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Extraction and separation of tea polyphenols and caffeine from tea leaves by mechanochemical pretreatment

[Extracción y separación de polifenoles del té y cafeína de hojas de té mediante pretratamiento mecanoquímico]

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Abstract: Mechanochemical pretreatment (MCPT) was applied to the extraction and separation of tea polyphenols (TP) and caffeine from tea leaves. Tea leaves were treated by mechanical activation with presence of Na_2CO_3 (MC) for 75 s. One gram of obtained powder was taken for extraction and separation to obtain the sample of MC (A) fraction and MC (B) fraction. According to caffeine and epigallocatechin gallate (EGCG) (a representative component of TP) yields in the sample, the optimum MCPT extraction conditions were as follows: 15% (w/w) Na_2CO_3 during milling, extracting caffeine with 40 mL 75% (v/v) ethanol and EGCG with 40 ml water at room temperature. Under these conditions, the caffeine EGCG yield in MC (A) fraction was 0.51 mg/g, and the EGCG yield in MC (B) fraction was 1.32 mg/g, with EGCG/caffeine ratio of 2.38. Compared with extraction with mechanical activation without presence of Na_2CO3 (M) and traditional extraction with no mechanical activation (CON), the MC extraction had obvious advantages. In conclusion, the MCPT is an effective method for the extraction and separation of TP and caffeine from tea leaves.

Keywords: Mechanochemical; Tea polyphenols; EGCG; Caffeine; Tea leaves.

Resumen: Se aplicó un pretratamiento mecanoquímico (MCPT) a la extracción y separación de polifenoles del té (TP) y cafeína de hojas de té. Las hojas de té se trataron mediante activación mecánica con presencia de Na2CO3 (MC) durante 75 s. Se tomó un gramo del polvo obtenido para la extracción y separación para obtener la muestra de la fracción MC (A) y la fracción MC (B). De acuerdo con los rendimientos de cafeína y galato de epigalocatequina (EGCG) (un componente representativo de TP) en la muestra, las condiciones de extracción MCPT óptimas fueron las siguientes: 15% (p/p) de Na2CO3 durante la molienda, extracción de cafeína con 40 mL de etanol al 75% (v/v) y EGCG con 40 ml de agua a temperatura ambiente. En estas condiciones, el rendimiento de cafeína EGCG en la fracción MC (A) fue de 0.51 mg/g, y el rendimiento de EGCG en la fracción MC (B) fue de 1.32 mg/g, con una relación EGCG/cafeína de 2.38. En comparación con la extracción con activación mecánica sin presencia de Na2CO3 (M) y la extracción tradicional sin activación mecánica (CON), la extracción MC tenía ventajas obvias. En conclusión, el MCPT es un método efectivo para la extracción y separación de TP y cafeína de hojas de té.

Palabras clave: Mecanoquímico; Polifenoles del té; EGCG; Cafeína; Hojas de té

INTRODUCTION

Tea is the second most consumed nonalcoholic beverage in the world. Tea polyphenols (TP) and alkaloids are the main bioactive components in the tea leaves (Astill *et al*., 2001). Catechins provide about 80% of TP (Tang *et al*., 2007). The chemical structures of the main catechin compounds are shown in Figure No. 1. Epigallocatechin gallate (EGCG) is the most abundant catechin and the component with the strongest biological activity in tea leaves (Rietveld & Wiseman, 2003). Several studies suggest that TP have the antioxidant, antimicrobial (Almajano *et al*., 2008), free radical scavenging, anticancer, cardiovascular protecting (Koo & Noh, 2007), anti-inflammatory (Nag Chaudhuri *et al*., 2005), and immunomodulatory activities (Zvetkova *et al*., 2001). As to the antioxidant property, EGCG and (-)-Epicatechin gallate (ECG) are considered the best substances, because they have the galloyl moieties structures which are absent in other catechins. On the other hand, the excessive caffeine ingestion from tea may cause the increase of cardiac frequency (Kaufman & Sachdeo, 2003), sleep deprivation (Shilo *et al*., 2002), cardiovascular and neuroendocrine activation (Lane *et al*., 2002), abortion and miscarriage (Giannelli *et al*., 2003). These side effects of caffeine make it hard to be broadly used.

There are many reports on decaffeination of tea extraction, including the extraction with organic solvents or hot water (Liang *et al*., 2007), lignocellulose column (Sakanaka, 2003), supercritical carbon dioxide extraction (Kim *et al*., 2008), and microbial and enzymatic treatments. However, these methods have many unacceptable deficiencies, such as needing the expensive equipment, using a large amount of solvents, causing the environmental pollution, and producing the solvent residue in products. Therefore, new methods of decaffeination should be extensively explored to minimize the use of toxic organic solvents, and avoid the change of labile or volatile compounds during lengthy extraction period at a high temperature.

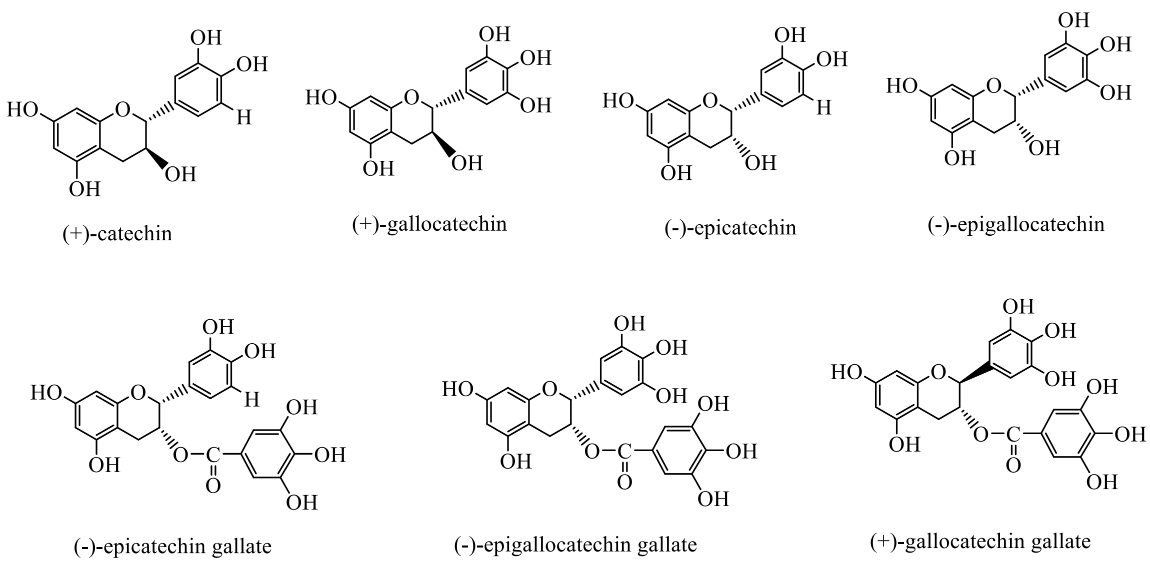

Figure No. 1
Chemical structures of main catechin compounds

Mechanochemistry is a branch of chemistry, and it deals with the chemical and physicochemical changes of substances in all states of aggregation under mechanical activation (Boldyrev & Tkáčová, 2000). Mechanochemical pretreatment (MCPT) is pretreatment process of extraction during which the impact-shift action on particles of material is accompanied not only by grinding, but also by destruction of cell wall. Therefore, in next extraction the diffusion of solvent is increased, resulting in an increased yield of targeted products (Lomovsky *et al*., 2003). MCPT may provide a new tool for obtaining bioactive substances from raw plant materials. Some researchers have used this technique to produce water-soluble forms of triterpene acids from fir needles (Korolev *et al*., 2003), extract phytoecdysteroids from *Serratula coronata* L. (Lomovsky *et al*., 2003), extract isofraxidin from *Eleutherococcus senticosus* (Liu *et al*., 2007), extract polyphenols from lotus seedpod (Bao *et al*., 2023) and extract active alkaloids from *Stephania tetrandra* S. Moore (Wang *et al*., 2019). It is well-documented that, compared to conventional extraction methods, the extraction with MCPT can obviously increase the extraction yield while reduce both extraction time and energy cost. The objective of this study was to investigate the technical feasibility of MCPT in efficient extraction and separation of TP and caffeine from tea leaves and to establish an appropriate procedure.

MATERIALS AND METHODS

Materials

Green tea leaves (*Camellia sinensis* L.) were obtained from Hangzhou Jiangnan Tea Market (Hangzhou, China), and were authenticated as Fanning Belas, China by Tea Research Institute of Chinese Academy of Agricultural Sciences (Hangzhou, China). The tea leaves were chopped in a laboratory blender to an average particle size less than 0.5 mm, and stored in the refrigerator until used. Caffeine (purity of 98%) was purchased from Sigma-Aldrich Co. (St. Louis, USA), TP (purity of 98%) and EGCG (purity of 98%) were purchased from Shanghai Winherb Medical Science Co., Ltd. (Shanghai, China). Analytical-grade Na_2CO_3, glacial acetic acid, phosphoric acid and ethanol were obtained from Gaojing Chemical Industry Co., Ltd. (Hangzhou, China). Other chemical reagents used were of HPLC grade (Tedia In., MA, USA) except stated otherwise.

MCPT equipment and procedure

MCPT was conducted using AGO-2 planetary centrifugal mill equipped with a water-cooled drum (ISSC, Novosibirsk, Russia). The stainless steel milling balls with 10 mm diameter were selected as milling media. The milling media load was 50% (v/v) of the mill chamber volume. The treatment duration was 75 s and the acceleration was 120-200 m/s^2. The mass ratio of milling media to material was 80:1 (w/w). The MCPT included two ways: mechanical activation with presence of Na_2CO_3 (MC) and mechanical activation without presence of Na_2CO_3 (M).

In MC extraction, 20 g tea leaves with Na_2CO_3 in different content were added into AGO-2 mill. After milling for 75 s, the powder was taken. One gram powdered sample was put into aqueous ethanol in a 100 mL glassy flask, followed by stirring for 20 min. Firstly, the extraction solution was filtered with “Double-Ring” 102 filter paper (aperture 50 μm; Xinhua Paper Industrial Co. Ltd., Hangzhou, China). The filtrate was obtained. The filter residue was washed twice with 5 mL of ethanol. After filtrating, the filtrate was obtained. The two filtrates were combined, and the pH was adjusted to 4 with glacial acetic acid. The solution was diluted to 100 mL to obtain the MC (A) fraction, followed by HPLC analysis. Secondly, other 40 ml distilled water was added to the filter residue, followed by stirring for 5 min. After centrifuging at 256 × g for 5 min, the supernatant was obtained, and was adjusted to pH 4 with glacial acetic acid and then diluted to 100 mL to obtain the MC (B) fraction, followed by high performance liquid chromatography (HPLC) analysis (Figure No. 2). The effects of Na_2CO_3 content in milling (10%, 15%, 25%, 30%), extraction solvent type (0%, 50%, 75% and 100% ethanol for MC (A) faction; water for MC (B) faction) and extraction temperature (room temperature, 50ºC, 75ºC, 100ºC) on EGCG and caffeine yields were investigated.

In M extraction, 20 g tea leaves were added into AGO-2 mill. After milling for 75 s, the powder was taken. One gram powdered sample was infused with 40 ml solvent (water or 75% (v/v) ethanol. The extraction was carried out at room temperature and reflux for 30 min, respectively. The extraction solution was filtered with filter paper. The filter residue was washed twice with 5 mL of 75% (v/v) ethanol. The combined filtrate was diluted with 125 mL of deionized water, and was filtrated through a 0.45 μm microporous membrane, followed by HPLC analysis.

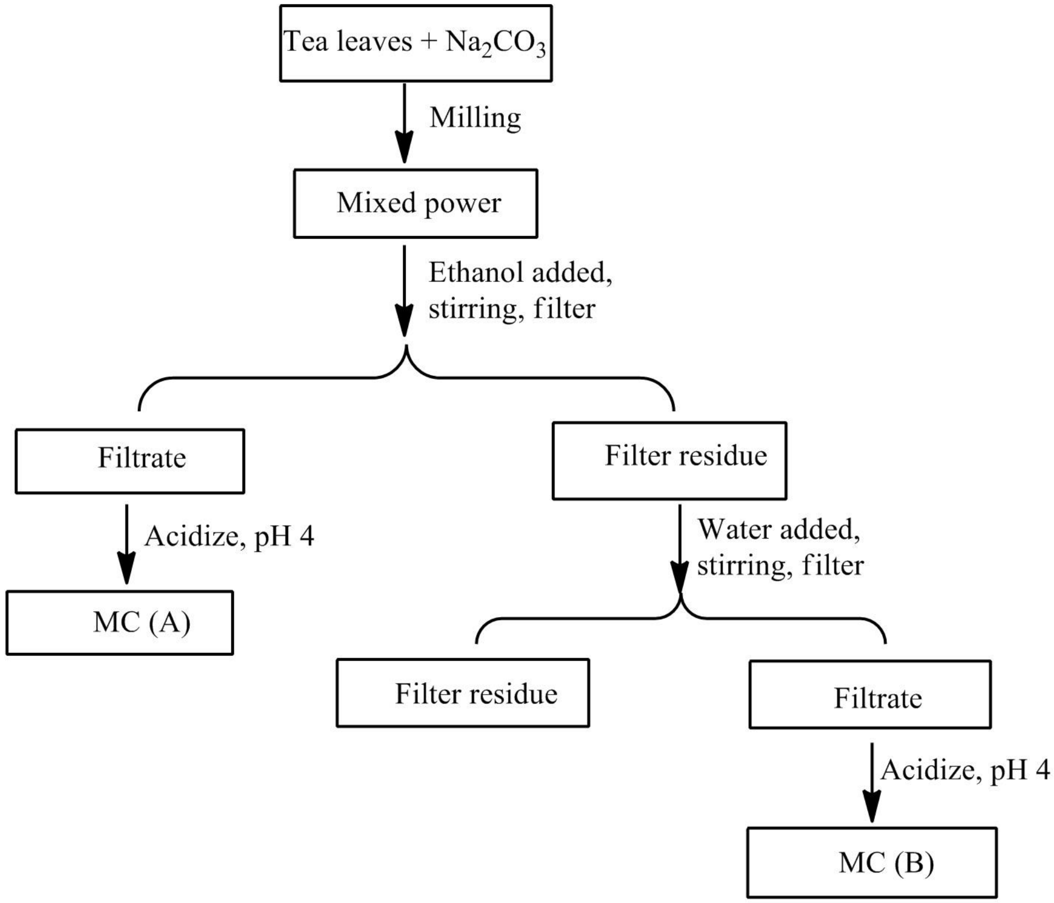

Figure No. 2
MC extraction procedure. MC, mechanical activation with presence of Na_2CO_3

Traditional extraction

To investigate the advantages of MC extraction, it was compared with the traditional extraction with no mechanical activation (CON). In CON extraction, the tea leaves were ground in a conventional laboratory pulverizer and passed through a 0.35 mm aperture sieve. The pulverized sample was obtained. One gram sample was infused with 80 mL 75% ethanol. The extraction was carried out at reflux for 30 min, respectively. The extraction solution was filtered with filter paper. The filter residue was washed twice with 5 mL of 75% (v/v) ethanol. The combined filtrate was diluted with 125 mL of deionized water, and was filtrated through a 0.45 µm microporous membrane, followed by HPLC analysis.

Analysis method

TP, EGCG and caffeine were analyzed by HPLC. Liquid chromatography was conducted on an Agilent 1100 (USA) chromatography system coupled to an Agilent 1100 VWD UV detector and equipped by a binary pump at a wavelength of 280 nm. The HPLC utilized a ZORBA × Extend-C18 reversed-phase column (150 mm×3.0 mm, i.d., 5µm, Agilent, USA). The column temperature was 35ºC. The mobile phase was: water-phosphoric acid (0.35:1000, v/v) for solvent A and acetonitrile for solvent B. The linear gradient elution system was changed from 92% A to 29% B during the first 25 min, B was maintained at 29% from 25 min to 30 min at a flow rate of 1 mL/min. The injection volume was 5 µL. Chromatographic peaks of samples were identified by comparing their retention times with those of the reference standard determined under the same chromatographic conditions. Working standard solutions were injected into the HPLC, and peak area responses were obtained. A standard graph of each component was prepared by plotting concentration versus area, the calibration curve for EGCG and caffeine were strictly linear (r^2 = 0.9999) in the concentration range from 0 to 1.6 mg/mL, 0 to 2.0 mg/mL, respectively. Quantification was carried out from the integrated peak area of the sample against the corresponding standard graph. Each sample was analyzed in triplicate.

According to the Perva-Uzunalic *et al*. (2006), reported method, the caffeine and EGCG contents in the tea were 26.36 mg/g and 24.87 mg/g, respectively. Water content in the green tea was measured using ORION AF8 volumetric Karl Fischer Titrator (Toledo, Spain).

Statistical analysis

Statistics were performed using 11.5 SPSS System for Windows. All tests in the present study were carried out in triplicate and the mean values were presented, based on dry weight of tea leaves.

RESULTS AND DISCUSSION

Component changes after MCPT

Figure No. 3 showed HPLC chromatograms of TP, caffeine and EGCG standards. The retention time of caffeine and EGCG standards was 4.0 min and 7.5 min, respectively. These retention times agreed well with the retention time of caffeine and EGCG extracted by CON, M and MC samples. Initial water content in the tea was determined to be 4.5%.

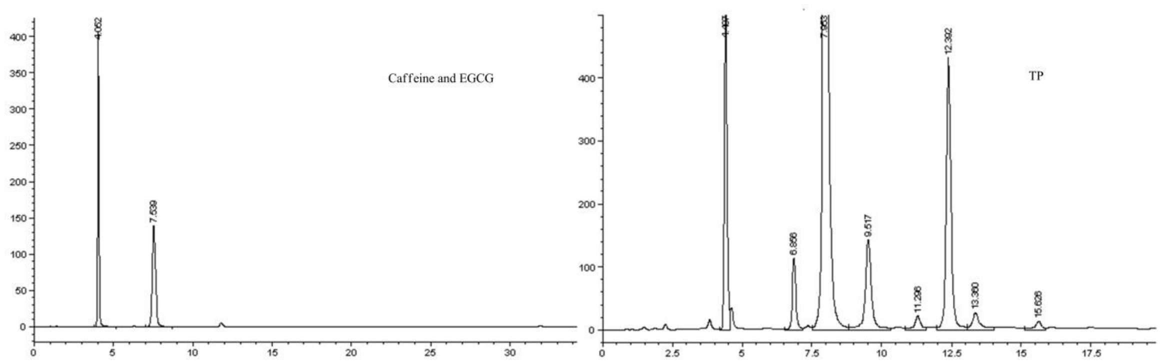

Figure No. 3
HPLC of EGCG, caffeine and TP. HPLC, high performance liquid chromatography; EGCG, epigallocatechin gallate; TP, tea polyphenols

In order to make sure whether components had been changed after different pretreatments, HPLC was applied to determine the prepared samples. The samples of CON, M and MC extractions were compared with the TP standard. Figure 4 showed that, the primary main components in CON and M samples were basically the same as the TP standard. The main components of MC (B) fraction were similar with TP standard, while the main component of MC (A) was similar with caffeine. Furthermore, the major component of TP was EGCG, which didn’t exist in MC (A) fraction while was found in the MC (B) fraction. This might be due to solubility change in some acid substances converted to the water-soluble substances reacted with alkali substance, resulting in the deceased solubility in high levels of ethanol. Caffeine showed a good solubility, suggesting that the main component of TP did greatly be changed during MCPT. Meanwhile, the fraction extracted by water with MCPT obviously contained lower caffeine content than that by the CON treatment. By contrast to the original caffeine concentration in dry weight tea leaves, only 1.9% caffeine concentration was retained in the fraction extracted by water. This confirmed that caffeine was removed well, and the TP extraction mainly consisted of EGCG and ECG (retention time was 11.7 min).

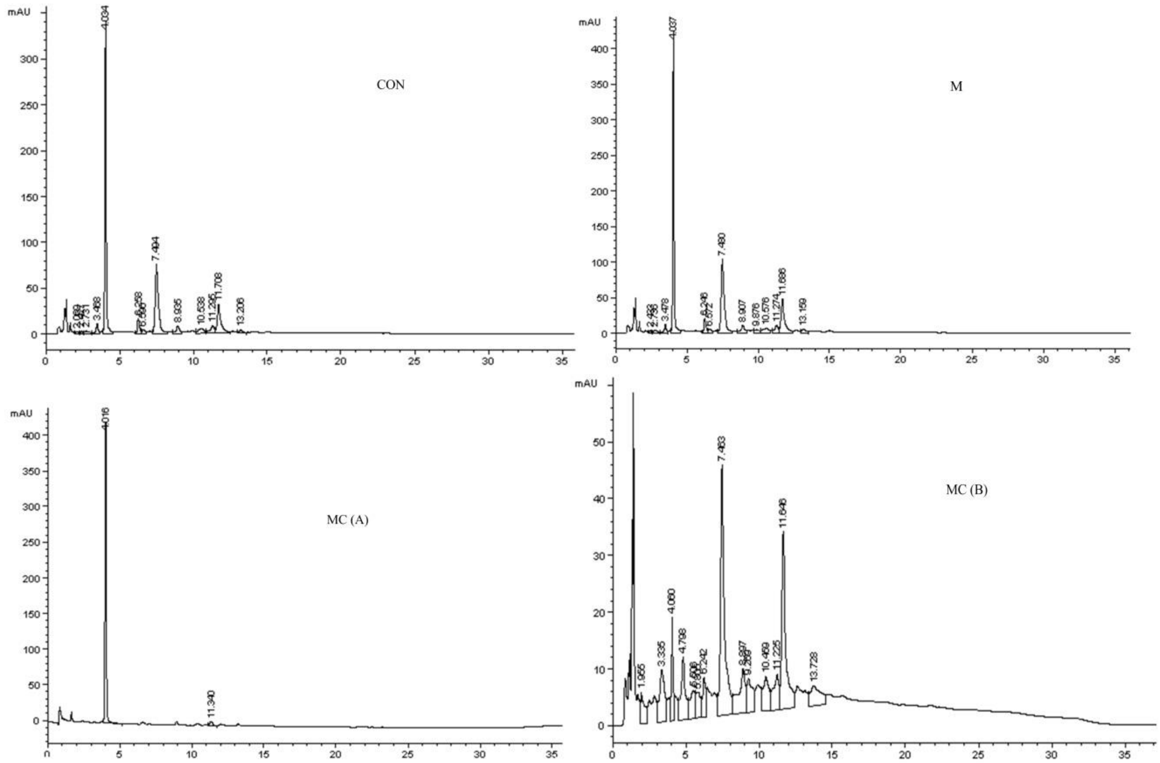

Figure No. 4
HPLC of CON, M, MC (A) and MC (B). HPLC, high performance liquid chromatography; CON, no mechanical activation; M, mechanical activation without presence of Na_2CO_3; MC, mechanical activation with presence of Na_2CO_3

Effect of Na_2CO_3 content on EGCG and caffeine yields in MC extraction

Minimal reagent content required for an extraction not only depends on the kinetics of the mechanochemical process, but also relies on the characteristics and the content of the target compounds in the plant material (Korolev *et al*., 2003). TP are known to be instable in neutral or alkaline solution (Liang & Xu, 2001). Therefore, the minimal Na_2CO_3 content in MC extraction was set 10%, 15%, 25% and 30% (w/w). The results suggested that 15% was the optimal Na_2CO_3 content for EGCG extraction from tea leaves. In this condition, EGCG yield in MC (B) faction was increased to 1.37 mg/g (Table No. 1). This may be due to the destruction of EGCG caused by the excess alkali residues in the extracts. The Na_2CO_3 content should be no less than 15%, otherwise the time of MC would be increased to an unacceptable duration. On the basis of these findings, Na_2CO_3 content of 15.0% was selected as the standard for further investigation of extraction conditions. In addition, it was noted that Na_2CO_3 content also influenced the yield of caffeine, but the effect was less than that on TP. The TP may be more sensitive to Na_2CO_3 content than caffeine.

Table No. 1
Effect of Na_2CO_3 content on EGCG and caffeine yields in MC extraction

Sample	Na_2CO_3 content (%)	EGCG yield (mg/g)	Caffeine yield (mg/g)	EGCG/caffeine
MC (A)	10%	0	25.55 ± 0.36	0
MC (B)	10%	1.10 ± 0.06	0.68 ± 0.01	1.62 ± 0.02
MC (A)	15%	0	24.91 ± 0.24	0
MC (B)	15%	1.37 ± 0.09	0.56 ± 0.08	2.44 ± 0.06
MC (A)	25%	0	25.50 ± 0.47	0
MC (B)	25%	0.93 ± 0.08	0.46 ± 0.04	2.05 ± 0.19
MC (A)	30%	0	24.93 ± 0.32	0
MC (B)	30%	0.75 ± 0.02	0.66 ± 0.02	1.13 ± 0.02

The extraction was performed at room temperature, with 40 mL 75% ethanol as solvent and stirring for 20 min for MC (A) and 40 water as solvent and stirring for 5 min for MC (B). Each value was expressed as mean ± standard deviation (n=3). EGCG, epigallocatechin gallate. MC, mechanical activation with presence of Na_2CO_3.

Effect of extraction solvent type on EGCG and caffeine yields in MC extraction

Solvent choice depends on the solubility of the compounds of interest, together with the interaction between solvent and target compound (Dabiri *et al*., 2005). Caffeine has been known to have good solubility in chloroform, dichloromethane and hot water, and almost insoluble in cold water, methanol, ethanol (at room temperature) and aqueous-alkaline solvents. Without chemical modification, TP are fully soluble in water, ethanol and aqueous-alkaline solvents. Table No. 2 showed the effect of extraction solvent on EGCG and caffeine yields in MC extraction. The caffeine yield in MC (A) faction increased with the ethanol concentration increased from 0 to 75% and decreased with the ethanol concentration increased from 75% to 100%. When the ethanol concentration was 75%, in MC (B) faction the EGCG yield was the highest, the caffeine yield was the lowest, and the EGCG/caffeine ratio was the highest. Therefore, 75% ethanol was selected to extract caffeine and water was selected to EGCG.

Table No. 2
Effect of extraction solvent type on EGCG and caffeine yields in MC extraction

Sample	**Solvent type**	**EGCG (mg/g)**	**Caffeine (mg/g)**	**EGCG/caffeine**
MC (A)	0% ethanol (water)	8.09 ± 031	22.10 ± 0.05	0.29 ± 0.00
MC (B)	water	0	1.86 ± 0.18	0
MC (A)	50% ethanol	3.14 ± 0.34	25.03 ± 0.99	0.13 ± 0.00
MC (B)	water	1.39 ± 0.01	0.76 ± 0.06	1.82 ± 0.25
MC (A)	75% ethanol	0	24.27 ± 0.13	0
MC (B)	water	1.42 ± 0.09	0.54 ± 0.03	2.62 ± 0.11
MC (A)	100% ethanol	0	12.66 ± 0.99	0
MC (B)	water	3.90 ± 0.27	11.79 ± 0.83	0.33 ± 0.05

The extraction was performed with 15% Na_2CO_3 in milling at room temperature, 40 mL solvent as solvent and stirring for 20 min for MC (A) and 40 solvent as solvent and stirring for 5 min for MC (B). Each value was expressed as mean ± standard deviation (n=3). EGCG, epigallocatechin gallate; MC, mechanical activation with presence of Na_2CO_3.

Effect of extraction temperature on EGCG and caffeine yields in MC extraction

Effect of extraction temperature (from room temperature to 100°C) on EGCG and caffeine yields in MC extraction was investigated. When the extraction temperature increased from room temperature to 100°C, the caffeine yield in MC (A) faction slightly increased from 24.87 to 26.33 whereas the EGCG yield in MC (B) faction decreased rapidly from 1.15 to 0.18, and the EGCG/caffeine ratio in MC (B) faction rapidly increased from 2.40 to 9.00. Considering the oxidation of EGCG at higher temperature, the optimal extraction temperature was the room temperature (Table No. 3).

Table No. 3
Effect of extraction temperature on EGCG and caffeine yields in MC extraction

Sample	Extraction temperature (ºC)	EGCG (mg/g)	Caffeine (mg/g)	EGCG/caffeine
MC (A)	Room temperature	0	24.87 ± 0.12	0
MC (B)	Room temperature	1.15 ± 0.01	0.48 ± 0.06	2.40 ± 0.06
MC (A)	50	0	25.03 ± 0.73	0
MC (B)	50	0.97 ± 0.22	0.34 ± 0.04	2.85 ± 0.02
MC (A)	75	0	26.30 ± 0.31	0
MC (B)	75	0.27 ± 0.15	0.04 ± 0.01	6.75 ± 0.02
MC (A)	100	0	26.33 ± 0.37	0
MC (B)	100	0.18 ± 0.02	0.02 ± 0.00	9.00 ± 0.02

The extraction was performed with 15% Na_2CO_3, 40 ml 75% ethanol and stirring for 20 min for MC (A) and 40 water and stirring for 5 min for MC (B). Each value was expressed as mean±standard deviation (n=3). EGCG, epigallocatechin gallate; MC, mechanical activation with presence of Na_2CO_3

Comparison among CON, M and MC extractions

Comparison among CON, M and MC extractions was performed under their respective optimum conditions. As shown in Table No. 4, in CON and M extractions, although more EGCG was extracted, more caffeine was also extracted, so the EGCG/caffeine ratio in the sample was very low. In MC extraction, although the EGCG yield in MC (B) faction was 1.32 mg/g, lower than that in other two methods, the caffeine EGCG yield was only 0.51 mg/g, so the EGCG/caffeine ratio was significantly increased compared with other two methods. Therefore, the MC extraction method is more suitable for separating caffeine and EGCG.

Table No. 4
Comparison among CON, M and MC extractions

Sample	Extraction temperature (ºC)	Solvent	EGCG yield (mg/g)	Caffeine yield (mg/g)	EGCG/caffeine
CON	Reflux	80 ml 75% ethanol	21.33 ± 1.03	26.05 ± 0.01	0.81 ± 0.05
M	Reflux	40 ml 75% ethanol	21.25 ± 2.07	26.32 ± 0.32	0.81 ± 0.10
	Room temperature	40 ml 75% ethanol	24.61 ± 0.77	25.14 ± 0.05	0.98 ± 0.03
	Room temperature	40 ml water	3.37 ± 0.04	20.26 ± 1.15	0.17 ± 0.01
MC (A)	Room temperature	40 ml 75% ethanol	0	24.58 ± 0.07	0
MC (B)	Room temperature	40 ml water	1.32 ± 0.09	0.51 ± 0.03	2.38 ± 0.01

Each value was expressed as mean±standard deviation (n=3). CON, no mechanical activation; M, mechanical activation without presence of Na_2CO_3; MC, mechanical activation with presence of Na_2CO_3; EGCG, epigallocatechin gallate

There are other methods in extraction of TP and caffeine or removal of caffeine from tea leaves. Ashraf *et al*. (2023), have used ultrasound-assisted method to extract TP and caffeine from the tea leaves and find that, the new method results in a higher extractive yield and a significant concentration of caffeine and polyphenols than traditional approach. Tang *et al*. (2010), have adopted ultrasonic-enhanced supercritical fluid to remove caffeine from tea leaves and find that, the caffeine of tea leaves was effectively removed and minished without damaging the structure of active ingredients. Perva-Uzunalic *et al*. (2006), have found that, the high-pressure extraction with CO_2 can effectively extract active ingredients from tea leaves, and 98% of caffeine was selectively isolated without significant impact on valuable catechins. Sökmen *et al*. (2018), have used sequential supercritical fluid extraction to obtain caffeine and catechins from tea leaves, and find that this method is effective in selective extraction of caffein and catechin from black tea waste and caffeindust. Results of our study indicate that, the MCPT is an effective method to extract and separate TP and caffeine from tea leaves. However, whether there are advantages of MCPT extraction to above methods should be investigated in further research.

CONCLUSIONS

MCPT was successfully applied to the extraction and separation of TP and caffeine from tea leaves. The optimum MCPT extraction conditions were as follows: 15% (w/w) Na_2CO_3 during milling, extracting caffeine with 40 mL 75% (v/v) ethanol and EGCG with 40 mL water at room temperature. Under these conditions, the caffeine EGCG yield in MC (A) fraction was 0.51 mg/g, and the EGCG yield in MC (B) fraction was 1.32 mg/g, with EGCG/caffeine ratio of 2.38. Compared with M extraction and CON extraction, the MC extraction had obvious advantages. The MCPT is an effective method for the extraction and separation of TP and caffeine from tea leaves.

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