Effect of Extraction Condition on the Content of EGCG and Caffeine of Green Tea: Comparison with the Inhibitory Activity on Pancreatic Lipase

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Abstract – Caffeine and epigallocatechin gallate (EGCG) are major constituents of green tea, the leaves of *Camellia sinensis* (Theaceae). Although EGCG is well known for diverse beneficial effect, caffeine is sometimes harmful with adverse effects. Therefore, the extraction efficiency was investigated using different extraction method such as extraction solvent, extraction time, extraction method, and repeated extraction. The content of EGCG and caffeine in green tea extract was quantitated by HPLC analysis. The extraction condition exerted difference on the extraction yield. The content of EGCG was also affected by different extraction condition. Especially, the extraction solvent greatly affected the content of EGCG in the extract. However, the content of caffeine was less affected compared to that of EGCG. The inhibitory effect of green tea extract on pancreatic lipase was almost similar regardless of extraction condition. Taken together, optimization of extraction condition will provide best efficacy for further development of green tea as anti-obesity therapeutics.
Keywords – Green tea, Quantitation, HPLC-DAD, Caffeine, EGCG, Pancreatic lipase.

Introduction

Green tea, the leaves of *Camellia sinsneis* (Theaceae), is a popular beverage in Asian countries and worldwide. Ancient document "Donguibogam" by Huh Jun says that green tea helps digesting, shortening their sleep, clearing the head and eyes and detoxifying the body. Recent study also revealed that pharmacological effects of green tea, including anti-cancer (Zhang *et al*., 2011), reducing cholesterol (Bornhoeft *et al*., 2012), protecting against allergy, diabetes, and detoxification (Rickman *et al*., 2010; Ramadan *et al*., 2009). Phytochemical investigations have reported epigallocatechin gallate (EGCG), catechin, epicatechin gallate (ECG) and caffeine as major constituents of green tea. Especially, EGCG is a characteristic constituent of green tea and is known to exert diverse effect of green tea (Du *et al*., 2012; Yu *et al*., 2010; Zhong *et al*., 2012; Steinmann *et al*., 2013). Caffeine is also contained in green tea and sometimes causes adverse effect due to the stimulatory action on central nervous system which results in disturbance of sleep and insomnia (Snel and Lorist, 2011). Therefore, the regulation of the content of

EGCG and caffeine is required for better efficacy with less adverse effect.

Recently, green tea is known to be beneficial for prevention and treatment of obesity (Hasumura *et al*., 2012; Yang *et al*., 2012). Because obesity has been steadily increasing and has become a serious health issue worldwide, anti-obesity effect of green tea is one of the important effects of green tea. Obesity is defined as abnormal or excessive fat accumulation that stems from a prolonged imbalance between the levels of energy intake and expenditure (Haslam and James, 2005). In particular, increased fat intake is highly associated with body weight gain, which can lead to obesity and other related metabolic (Kopelman, 2000; Lefterova and Lazar, 2009). Fat is absorbed in the small intestine after being hydrolyzed by pancreatic lipase. Thus, block of fat absorption by inhibiting pancreatic lipase is important therapeutic for obesity (Birari and Bhutani, 2007; Yun, 2010).

For the optimization of extraction condition with great extraction efficacy and anti-obesity effect, the content of caffeine and EGCG in green tea extraction from different extraction condition was quantitated using HPLC analysis. The inhibitory effect on pancreatic lipase activity was also investigated.

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Experimental

Materials – Dried green tea, the leaves of *Camellia sinensis*, was provided purchased from the local herbal market, Chungbuk, Korea in November 2012. They were identified by the herbarium of College of Pharmacy at Chungbuk National University, where a voucher specimen was deposited (CBNU201211-GT). Caffeine and EGCG were purchased from Sigma-Aldrich Chemical Co.

General experimental procedures – Analysis was performed using a Waters HPLC system equipped with Waters 600 Q-pumps, a 996 photodiode array detector, and Waters Empower software using Gemini-NX ODS-column (2.6 μm, 4.6 × 100 mm) for quantitation.

Preparation of standard solution – Stock standard solution of caffeine and EGCG was prepared in methanol at a concentration of 1 mg/mL, respectively. The purity of each compounds was > 98%, as determined by HPLC analysis. The appropriate amount of every standard solution was mixed and diluted with methanol as indicated.

Sample preparation for HPLC – The powdered green tea (each 100.0 mg) was weighed accurately and extracted with 1 mL of ethanol-water (50 : 50) for 30 min at room temperature using sonic apparatus. This extract was filtered through 0.45 μm membrane filter (Millipore, Nylon, 170 mm) and used for HPLC analysis. Some extract was evaporated in vacuum, and then weighed. For pancreatic lipase assay, the evaporated extract was dissolved in dimethyl sulfoxide (DMSO) and diluted with water.

Extraction using different condition – For evaluation of solvent effects, powdered green tea (100.0 mg) was weighed accurately and extracted with the mixture of ethanol-water (100, 75, 50, 25 and 0% ethanol in water) for 30 min using sonic apparatus. Effects of extraction time were evaluated by extracting powdered green tea (100.0 mg) with 50% ethanol in water for different time periods such as 5, 10, 20, 60, 120 and 180 min using sonic apparatus. For the optimization of extraction method, powdered green tea was extracted at RT or 70 °C or using ultrasonic apparatus for 30 min. For comparison of efficiency of repeated extraction, dried green tea was extracted with 50% ethanol in water for each 10 min four times and each extract was collected separately. Each sample solution was filtered through 0.45 μm membrane filter before HPLC analysis.

Development of HPLC methods – For simultaneous determination of caffeine and EGCG in green tea, the chromatographic condition was investigated using various mixtures of water, methanol, and acetonitrile in combination with acetic acid as a mobile phase. The linearity of calibration curves was calculated according to the International Conference on Harmonization (ICH) guidelines. Four concentrations of each compound (0.0625, 0.125, 0.25 and 0.5 mg/mL) were prepared and analyzed in triplicate.

Assessment of pancreatic lipase activity – Pancreatic lipase inhibitory activity was evaluated as previously reported (Ahn *et al*., 2012). Briefly, enzyme solution was prepared by the reconstitution of porcine pancreatic lipase (Sigma, St. Louis, MO) in 0.1 M Tris-HCl buffer (pH 8). Then, test sample was mixed with enzyme buffer, and incubated for 15 min at 37 °C. After incubation, 10 mM *p*-nitrophenylbutyrate (*p*-NPB) was added and the enzyme reaction was allowed to proceed for 15 min at 37 °C. Pancreatic lipase activity was determined by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol at 405 nm using a microplate reader. Relative pancreatic lipase activity (%) was calculated as (activity of compound w/substrate – negative control of compound w/o substrate) / (activity of w/o compound and w/substrate – negative control of w/o compound and substrate) × 100.

Results

Development of HPLC method – For simultaneous determination of caffeine and EGCG in green tea extract, the chromatographic condition was first investigated. Various mixtures of water, methanol, and acetonitrile in combination with acetic acid were tested as a mobile phase. In our chromatographic condition, mixture of water and methanol showed favorable separation and addition of acetic acid did slightly improve the peak resolution. The wavelength for detection was set at 270 nm, where caffeine and EGCG showed the maximum absorption as measured by DAD. As a result, the optimal mobile phase consisting of 23% methanol in water containing 0.1% acetic acid was subsequently employed for the analysis of green tea. Under this chromatographic condition, caffeine and EGCG have retention times of 8.3 and 11.7 min, respectively (Figs. 1A and 1B). Specificity was evaluated using DAD and its corresponding computer software, which confirms the singularity of the peak component. Calibration curves were linear in relatively wide range of concentrations and all showed good linear regressions with high correlation coefficient values ($r^2 >$ 0.9996) between peak area (y) and amount of each compound (x) (Fig. 1C).

Pancreatic lipase inhibitory activity of green tea extract, caffeine and EGCG – The inhibitory activity green tea extract, caffeine and EGCG were assessed by
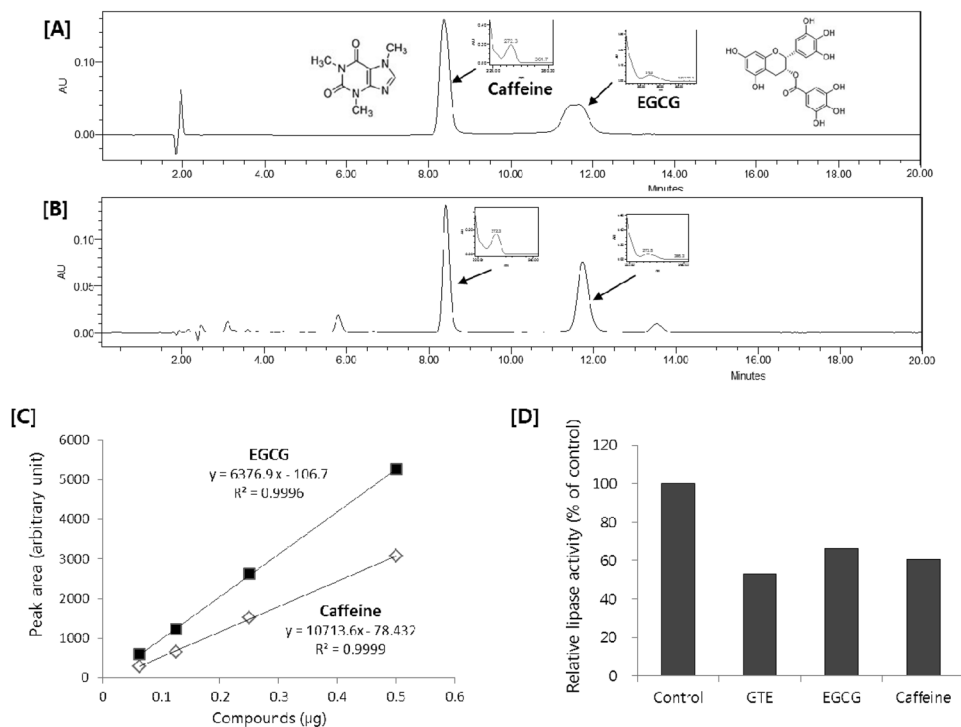
Fig. 1. (A) HPLC chromatogram of EGCG and caffeine, (B) HPLC chromatogram of green tea extract, (C) Calibration curves of EGCG and caffeine, and (D) inhibitory effect on pancreatic lipase. GTE: green tea extract, 100 μg/mL; caffeine and EGCG: 100 μM.
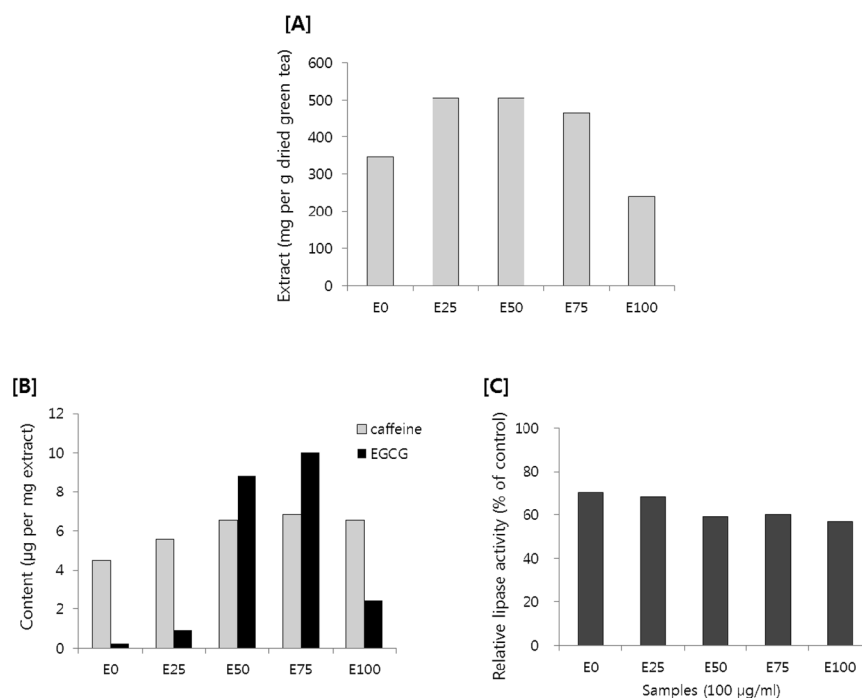

Fig. 2. Effect of extraction solvent on (A) extraction yield, (B) the content of EGCG and caffeine, and (C) inhibitory effect on pancreatic lipase. E0 – water; E25 – 25% ethanol in water; E50-50% ethanol in water; E75-75% ethanol in water; E100-100% ethanol.

porcine pancreatic lipase using *p*-NBT as a substrate. As shown in Fig. 1D, green tea extract inhibited pancreatic lipase activity up to 47.1% at the concentration of 100 μg/mL. The two major constituents, caffeine and EGCG, inhibited pancreatic lipase activity by 40.0 and 34.9%, respectively, at the concentration of 100 μM.

Effect of extraction solvent on extraction efficiency – Extraction condition greatly affects the extraction efficiency (Kim *et al*., 2009; Borrás *et al*., 2011; Chen *et al*., 2012; Liu *et al*., 2012). For the optimization of extraction condition, established method has been applied to the quantitation of green tea samples from different preparation using different solvent composition, extraction time, and extraction method..

The effects of extraction solvent on extraction efficiency were first evaluated. Green tea was extracted with the mixture of ethanol and water (100%, 75%, 50%, 25% and 0% ethanol in water) and analyzed. As shown in Fig. 2A, the extraction yield was higher at extraction with the mixture of ethanol and water, such as 25% ethanol, 50% ethanol and 75% ethanol than those of ethanol or water only. The content of caffeine and EGCG in green tea extract was greatly affected by the extraction solvent. The content of EGCG in the extract was dramatically increased by the addition of ethanol to extraction solvent, while decreased at 100% ethanol extraction. The extract using 75% ethanol extraction contained almost 40 times higher content compared to those from water extraction. The content of caffeine was slightly increased at 50% ethanol and 75% ethanol extraction but not dramatic compared to EGCG (Fig. 2B).

Next, the inhibitory activity of these green tea extracts was measured. Interestingly, the inhibitory activity of

green tea extract was not greatly affected by the extraction solvent (Fig. 2C).

Effect of extraction time on extraction efficiency – Next the effects of extraction time on extraction efficiency were evaluated. Dried green tea was extracted with 50% ethanol using ultrasonic apparatus for different times.The extraction time also affected the extraction yield. Extraction needed longer extraction time for maximum yield. The extraction yield showed steady increase at longer extraction up to 180 min (Fig. 3A). The content of caffeine reached maximum as early as 10 min extraction. However, the content of EGCG was gradually increased up to 60 min extract and decreased after that time (Fig. 3B). However, the effect on pancreatic lipase activity was little increased at longer extraction (Fig. 3C).

Effect of extraction method on extraction efficiency – For the comparison of extraction method, powdered green tea was extracted with 50% ethanol at room temperature, 70 °C or by using ultrasonic apparatus for 30 min. The extraction yield and the content was the highest at the extraction using ultrasonic apparatus (Figs. 4A and 4B). However, the effect on pancreatic lipase activity showed little variance in three extraction method (Fig. 4C).

Effect of repeated extraction on extraction efficiency – Green tea was usually extracted repeatedly for tea or industrial process. Therefore, the extraction efficiency of repeated extraction was tested. Powdered green tea was
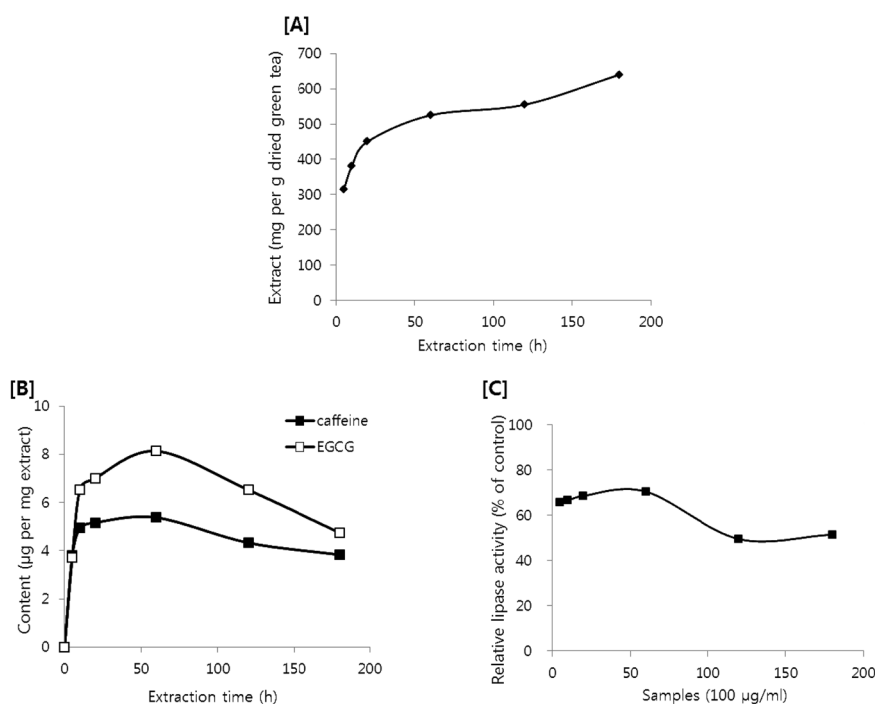

Fig. 3. Effect of extraction time on (A) extraction yield, (B) the content of EGCG and caffeine, and (C) inhibitory effect on pancreatic lipase.
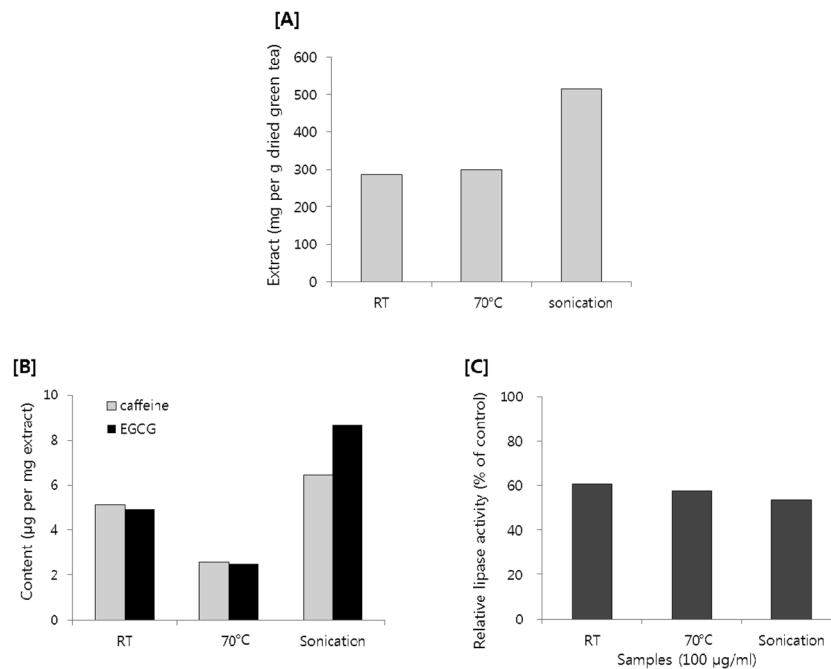
[A]

[B]

[C]

Fig. 4. Effect of extraction method on (A) extraction yield, (B) the content of EGCG and caffeine, and (C) inhibitory effect on pancreatic lipase. RT-extraction at room temperature; 70 °C-extraction at 70 °C; Sonication-extraction using ultrasonic apparatus.
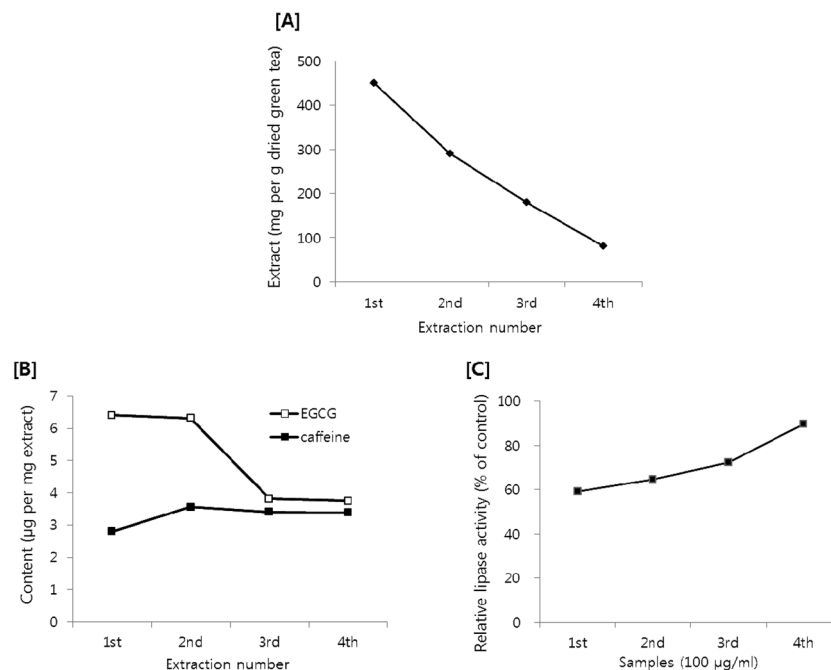
[A]

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Fig. 5. Effect of repeated extraction on (A) extraction yield, (B) the content of EGCG and caffeine, and (C) inhibitory effect on pancreatic lipase.

extracted repeatedly with 50% ethanol using ultrasonic apparatus for each 10 min. The extraction yield greatly decreased at repeated extraction. The yield of fourth extraction was only 17% compared to first extraction (Fig. 5A). Interestingly, however, the content of caffeine was similar or even slightly increased at repeated extraction, whereas that of EGCG was decreased at repeated extraction (Fig. 5B). The inhibitory activity on pancreatic lipase was also decreased by repeated extraction (Fig. 5C).

Discussion

Green tea has been used as a healthy beverage for a long time, especially in Asian countries. Green tea is now popular worldwide. Extensive studies have revealed diverse beneficial effect of green tea. Anti-obesity effect of green tea is one of the important functions in these days, as also demonstrated in our present study. Our study showed the anti-obesity effect of green tea extract, EGCG, and caffeine by inhibition of fat absorption (Fig. 1D). For these reasons, green tea is widely consumed as a tea or a functional food.

Green tea contains EGCG, ECG, catechin and caffeine with diverse other constituents. Among them, EGCG is a major constituent and is well-known for diverse beneficial effects, while caffeine often causes adverse effects such as disturbance of sleep. For this reason, several analytical methods have been developed for the quantitation of catechin derivatives and caffeine. The content of EGCG quite differs in various tea cultivars and commercial tea samples (El-Shahawi *et al.*, 2012; Maeda-Yamamoto *et al.*, 2012). The extraction condition also affected the content of EGCG and caffeine (Ziaedini *et al.*, 2010; Hu *et al.*, 2009). In addition, the variation of EGCG derivatives ultimately resulted in the difference of biological activity (Maeda-Yamamoto *et al.*, 2012). Therefore, optimized condition to maximize EGCG and to minimize caffeine was investigated using diverse extraction method. The effect on pancreatic lipase activity was also investigated to maximize the anti-obesity effect.

We compared different extraction condition such as extraction solvent, extraction time, extraction method, and repeated extraction. As expected, the extraction efficiency was greatly affected by different extraction condition. As extraction solvent, the mixture of ethanol and water is better than single solvent (Fig. 2A). The longer extraction also increased extraction efficiency (Fig. 3A), whereas repeated extraction rapidly decreased the extraction efficiency (Fig. 5A). As extraction method, extraction using ultrasonic apparatus increased the extraction efficiency (Fig. 4A). Therefore, extraction with the mixture of ethanol and water using ultrasonic apparatus more than 60 min is suggested as extraction condition for great extraction efficiency.

Next, we analyzed the content of EGCG and caffeine in green tea extract using HPLC analysis (Figs. 1A and 1B). The content of EGCG was greatly affected by different extraction condition. The content of EGCG was the highest in the extract using 75% ethanol and 50% ethanol, and followed by 100% ethanol > 25% ethanol > water.

The content of EGCG in water extraction was only 2.5% of 75% ethanol extraction (Fig. 2B). The extraction time also affected the content of EGCG. The content of EGCG was increased up to 60 min extraction (Fig. 3B). However, the content of EGCG was decreased as extraction was repeated. The content of EGCG was similar at first two extractions, however, decreased greatly from third extraction (Fig. 5B). Contrary to EGCG, the content of caffeine was little affected by different extraction condition. Although the extraction with the mixture with ethanol and water increased the content of caffeine, the change was not dramatic (Fig. 2B). Related to extraction time, the content of caffeine was almost steady from 20 min extraction (Fig. 3B). The content of caffeine was not affected by repeated extraction (Fig. 5B).

Although the content of EGCG and caffeine showed variances in different extraction method, the inhibitory effect on pancreatic lipase was almost similar. Therefore, the inhibitory effect of green tea extraction might be achieved by the combination of diverse constituents as well as EGCG and caffeine.

Taken together, our study suggested that extraction with the mixture of ethanol and water for 60 min using ultrasonic apparatus is good extraction condition for maximum content of EGCG and minimum content of caffeine. In addition, extraction of green tea less than twice is suggested for great efficiency.

Acknowledgements

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