An improved solvent extraction method for the analysis of catechins and caffeine in green tea

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Summary
The aims of this research were to improve the stability of green tea catechins (GTC) in order to make the HPLC analysis more accurate. Five solutions were selected to study the efficiency of GTC extraction: hot water (90 °C), room-temperature water (22 °C), methanol, ethanol and 75% ethanol. The study showed that the most efficient solvent for extraction of GTC was methanol followed by 75% (v/v) ethanol, hot water, room-temperature water and ethanol. In addition, it was found that (–)-epigallocatechin-3-gallate (EGCG) was unstable in hot water infusion as the EGCG content sharply decreased after 30 min at room temperature. In contrast, caffeine and other GTC were more stable in hot water infusion and their contents did not significantly change until the end of the experiment (90 min). However, the study revealed that when the hot water infusion was cooled on ice, the quantity of GTC was 10% higher than for methanol, and when the pH was adjusted to below 4 after cooling, the quantity of GTC was 20% higher than for methanol. In conclusion, placing the infusions on ice and adjusting the pH to below 4 dramatically improved the HPLC analysis of GTC and caffeine in green tea.

Keywords
green tea; extraction; catechins; caffeine; (–)-epigallocatechin-3-gallate; HPLC

Green tea is rich in compounds called catechins. Many studies indicate that green tea catechins have a number of health-promoting properties that include anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-bacterial, anti-obesity and anti-atherosclerotic (heart disease and stroke) effects [1]. Green tea catechins have the potential to lower blood cholesterol, a major risk factor for heart disease and strokes, by reducing dietary cholesterol and fat absorption from the gastrointestinal tract [2], as well as by decreasing cholesterol synthesis and increasing the expression of the LDL receptor, which is involved in clearing cholesterol from the blood vessel [3]. The composition of green tea catechins varies with species, harvesting season, picking maturity (plucking position), climate and horticultural practices [4], all of which may make up to 30% of the total dry weight. The major catechins in green tea are: (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatecatechin (EGC), (–)-epicatechin (EC) and (–)-epicatechin-3-gallate (ECG). High performance liquid chromatography (HPLC) is one of the most widely used techniques to separate and quantify the catechins in green tea. However, according to a study in 2002 [5], it was indicated that the four main green tea catechins including EGCG, EGC, EC and EGC were unstable at high temperatures and in alkaline solutions. Therefore, to improve the accuracy of the HPLC technique, increasing the stability of catechins had to be established. So a water extraction method was selected for this research project because water does not leave behind any hazardous residue compared to other solvents such as methanol, and because it is environmentally friendly [6].

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MATERIAL AND METHODS

Tea samples and chemicals

The Chinese green tea (Temple of Heaven) from the Shanghai Tea Import and Export Company (Shanghai, China) was purchased from an Asian supermarket in Ashfield, Australia, and the HPLC grade methanol and ethanol used for the extraction of the tea samples were purchased from Merck (Kilsyth, Australia). Solvents and organic modifiers used in the HPLC mobile phases, acetronitrile, ortho-phosphoric acid and tetrahydrofuran were of HPLC grade from Sigma (Castle Hill, Australia) and AJAX Finechem (Baulkham Hill, Australia), respectively. Milli-Q de-ionized water was prepared daily using Millipore purification system (Millipore Australia, North Ryde, Australia). The four major catechins EC, EGC, EGCG and ECG were purchased from Sigma and used as external standards, which were more than 98% pure. The internal standard, 4-amino salicylic acid was purchased from Sigma-Aldrich.

Extraction of tea components

Green tea (1.0 g) and 400 μl internal standard solution (0.125 mol·l^{-1} 4-amino salicylic acid) were infused for 30 min with 100 ml of water at room temperature (RT, 22 °C), ethanol, 75% ethanol (v/v) or methanol at 50 °C or water heated to 90 °C.

Procedures after the extractions

The preparation of the extracts for HPLC analysis was performed using three different procedures. The first procedure was to immediately filter the infusion through a disposable 0.45 μm syringe filter unit (Altech Associate, Baulkham, Australia) into 4 ml vials for injection onto the HPLC system. The second procedure was to immediately place the infusion on ice for 10 minutes to bring the temperature of the infusion down to 4 °C before filtering and injecting onto the HPLC system. The third procedure involved placing the infusion on ice as in the second procedure, but after cooling the pH of the infusion was adjusted to below 4 using 0.2% phosphoric acid, before filtering and injecting onto the HPLC system. All procedures were done three times.

The HPLC system

The method for analysing the amount of catechins and caffeine in the green tea was developed by YOSHIDA et al. [7]. A Shimadzu HPLC system (Shimadzu Scientific Instruments (Oceania), Rydlmere, Australia) consisted of a computer-controlled system with VP 5.03 software, SCL-10A VP system controller, GT-154 degasser, FCV-10AL mixer, LC-10AD liquid chromatography pump, automatic injector SIL-10AXL VP with a 20 μl loop, SPD-10A UV-VIS detector, CTO-10Avp column oven and CBM-10A communication BUS module. The chromatographic separation was performed on a C_{18} reversed phase. Mobile phase A consisted of 0.2% (v/v) phosphoric acid 86% (v/v), acetonitrile 12% (v/v) and tetrahydofuran 1.5% (v/v) and mobile phase B consisted of 0.2% (v/v) phosphoric acid 73.5% (v/v), acetonitrile 25% (v/v) and tetrahydrofuran 1.5% (v/v). The flow rate of the mobile phase was 1 ml·min^{-1}. The mobile phase compositions used were 100% of mobile phase A during the first 30 min; then mobile phase B was increased from 0% to 100% over the next 10 min and allowed to continue at 100% for further 20 min. After that, mobile phase B was decreased from 100% to 0% for 10 min and, finally, mobile phase A was used again for 20 min before the next injection.

Standard curve

The external standard stock solutions, which included caffeine, EGC, EC, EGCG and ECG, were prepared in de-ionized water (all 200 μg·ml^{-1} of de-ionized water) and stored at 4 °C until use. Six working standard solutions were prepared by serially diluting the stock solutions (1:1; stock solution: de-ionized water) five times. All of the working standard solutions were injected onto the HPLC three times and the average areas for each constituent were divided by the average area for the internal standard plotted against each external standard concentration to generate the standard curves.

Data analysis

Each extraction or procedure was performed thrice, with final values expressed as the mean (gram of compound per kilogram of green tea) $\pm$ the standard error (SEM) for each triplicate. The equations used to calculate the amount of caffeine and catechins are stated in the Tab. 1.

Tab. 1. Equations used to calculate the amount of caffeine and catechins.

	Equation
Caffeine	$y = 7.350x + 1.111$ ($R^2 = 0.997$)
EGC	$y = 12.90x + 0.646$ ($R^2 = 0.996$)
EC	$y = 5.846x + 0.001$ ($R^2 = 0.999$
EGCG	$y = 10.29x - 0.014$ ($R^2 = 0.998$)
ECG	$y = 21.06x - 0.410$ ($R^2 = 0.998$)

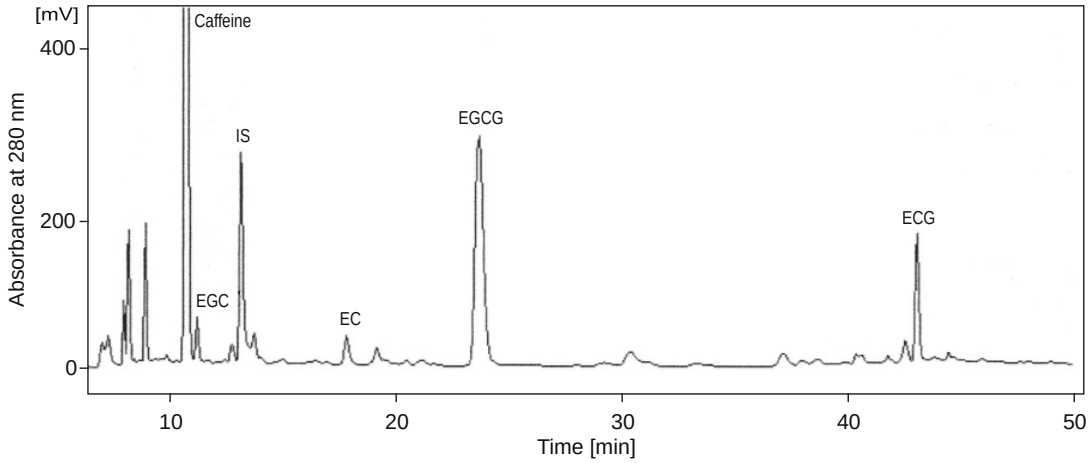

Fig. 1. Chromatogram of green tea infusion.

Green tea (1 g) was infused in 100 ml of hot water (90 °C) for 30 min. Internal standard (0.5 mmol·l^{-1} 4-amino salicylic acid) is involved.

Linear regression analysis of the external standard curves was carried out by Excel 6.0. (Microsoft, Redmond, Washington, USA). The SPSS 9 program (SPSS, Chicago, Illinois, USA) was used to compare the mean level of the components in the tea infusion using One-way ANOVA and the Post-Hoc Bonferroni test with statistical significance $P<0.05$.

RESULTS

Quantitative extraction of catechins and caffeine using different solvents

The chromatogram in Fig. 1 is an illustration of a typical separation of a Chinese green tea constituents, which was extracted using water at 90 °C for 30 min. All major catechins and caffeine were well separated.

Fig. 2 shows the quantity of catechins and caffeine extracted from green tea using different solvents. The graph indicates that the most efficient solvent for extraction of catechins (total for EGC, EC, EGCG and ECG) from green tea was methanol (38.0 g·kg^{-1} ± 1.9 g·kg^{-1}) followed by 75% (v/v) ethanol (31.8 g·kg^{-1} ± 1.2 g·kg^{-1}), hot water (26.4 g·kg^{-1} ± 1.5 g·kg^{-1}), room temperature water (11.3 g·kg^{-1} ± 0.8 g·kg^{-1}) and ethanol (9.4 g·kg^{-1} ± 0.6 g·kg^{-1}). In addition, Fig. 2 shows that the most efficient solvent for extraction of caffeine was 75% (v/v) ethanol (19.1 g·kg^{-1} ± 0.8 g·kg^{-1}) and hot water (19.0 g·kg^{-1} ± 0.2 g·kg^{-1}) followed by room temperature water (16.3 g·kg^{-1} ± 1.9 g·kg^{-1}), methanol (14.7 g·kg^{-1} ± 0.8 g·kg^{-1}) and ethanol (7.3 g·kg^{-1} ± 0.7 g·kg^{-1}).

Stability of green tea infusions extracted with hot water

Fig. 3 illustrates the stability of catechins and caffeine in green tea infusions left to cool down at room temperature after extraction at 90 °C. The graph indicates that EGCG was stable in the cooling infusion for up to 30 min, and then the content declined until the end of the experiment (90 min).

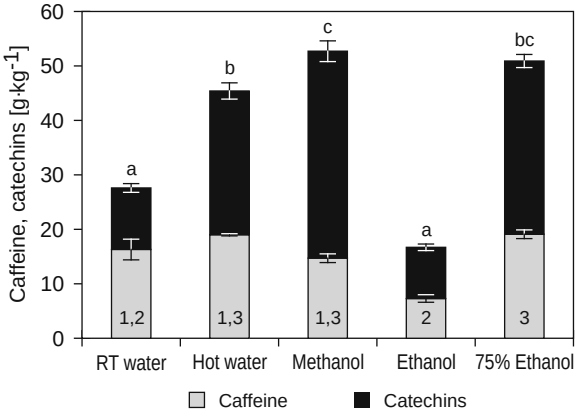

Fig. 2. Comparison of the extraction of caffeine and total catechins from green tea using different solvents.

Caffeine and total catechins were measured by HPLC after infusion for 30 min of 1 g of green tea with water at room temperature (RT water), water at 90 °C (hot water), methanol at 50 °C (methanol), ethanol at 50 °C (ethanol) and 75% (v/v) ethanol at 50 °C (75% ethanol). The values are means + SEM of triplicate extractions and values for total catechins not sharing a letter and those for caffeine not sharing a number are significantly different (P < 0.05). Values are expressed in grams per kilogram of tea.

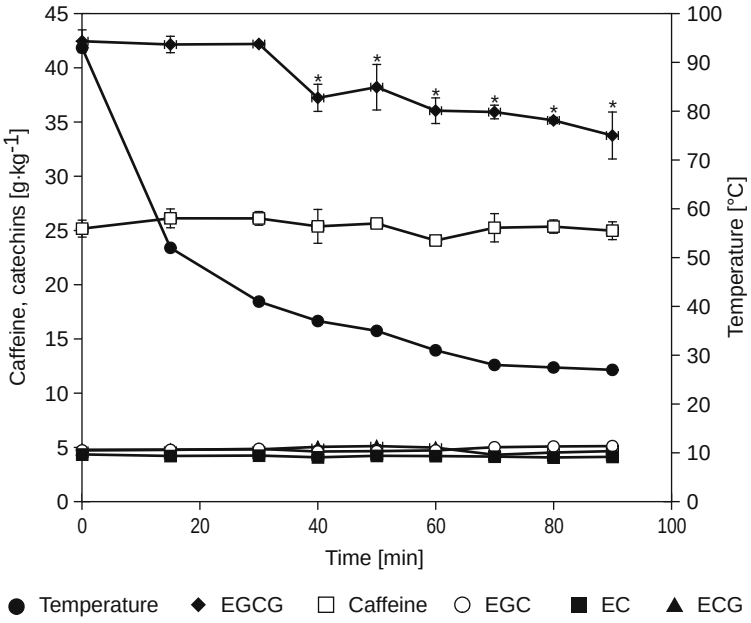

Fig. 3. Stability of caffeine and catechins in a hot water infusion of green tea.

One gram of green tea and internal standard (0.5 mmol·l^{-1} 4-amino salicylic acid) were infused with 100 ml of hot water at 90 °C for 30 min and then left to cool down to room temperature. Temperature, EGCG, caffeine, EGC, EC and ECG were measured at the indicated times. The values are means + SEM of triplicate experiments. The * symbol indicates that the values for EGCG from 40 min onwards were significantly different from the EGCG values at 0, 15 and 30 min of cooling time at room temperature ($P < 0.05$). Values are expressed in grams per kilogram of green tea.

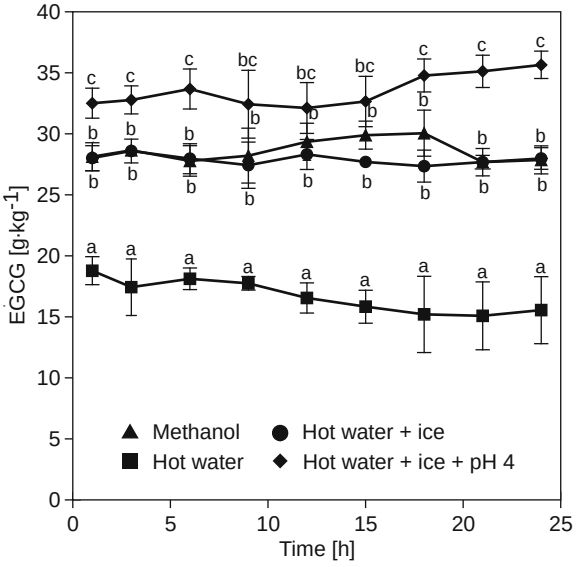

Fig. 4. Comparison of the quantity of EGCG from green tea infusions prepared using different extraction procedures.

One gram of green tea and internal standard (0.5 mmol·l^{-1} 4-amino salicylic acid) were infused with 100 ml of methanol at 50 °C for 30 min (Methanol), hot water at 90 °C for 30 min (Hot water), hot water at 90 °C for 30 min followed by 10 min on ice (Hot water + ice) or hot water at 90 °C for 30 min followed by 10 min on ice and pH adjusted to 4 (Hot water + ice + pH 4). The samples were then placed on the HPLC automatic injector tray (21 °C) and EGCG was determined at the indicated times after the end of the 30 min infusion period. The values are means + SEM of triplicate experiments and values not sharing a letter are significantly different ($P < 0.05$). Values are expressed in grams per kilogram of green tea.

In addition, the study showed that the amount of EGCG started to decline when the temperature decreased to 35–37 °C. In contrast, caffeine and other catechins were more stable than EGCG, as the amounts of these barely changed throughout the experiment.

Fig. 4 shows the stability of EGCG at room temperature for up to 24 h when extracted using different procedures. It was found that the contents of EGCG continuously decreased in the infusions for up to 24 h when it was extracted from green tea using hot water (90 °C) for 30 min and then left at room temperature. In contrast, EGCG was not lost from infusions when it was extracted using either methanol at 50 °C, nor when the hot water infusions were placed on ice for 10 min immediately after 30 min at 90 °C.

Consistently with Fig. 4, the quantity of EGCG measured in the hot water extract left to cool down at room temperature was about 30% less than the EGCG in the methanol extract. In contrast, EGCG in the hot water infusion placed on ice for 10 min after 30 min at 90 °C, was not significantly different from the amounts measured in the methanol extract. Furthermore, when the pH of the water infusions was adjusted to less than 4 after the 10 min on ice, the quantity of EGCG was approximately 16% higher than for the methanol extract.

In Fig. 5, the stability of the other three main catechins and caffeine over 24 h is shown. Consistent with Fig. 3, there were no major losses of these

catechins and caffeine in any of the infusions. However, as for EGCG, the quantity of ECG in the hot water extract left to cool down at room temperature was about 55% less than ECG in the methanol extract or in the hot water infusions that were placed on ice for 10 min immediately after 30 min at 90 °C. However, EC, EGC and caffeine were more efficiently extracted when the green tea was infused with hot water than when methanol was used.

Quantitative extraction of catechins and caffeine using different procedures

Fig. 6 shows the quantity of catechins and caffeine extracted from green tea using different procedures. It was found that the combination of infusing the green tea with water at 90 °C for 30 min followed by 10 min on ice and then adjusting the pH to below 4, was the most efficient method for extracting the catechins and caffeine. The quantity of catechins extracted in this manner was approximately 10% higher than for water infusions not pH-adjusted after cooling on ice for 10 min, 20% higher than for methanol and 30% higher than for water infusions left to cool at room temperature.

DISCUSSION

In order to improve the method for analysing caffeine and catechins in green tea, a HPLC procedure was used for the simultaneous determina-

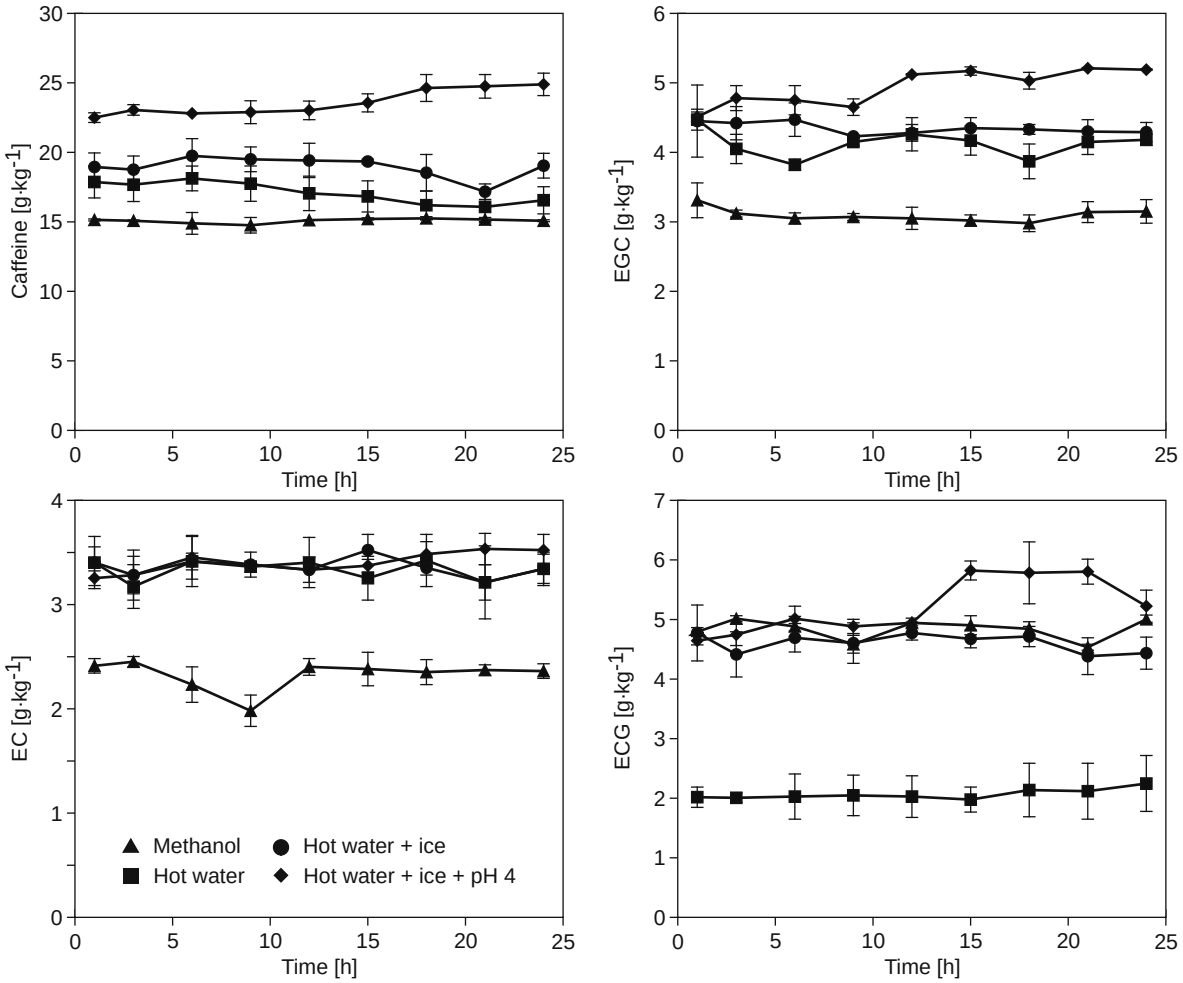

Fig. 5. Comparison of the quantity of caffeine, EGC, EC and ECG from green tea using different solvents and extraction procedures.

One gram of green tea and internal standard (0.5 mmol·l^{-1} 4-amino salicylic acid) were infused with 100 ml of methanol at 50 °C for 30 min, hot water at 90 °C for 30 min, hot water at 90 °C for 30 min followed by 10 min on ice or hot water at 90 °C for 30 min followed by 10 min on ice and pH adjusted to 4. The samples were then placed on the HPLC automatic injector tray (21 °C) and measurements made at the indicated times after the end of the 30 min infusion period. The values are means + SEM of triplicate experiments.

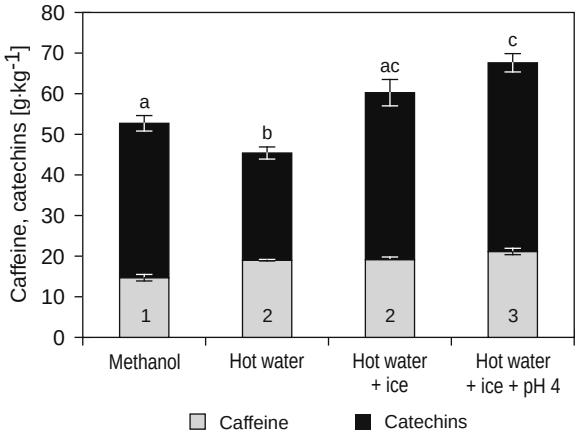

Fig. 6. Comparison of the extraction of caffeine and total catechins from green tea using different solvents.

Caffeine and total catechins were measured by HPLC after infusion for 30 min of 1 g of green tea with methanol at 50 °C (Methanol), water at 90 °C for 30 min and cooled at room temperature (Hot water), water at 90 °C for 30 min and cooled on ice for 10 min (Hot water + ice), water at 90 °C for 30 min cooled on ice and the pH adjusted to less than 4 (Hot water + ice + pH 4). The values are means + SEM of triplicate extractions and values for total catechins not sharing a letter and those for caffeine not sharing a number are significantly different ($P < 0.05$). Values are expressed in grams per kilogram of green tea.

tion of caffeine and catechins (EGC, EC, EGCG and ECG). The extraction efficiency by different solvents was determined by comparing the quantity of caffeine, EGC, EC, EGCG and ECG. Methanol performed the best, being the most efficient extraction solvent. However, the highest quantity of caffeine was obtained from the water extraction, a result that agrees with the study of PERVA-UZUNALIĆ et al. [8]. The addition of water (25% by volume; MilliQ de-ionized water) to ethanol resulted in an increase of the extraction efficiency compared to pure ethanol, illustrated by respective increase of 161% and 238% in the extracted quantities of caffeine and catechins, a finding similar to results of a study by YUNG-SHEN et al. [9]. A possible reason for this is that polarities of catechins and caffeine are closer to water than ethanol. Regarding the stability of catechins and caffeine in hot water in the first 90 min after extraction, the studies showed that epigallocatechin gallate (EGCG) accounted for most of the losses in catechins when the hot water was allowed to slowly cool down to room temperature (22 °C). In addition, the continuing studies showed that EGCG content continually declined until the end of the experiment (24 h). In contrast, caffeine and the other catechins (EGC, EC and ECG) were more stable and their concentrations changed very little during 24 h after extraction. The reason why EGCG was less stable than other catechins is not clear. However, according to study by ZHU et al. [10], the three adjacent hydroxyl groups at position 3', 4' and 5' in EGCG are more vulnerable to destruction than the two adjacent hydroxyl groups at position 3' and 4' in ECG and EC.

CONCLUSIONS

With the aim of improving extraction efficiency in sample preparation for the analysis of caffeine and catechin concentrations by HPLC, three different post-extraction sample preparation methods were investigated. The results showed that when a hot water infusion was filtered immediately after extraction and analysed by HPLC, the total quantity of catechins measured was 30% less than for methanol. However, when the hot water infusion was cooled on ice, the quantity of catechins measured was 10% higher than for methanol, and when the pH was adjusted to below 4 after cooling, the quantity of catechins measured was 20% higher than for methanol. Although decreasing pH to below 5 and rapid cooling of solution did not influence the diffusion of EGCG into the water, it slowed down the oxidation activity of EGCG. In other words, it increased the stability of EGCG. Therefore, higher amounts of EGCG were found [11].

Further studies showed that epigallocatechin gallate (EGCG) accounted for most of the losses in catechins when the hot water infusion was allowed to slowly cool down to room temperature (22 °C). This study showed that placing the infusions on ice and adjusting the pH to below 4 after cooling dramatically improved the HPLC analysis of catechins extracted from green tea using hot water. This prevented the loss of EGCG during the cooling process when the hot water infusion was left to slowly cool down to room temperature.

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