 20.2% of total polyphenol content. In order to confirm the structure-antioxidant capacity relationship additional correlations were performed between the content of EGCG, determined in all tested teas and infusions prepared at 100 °C, and their antioxidant capacity, and a high correlation was obtained (r_{ABTS} = 0.900 and r_{DPPH} = 0.750). According to these results, EGCG is the most efficient radical scavenger and contributes the most to the antioxidant potential of the tested teas, which agrees with previous findings (Guo et al., 1999; Rice-Evans, Miller, & Paganga, 1996).

4. Conclusion

This study provides a reliable set of informations regarding composition, polyphenolic content and antioxidant capacity of teas frequently consumed all over the world. Using a combination of spectrophotometric methods, FIA method for the evaluation of antioxidant capacity, HPLC analysis, and varying the extraction conditions, white, green, Oolong and black tea extracts as well as linden and chamomile infusions were characterised in terms of their polyphenolic content and antioxidant properties. Green tea was recognised as the richest source of both total phenols and flavonoids, whilst herbal infusions, especially linden contained the lowest TPC and TFC. By increasing the water temperature for the extraction, higher TPC and TFC were obtained, reaching their maximum when boiling water was used (100 °C), whilst multiple extractions of individual tea samples yielded significantly lower TPC and TFC: 1st extraction > 2nd extraction ⩾ 3rd extraction. EGCG was recognised as the most abundant phenolic compound, whilst caffeine was quantitatively the major constituent of tea infusions, except herbal infusions where it was not detected. The highest content of these compounds was extracted at 100 °C. The values of the antioxidant capacity of the extracts obtained by both DPPH and ABTS assays were in accordance with the TPC and TFC. The addition of lemon juice to tea extracts did not show a significant effect on their antioxidant capacity, whilst the addition of milk to the tea extracts caused a significant decrease in the antioxidant capacity from 3% (Oolong tea) to 35% (green tea).

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