Tea Polyphenols and Alkaloids Content Using Soxhlet and Direct Extraction Methods

Fui-Seung Chin, Khim-Phin Chong, Atong Markus and Nyet Kui Wong

School of Sustainable Agriculture, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu Sabah, Malaysia
School of Science and Technology, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu Sabah, Malaysia

Abstract: Extraction efficiency of extraction methods on the content of polyphenols (catechins and gallic acid) and alkaloids (caffeine, theobromine and theophylline) from tea leaves is studied. Level of polyphenols and alkaloids in samples (leaf buds, young leaves, old leaves and Sabah black Tea) from Sabah Tea Plantation were evaluated by HPLC. Leaf buds were found to have a higher polyphenol and alkaloid contents compared to young and old leaves. Fermentation process during manufacturing of Sabah Tea significantly reduced the levels of catechins, gallic acid and alkaloids by approximately 2-fold in comparison to fresh tea leaves. Degradation of catechins was also observed at high extraction temperatures. By increasing the heating temperature from 40°C in direct extraction to 70°C in soxhlet extraction, concentrations of total polyphenols and total alkaloids decreased. However, no significant change was observed in the Sabah Black Tea, with the only exception for epigallocatechin that was 3-fold lower in soxhlet extraction. It is recommended that for preparation of oxidation-sensitive plant samples like tea, extraction should be carried out at 40°C or lower using direct extraction method couple with a multi-step extraction procedure, rather than percolating with high boiling point (> 40°C) organic solvents that are routinely used in plant extraction.

Key words: Tea leaves • Soxhlet extraction • Direct extraction • Polyphenols • Alkaloids

INTRODUCTION

In tea (Camellia sinensis L.) plant, polyphenols and alkaloids have been considered as the main active components in many pharmacological studies [1-5]. Lost of potential activities in plant extracts after prolong extraction and heating were usually reported and that would be barrier to further study the functionality and potential of these extracts. Hence, a good extraction method must be able to extract compounds of interest completely and simultaneously avoid chemical modification [3]. Hot water (70–100°C) was frequently used in tea extraction in many studies [1, 2, 4-6]. However, the purpose of these studies focused more on the sensory evaluation of tea quality by colour, flavour and taste [7], provided little quantitative information about the actual polyphenols and alkaloids present in the solid samples. Many attempts have been made to investigate the factors attributed to extraction yields and great progress has been made. Yield of polyphenols and alkaloids in different extraction parameters such as heating temperatures, extraction solvents, pH and filter membranes have been analyzed [6, 8-12]. However, comparison of difference extraction methods for the extraction yields of tea leaf polyphenol and alkaloid contents from different maturation stages has not been reported.

In the present paper, the changes in polyphenol and alkaloid components in tea leaves (leaf buds, young leaves and old leaves) and in the commercial Sabah Black Tea (SBT) in two different extraction methods are examined.

MATERIALS AND METHODS

Chemicals: Authentic (+)-catechin hydrate (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), gallic acid (GA) and caffeine (Caf) were bought
from Sigma Chemical Co. (St. Louis, MO). Acetonitril (ACN) and methanol (MeOH) were HPLC grade. Dichloromethane (DCM) and n-hexane (Hex) were GC grade. Ortho-phosphoric acid-85% (H₃PO₄) was reagent grade chemical. High-purified water was from Millipore (Billerica, MA, USA).

**Samples Preparation**: Fresh tea leaves of the variety assamica were harvested from the field of Sabah Tea Plantation (STP). They were categorized into three main groups, consisting of tea leaf buds (LB), young leaves with light green in colour (YL) and old leaves with dark green in colour (OL). Commercial SBT was from the factory of STP. They were transferred to Laboratory in the same day and cleaned before being dried in a ventilated oven at 40°C for 48 hours. Dried plant samples were pulverized into powders and were used throughout in this study.

**Identification and Quantification of Tea Polyphenols and Alkaloids**

**Analytical Condition of High Performance Liquid Chromatography**: An Agilent Technologies 1200 series high performance liquid chromatography (HPLC) system was used. The column used was an Eclipse XDB-C18 reversed phase (5 µm, 250 x 4.6 mm) with a C18 (5 µm, 30 x 4.6 mm) guard column. Analytical conditions were optimized based on the methods of Yao et al. [13] and Owuor and Obanda [14]. The mobile phase composition started at 92% solvent A (0.1% aqueous H₃PO₄) and 8% solvent B (100% ACN) and linearly increased to 18% solvent B in 25 minutes, following by a post-run of 3 minutes before the next analysis cycle. Column temperature was set at 35°C and the effluent was monitored at 280 nm. All standards and samples were filtered through 0.20 µm PVDF membrane filters and aliquot of 20 µL was injected into the HPLC system.

**Establishment of Calibration Curves**: Quantification of catechins (C, EC, ECG, EGC and EGCG), caffeine (Caf) and gallic acid (GA) in sample extracts were carried out using HPLC with external standard. Stock standard solutions of 1 mg.mL⁻¹ of catechins, GA and Caf were prepared. A 10 different concentration of each authentic standard (0.05-50.0 µg.mL⁻¹) was generated by plotting linear regression of peak area in relation to known concentration (µg.mL⁻¹) injected into the HPLC system. The correlation coefficient (R²) of linear regression curve for all of the standards was then calculated using Microsoft Excel. Authentic standards of minor catechin [(-)-catechin gallate (CG)] and minor alkaloids [theobromine (Tb) and theophylline (Tp)] were not available in this study, the concentration of CG was quantified as the equivalent of its epimer ECG, while concentration of Tb and Tp were quantified as the equivalent of Caf.

**Extraction Methods**

**Soxhlet Extraction (SE)**: Each plant samples (50 g) were separately filled in four sets of soxhlet apparatus, each containing 350 mL of Hex as the first extraction solvent. The temperature was brought to boiling point (69°C) and percolation was carried out for 24 h. On the next day, Hex extract was discharged and all plant materials were allowed to dry before the second solvent DCM (350 mL) was refilled in and percolation was continued for another 24 h. The same process was repeated for MeOH (350 mL). At the end of extraction period, DCM and MeOH extracts of LB, YL, OL and SBT were individually distilled off at 40°C under reduced pressure in rotary evaporator. Once concentrated to a small volume, each extract was transferred to pre-weighed beaker and was allowed to dry completely in a bench top oven (30°C) overnight. A final concentration of 1 mg.mL⁻¹ of each DCM and MeOH extracts of LB, YL, OL and SBT were individually distilled off at 40°C under reduced pressure in rotary evaporator. The mean concentration was taken from 3 subsequent extractions of each sample in 3 replications to indicate the final concentration (mg.g⁻¹ DW⁻¹) of individual compound.

**Direct Extraction (DE)**: Direct extraction of plant samples (LB, YL, OL and SBT) were carried out with 20% aqueous ACN under continuous stirring (120 rpm) for 24 h in the dark. Ten grams of each plant sample were placed in 200 mL capped bottles, each of which was extracted with 100 mL x 3 of 20% aqueous ACN. In the fourth day, all 20% aqueous ACN extracts of LB, YL, OL and SBT were filtered prior to HPLC analysis. The mean concentration was taken from 3 subsequent extractions of each sample in 3 replications to indicate the final concentration (mg.g⁻¹ DW⁻¹) of individual compound.

**Data Analysis and Statistics**

**Statistical Analyses**: Statistical Package for Social Science software (SPSS) 12.0 Version for Windows was chosen in this study. The means and standard deviation of all the studied parameters within the samples were calculated with Microsoft Excel. Statistical significance differences of the extraction yields between SE and DE were analyzed with one-way ANOVA (analysis of variance) and the difference of means between groups was analyzed with post hoc test Tukey HSD. A p-value of 0.05 or less was considered statistically significant.
RESULTS AND DISCUSSION

Analytical Conditions: Complete baseline separation of tea polyphenols (C, EC, CG, ECG, EG, EGCG, and GA) and alkaloids (Caf, Tb, and Tp) was achieved in 25 min. All calibration curves obtained were linear over the concentration ranges indicated in Section 2.3.2, with R² ranged from 0.9977 to 1.0000 (results not shown). The HPLC chromatogram of a standard (C, EC, ECG, EGCG, GA, and Caf; 3 µg per standard) and a typical chromatogram of LB are shown in Fig. 1a and 1b, respectively. The major polyphenols and alkaloids present are labeled in Fig. 1a and 1b. The additional 3 peaks were unknown but they are most probably the minor alkaloid theobromine (Tb), theophylline (Tp) and minor catechins (-)-catechin gallate (CG) [13, 15, 16].

Yields of Individual Polyphenol from Soxhlet and Direct Extraction Methods: The SE-DCM, SE-MeOH and 20% ACN aqueous DE extractions from all samples (LB, YL, OL, and SBT) were subjected to HPLC to determine their concentration of polyphenol and alkaloid contents and were shown in Fig. 3. The largest portion of total polyphenols content was EGC, with LB (DE, 43.3 mg·g⁻¹; SE-MeOH, 10.8 mg·g⁻¹) and YL (DE, 35.8 mg·g⁻¹; SE-MeOH, 12.8 mg·g⁻¹) being the highest. The second largest portion of total polyphenols is EGCG, with LB and YL contained the highest (DE, 22.0-37.9 mg·g⁻¹; SE, 12.8-24.8 mg·g⁻¹). Major catechin ECG made up the third largest portion in the total polyphenols content; with LB contained the highest in concentration (DE, 22.0 mg·g⁻¹; SE-MeOH, 12.9 mg·g⁻¹), followed by YL (DE, 15.0 mg·g⁻¹; SE-MeOH, 7.9 mg·g⁻¹).
of Individual Alkaloid from Soxhlet and Direct Extraction Methods: Yield of total Caf from both SE and DE methods remains in the range of 54.9-58.8 mg·g⁻¹ in LB, 34.4-37.4 mg·g⁻¹ in YL and 19.9-22.2 mg·g⁻¹ in OL; with highest content obtained from SE method. In contrast, Caf content obtained from SBT was higher in the method of SE (44.5 mg·g⁻¹) in comparison to DE (30.5 mg·g⁻¹). For the minor alkaloids Tb and Tp, their present was in much lower quantities than Caf. The Tb contents in leaf samples were found to be approximately 10-fold lower than major alkaloid Caf, with a relative ratio of 11.0 in LB, 11.6 in YL, 8.5 in OL and 9.5 in SBT, whereas Tp was found to decrease from LB > YL > SBT > OL. Overall, total Caf content in all of the samples were mainly constant irrespective to maturity stages.

Contents of Polyphenol and Alkaloid in Sample Extracts: Fig. 3 shows the total concentration of all types of polyphenols and alkaloids obtained. Tea leaves were high in both polyphenols and alkaloids in general. Method of DE consistently yields higher and greater polyphenols and alkaloids in comparison to SE. Polyphenols content in LB, YL and OL obtained from DE method were 61.4-127.1 mg·g⁻¹, but decreased by 53.6-68.1% when SE method was used. The impact of extraction methods on SBT was small but yet significant, with a 25.4% and 22.0% decrease in polyphenol and alkaloid contents, respectively. Total alkaloid contents in LB, YL and OL were varied between 26.6-74.6 mg·g⁻¹ in DE and 20.8-58.1 mg·g⁻¹ in SE. Interestingly, the percentage variation of alkaloid contents from DE method to SE method in YL and OL were 64.3% and 64.2%, respectively. Prolong percolation of LB at high temperature (b.p. MeOH 65°C) in the SE method yielded polyphenols content that is closed to the content obtained in SBT, 59.0 and 51.5 mg·g⁻¹ respectively.

CONCLUSIONS

This study has demonstrated that direct extraction can extract polyphenol and alkaloid which its content is as similar to green tea, but when it is using soxhlet extraction, the polyphenol content is as similar to black tea. The yields of both polyphenol and alkaloid were significantly higher in the extract obtained by direct extraction method using 20% aqueous acetonitril than to the soxhlet extraction method using dichloromethane and methanol solvents. This indicated that total polyphenol and alkaloid contents in tea extracts are affected by the extraction methods used in sample preparation as well as maturity stages of the tea leaves used, with their contents decreased in the order of LB > YL > SBT > OL for polyphenol, while alkaloid follows the order of LB > SBT > YL > OL.

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