Effect of multiple extractions and water-ethanol ratio on the bioactive composition and antioxidant capacity of Yinzhen tea

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Abstract

Tea is one of the most widely consumed beverages in the world. Its health benefits attributed to antioxidant, antitumor, and anti-inflammatory activities are the results of a rich polyphenolic content and composition. This study examined the effect of extraction solvents (water, 10%, 25%, 50%, 75% and 100% ethanol) and multiple extractions (1st, 2nd, 3rd) on the composition and antioxidant capacity of Yinzhen tea in loose leaf form. Total polyphenol and flavonoid contents, as well as the antioxidant capacity were measured spectrophotometrically, while individual catechins and methylxanthines were quantified using HPLC-PDA. The highest polyphenol content and antioxidant capacity were determined in 75% ethanol tea extract. The most abundant catechin was EGCG, with the highest content determined in 50% ethanol extract (429.82 mg/L). The application of lower ethanol concentration (50% or less) was the most efficient for extraction of catechins.

Keywords
Extraction solvent, HPLC, Polyphenols, Methylxanthines, Multiple extraction, Yellow tea

1. Introduction

Tea is one of the most popular beverages consumed worldwide. Its production and consumption grows rapidly, not only because of the health promoting effects, but also due to stimulative effects and desirable sensory properties. In the reports of FAO [1] world tea consumption increased from 3.8 million in 2009 to 4.4 million tons in 2010, while world tea production from 3.60 million in 2006 to 4.12 million tons in 2010. Projections from 2012 for the next 10 years indicate that the world production of black tea will grow at 1.87 % annually to reach 3.29 million tons, while the world production of green tea is expected to grow at a considerably faster rate of 7.2 % annually to reach 2.60 million tons [1].

Yellow tea is minimally fermented tea, produced only in China, but slowly gaining recognition in Western countries. It is very often equalized with green tea because of the very similar antioxidant content. Leaves for the production of yellow tea are harvested in the same manner as the leaves for green tea, but the aroma of yellow tea extract is flowery, fresh, and mild [2]. Apart from the attractive aroma profile, tea is popular due to its chemical composition responsible for health benefits. Its chemical composition is very complex, since it contains polyphenols, methylxanthines, amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, minerals such as manganese and potassium [3] etc. Horžić et al. [4] studied the effect of extraction techniques on yellow tea composition. Although the quantity of polyphenols and methylxanthines in this type of tea varied with the extraction procedure and extraction conditions (solvent, extraction duration, and methods), yellow tea was proved to be a rich source of bioactive compounds, as well as other, extensively examined types of tea [4]. It is particularly rich in polyphenols, especially flavonoids, among which the flavan-3-ols are generally the most abundant. The epigallocatechin gallate (EGCG) is the most prominent compound in this group. Methylxanthines were also quantified, with the highest content of caffeine [4]. Since polyphenols and methylxanthines are the main bioactive compounds in tea, significant effort has been put into finding the efficient extraction procedure. Most extraction
techniques manipulate the solvents physical properties in order to reduce the surface tension, increase the solutes solubility, promote a higher diffusion rate, and sometimes to change the solvent polarity. Besides simple solid-liquid extraction of tea polyphenols and methylxanthines, previous studies reported microwave assisted extraction [5], ultrasound-assisted extraction [6] or usage of supercritical carbon dioxide [7]. Water has been used to simulate household brewing conditions for a cup of tea [5, 8], alcoholic solvents [5] and other organic solvents have also been used for this purpose. Although alcoholic solvents are not highly selective for polyphenols, ethanol is often used because it is a food grade solvent, acceptable in small residual percentages. According to good manufacturing practice (GMP), ethanol extracts polyphenols better than water, it can be mixed with water in different ratios and can be much easily evaporated. Due to the lack of data on yellow tea, the aim of this study was to find the most effective water-ethanol ratio for the extraction, as well as to determine the effect of multiple extractions on polyphenols and methylxanthines of Yinzhen tea in loose leaf form.

2. Materials and methods

2.1. Chemicals
Folin-Ciocalteu, iron (III) chloride hexahydrate, and formic acid were supplied from Kemika (Zagreb, Croatia). DPPH (2,2-diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), and TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) were supplied from Fluka (Buchs, Switzerland). ABTS (2,2-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid)diammonium salt), authentic standards of flavan-3-ols and theaflavine were obtained from Aldrich (Sigma–Aldrich Chemie, Germany).

2.2. Sample preparation
Yinzhen tea analyzed in this study was purchased in a specialized tea store (House of tea, Zagreb, Croatia). The extractions were conducted using tea leaves in loose leaf (2.0 g) and pouring them with 200 mL of solvent heated to boiling temperature, stirred for 3 minutes, and then the extracts were filtered through a tea strainer. In order to determine the effect of multiple extractions, loose leaves of tea were extracted three times, under the same conditions (boiling temperature/3 min) to produce first, second and third extract.

2.3. Determination of total polyphenol (TPC), total flavonoid (TFC) and total nonflavonoid content (TNC)
Total polyphenol content was measured spectrophotometrically using Folin-Ciocalteu’s reagent according to a modified method of Lachman et al. [9]. Total flavonoids content was determined using the formaldehyde precipitation assay. Precipitated flavonoids were separated from the solution by filtration and nonflavonoids remained in the filtrate. Total flavonoid content was calculated as the difference between TPC and TNC. All measurements were performed in triplicate and expressed as mg/L of gallic acid equivalents (GAE).

2.4. HPLC analysis of polyphenolic 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. Separation was performed using a reversed-phase Pinnacle II-C18 column (250 mm x 4.6 mm x 5 µm). The samples were filtered through a 0.45 µm membrane filter and 20 µL was injected for analysis. The solvent compositions used were 2% formic acid (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 mL/min. Chromatograms were recorded at 278 nm. PDA detection was performed by recording the absorbance of the eluate between 200 and 400 nm. Compounds were identified by comparison of the retention times and spectral data with those of authentic standards. All analyses were repeated three times.

2.5. Determination of antioxidant capacity
Determination of free radical scavenging ability by the use of DPPH radical is a method reported by Brand-Williams et al. [10] based on the reduction of DPPH radical by methanolic solution of antioxidants. The results are expressed as mmol/L Trolox equivalents. The free radical-scavenging activity of tea extracts was also determined using the ABTS radical cation decolourization assay according to Re et al. [11]. The results of both assay were expressed as mmol/L Trolox, derived from a calibration curve determined for this standard. The ferric reducing/antioxidant power (FRAP) assay was carried out according to Benzie & Strain [12], and the results were expressed as mmol/L Fe⁺. All determinations were performed in triplicate.

3. Results and discussion
The efficiencies of multiple extractions (1st, 2nd, 3rd), and solvent (water, aqueous ethanol, 100% ethanol) on the content of total polyphenols (TPC), flavonoids (TFC) and methylxanthines from loose leaves of yellow Yinzhen tea
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were examined. The antioxidant capacity (AC) was measured and quantitative HPLC analyses of polyphenols and methylxantines in these extracts were also performed. Figure 1 displays the TPC and TFC of *Yinzhen* tea extracts, affected by multiple extractions, and water-ethanol ratio in the solvent. TPC of 1st extracts varied between 36.66 mg/L GAE and 1098.79 mg/L GAE, while TFC ranged from 32.45 mg/L GAE to 654.55 mg/L GAE. Multiple extractions exhibited a decreasing trend of both TFC and TPC: 1st extraction > 2nd extraction > 3rd extraction. The obtained results confirmed previously published data on green, black, white, oolong tea and herbal infusions: 2nd extraction still yields a certain TPC and TFC and can be used very well, while in the 3rd extract the ratio of flavonoids is negligible [13]. Based on the obtained results, the practice of reusing tea samples is not a good way of drinking since most of health-enhancing compounds are already previously extracted. 75% ethanol is the most effective extraction solvent during multiple extractions, even better than water, while 100% ethanol was the least effective for the preparation of polyphenol and flavonoid-rich extracts. Analysis of variance points out a mutual significant influence (p < 0.05) of multiple extractions and ethanol concentration on TFC and TPC.

![Figure 1: Changes in total flavonoid (TFC) and nonflavonoid (TNC) content of *Yinzhen* tea extracts affected by multiple extractions (1st, 2nd, 3rd) and the solvent (A= water, B= 10% ethanol, C= 25% ethanol, D= 50% ethanol, E= 75% ethanol, F= 100% ethanol).](image)

Generally, the process of extraction is rather complicated, and the yield of extraction depends on the type of solvents with varying polarities and pH, extraction time, and temperature, as well as on the chemical compositions and physical characteristics of the sample. Under the same condition of extraction time and temperature, the solvent used and the chemical properties of the sample are two most important factors. Some of the liquids used in solvent extraction, especially water, interact by means of hydrogen bonding. If molecules of substance can both donate and accept a hydrogen bond, a new entity, less polar than the substance, may be formed as a result. Although water is more effective for extraction purposes due to its higher polarity and shorter chain, the main drawback of the aqueous extraction is the low yield in antioxidants with low polarity or liposoluble antioxidants, as well as methylxanthines [14]. On the other hand, the usage of pure solvent, not only water, but also some other solvents such as ethanol or acetone for extraction of bioactive compounds is not preferable. Previous studies on plant materials have shown that the lowest amounts of recovered biologically active compounds were obtained with the usage of pure solvents including absolute ethanol [15] which is in agreement with the results obtained in this study. As it was previously mentioned, since water exhibits higher polarity than ethanol, the addition of a certain amount of water improves the extraction efficiency, due to the potential of water to increase the polarity of other solvent (ethanol), which also improves its extractability. The second explanation lies in the fact that ethanol as less polar solvent is more efficient in cell wall degradation, that are of nonpolar character, and cause the release of polyphenols from cells [16]. The addition of water causes the increment in swelling of plant material by water, which also enables better contact surface area between ethanol and matrix and further actions of ethanol. During extraction the content of ethanol lower than 75% water contributes to increased extraction of other compounds and, as a consequence a lower content of TPC and TNC is present in the extracts [17].
Catechins (flavan-3-ols) are the major polyphenolic constituents of tea and contribute to the overall antioxidant capacity of tea extracts. Table 1 summarizes the content of flavan-3-ols and gallic acid (GA), as the predominant phenolic acid in tea, that were quantitatively analyzed by HPLC, after tea leaves were multiply extracted. From the group of flavan-3-ols (-)-epigallocatechin-3-gallate (EGCG), (+)-gallocatechin (GC), (+)-catechin (C) and (-)-epicatechin (EC) were identified. The content of flavan-3-ols and GA gradually decreased (although not significantly) in subsequent extractions, producing the lowest content in 3rd extracts.

Table 1: Content of flavan-3-ols; (+)-gallocatechin (GC), (-)-epigallocatechin (EC), (-)-epigallocatechin-3-gallate (EGCG), (+)-catechin (C) and gallic acid (GA), and methylxanthines: theobromine (TB), theophylline (TP) and caffeine (CF), affected by the solvent (A= water, B= 10% ethanol, C= 25% ethanol, D= 50% ethanol, E= 75% ethanol, F= 100% ethanol) and multiple extractions (1st, 2nd, 3rd).

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<th>1st</th>
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<tr>
<td></td>
<td>mg/L</td>
<td>mg/L</td>
<td>mg/L</td>
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<tr>
<td></td>
<td>GC</td>
<td>EGC</td>
<td>EGCG</td>
</tr>
<tr>
<td>A</td>
<td>46.07±0.15</td>
<td>99.12±0.23</td>
<td>220.72±2.27</td>
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<tr>
<td>B</td>
<td>48.06±0.23</td>
<td>163.64±2.52</td>
<td>365.48±4.84</td>
</tr>
<tr>
<td>C</td>
<td>59.62±1.50</td>
<td>121.89±1.32</td>
<td>300.67±2.66</td>
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<tr>
<td>D</td>
<td>36.29±0.30</td>
<td>126.32±0.93</td>
<td>429.82±4.32</td>
</tr>
<tr>
<td>E</td>
<td>24.59±0.51</td>
<td>108.33±0.70</td>
<td>427.07±5.84</td>
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<tr>
<td>F</td>
<td>n.d.</td>
<td>8.81±0.02</td>
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<td>13.24±0.50</td>
<td>26.58±0.23</td>
<td>45.54±1.23</td>
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<td></td>
<td>9.84±0.51</td>
<td>45.90±1.08</td>
<td>152.93±2.23</td>
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<td>14.12±0.98</td>
<td>60.56±1.48</td>
<td>129.38±2.12</td>
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<td></td>
<td>6.79±0.21</td>
<td>48.30±1.56</td>
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<tr>
<td></td>
<td>3.84±0.16</td>
<td>13.32±0.87</td>
<td>148.11±2.69</td>
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<tr>
<td></td>
<td>n.d.</td>
<td>14.48±0.23</td>
<td>34.42±0.56</td>
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n.d. - not detected

However, exceptions were observed, since the 2nd extract had higher flavan-3-ols or GA content in relation to the 1st extract. The same was observed in white and Oolong tea infusions examined by Horžič et al. [13]. The most abundant catechin was EGCG, ranging from 24.34 to 429.82 mg/L with the highest content in 50% ethanol extract. Solvents with ethanol percentage lower than 50% are preferable for recovery of other identified catechins. This is in accordance with their extremely polar and hydrosoluble nature. Only catechin (C), the least polar catechin, was determined in higher amounts in 75% ethanol extract, while absolute ethanol exhibited the lowest extraction efficiency of polyphenols. Compared to the content of flavan-3-ols, the content of gallic acid was significantly lower, and the highest was detected in 1st extraction performed with 50% ethanol.

Besides polyphenols, methylxanthines are important biologically active compounds of tea contributing the tea alkaloids. The main methylxanthines (Table 1), caffeine (CF), theobromine (TB) and theophylline (TP), contribute
to the quality of tea. The content of methylxanthines was significantly affected by multiple extractions (p < 0.05), pointing again to a decreasing trend (1st > 2nd > 3rd) in subsequent extractions. CF was quantitatively the most abundant catechin whose highest content was determined in 50% ethanol extract, in which was present with TP activity in Yinzhen leaves. This makes them more soluble in water, and as the consequence, their higher content is found in extracts with lower ethanol percentage and/or pure water.

Numerous studies have demonstrated that tea catechins and polyphenols are effective scavengers of physiologically relevant reactive oxygen and nitrogen species \textit{in vitro} [18]. Since Fukumoto & Mazza [19] suggested that AC should be measured using more than one method, by detecting the primary and secondary oxidation products, in order to obtain the most relevant data about antioxidant activity of the extracts, three different methods were used in this study. Table 2 shows AC of extracts affected by multiple extractions and different water-ethanol ratio for loose leaf extracts obtained by FRAP, DPPH and ABTS assays. According to these results a significant decrease (p < 0.05) of AC was consistent with the number of repeated extractions and solvent used. Equally as for TPC and TFC, the difference in solvent polarity alters its ability to dissolve a selected group of antioxidant compounds and influences the content of bioactive compounds, as well as AC estimation. The values of the AC of DPPH, ABTS and FRAP assays are in accordance with TPC and TFC content, which is confirmed by a significant linear correlation obtained between the results (r > 0.80). This implies that the AC of samples is directly related to their polyphenolic content.

Table 2: Antioxidant capacity of Yinzhen tea extracts affected by the solvent (A= water, B= 10% ethanol, C= 25% ethanol, D= 50% ethanol, E= 75% ethanol, F= 100% ethanol) and multiple extractions (1st, 2nd, 3rd)

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<th>1st</th>
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<tr>
<td>DPPH</td>
<td></td>
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<tr>
<td>mmol/L</td>
<td>mmol/L</td>
<td>mmol/L Fe²⁺</td>
<td>mmol/L</td>
</tr>
<tr>
<td>Trolox</td>
<td>Trolox</td>
<td>Fe²⁺</td>
<td>Trolox</td>
</tr>
<tr>
<td>A</td>
<td>12.03 ± 0.11</td>
<td>9.43 ± 0.07</td>
<td>13.60 ± 0.33</td>
</tr>
<tr>
<td>B</td>
<td>13.40 ± 0.15</td>
<td>10.95 ± 0.27</td>
<td>13.06 ± 0.23</td>
</tr>
<tr>
<td>C</td>
<td>10.00 ± 0.14</td>
<td>10.92 ± 0.24</td>
<td>12.02 ± 0.17</td>
</tr>
<tr>
<td>D</td>
<td>13.41 ± 0.08</td>
<td>13.48 ± 0.06</td>
<td>15.02 ± 0.13</td>
</tr>
<tr>
<td>E</td>
<td>15.31 ± 0.10</td>
<td>13.29 ± 0.18</td>
<td>15.39 ± 0.19</td>
</tr>
<tr>
<td>F</td>
<td>0.55 ± 0.05</td>
<td>0.45 ± 0.03</td>
<td>0.63 ± 0.03</td>
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4. Conclusions
Extraction conditions (solvent used and multiple extractions) significantly affected TPC, TFC, methylxanthines content and AC of yellow Yinzhen tea extracts. Regardless of the solvent used the bioactive content, as well as AC decrease with the number of repeated extractions. 75% ethanol is the most effective extraction solvent during multiple extractions, even better than water, while absolute ethanol is the least effective for TPC and TFC recovery. EGCG is the most abundant catechin whose highest content was determined in 50% ethanol extract, in which was also the highest content of gallic acid found. Solvents with ethanol percentage lower than 50% are preferable for recovery of other identified catechins. The higher water content is preferable for the extraction of methylxanthines. The AC of the examined infusions correlated with their TPC and TFC, as well as with the content of EGCG.

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