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Characterization of polyphenols from green tea leaves using a high hydrostatic pressure extraction

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ABSTRACT

A new extraction technique, high hydrostatic pressure extraction (HHPE), was used to obtain polyphenols from green tea leaves. Various experimental conditions, such as different solvents (acetone, methanol, ethanol and water), pressure (100, 200, 300, 400, 500, 600 MPa), holding time (1, 4, 7, 10 min), ethanol concentration (0–100% mL/mL), and liquid/solid ratio (10:1 to 25:1 mL/g) for the HHPE procedure, were investigated to optimize the extraction. The optimal conditions were as follows: 50% (mL/mL) of ethanol concentration, 20:1 (mL/g) of liquid/solid ratio and 500 MPa of high hydrostatic pressure for 1 min. Under such conditions the extraction yield of polyphenols was up to 30 ± 1.3%. The extraction yields of polyphenols with HHPE for only 1 min were the same as those of extraction at room temperature for 20 h, ultrasonic extraction for 90 min and heat reflux extraction for 45 min, respectively. On the basis of the extraction yields of polyphenols, extraction time and the percentages of polyphenols in extracts, the HHPE is more effective than the conventional extraction methods studied.

1. Introduction

Tea is the second most popular beverage after water in the world. In addition to its attractive aroma and good taste, tea has been recognized to have health-promoting properties, including antioxidant activity, anticarcinogenic and antihypertensive effects (Rice-Evans et al., 1997). Green tea leaves (*Thea sinensis* L.) contain polyphenols, caffeine, amino acids, saponins, tannins, etc. In general, dry green tea leaves contain about 10–35% (w/w) polyphenols and 2–5% (w/w) caffeine (Pan et al., 2003). Polyphenols include catechines, flavanols, flavanones, phenolic acids, glycosides and the aglycons of plant pigments, which are easily dissolved in water, ethanol, methanol, acetone, etc. Polyphenols isolated from green tea leaves are natural antioxidants (Tanizawa et al., 1984), and have a scavenging effect on active oxygen radical (Zhao et al., 1989). Polyphenols have a stronger anti-oxidative activity than butylated hydroxyanisole, butylated hydroxytoluene and DL-α-tocopherol does, and the toxicity of tea polyphenols is lower than that of butylated hydroxyanisole, butylated hydroxytoluene and DL-α-tocopherol (Chen and Wan, 1994). Nowadays polyphenols have been widely applied to food and medicinal industries.

High hydrostatic pressure (HHP), which means cold isostatic superhigh hydraulic pressure that ranges from 100 to 800 MPa or even higher (US FDA, 2000), is currently considered as an attractive innovative non-thermal process that can effectively inactivate microorganisms and preserve fresh-like food products (Knorr, 1993). Exploring the effects of HHP in biotechnology has received increased interest during the last decade (Mozhaev et al., 1994). The work of compression during HHP treatment will increase the temperature of biomaterial through adiabatic heating approximately 3 °C per 100 MPa, depending on the composition of the biomaterial (US FDA, 2000). Biomaterial cool down to their original temperature on decompression, if no heat is lost to or gained from the walls of the pressure vessel during the hold time at pressure (US FDA, 2000). Fig. 1 shows typical temperature rises for water and fat as a function of compression pressures (US FDA, 2000). So HHP operation has no any heating process, except for the minor temperature rise resulting from the compression.

Mass transport phenomena can be enhanced by changes in concentration gradients, diffusion coefficients or boundary layer (Liang, 1993). HHP is a novel method to enhance mass transport phenomena (Rastogi et al., 2003). Higher caffeine extraction yields from coffee and a higher carotenoid content in tomato puree have been demonstrated when extractions were assisted by HHP (Knorr, 1999; Sanchez-Moreno et al., 2004). More recently, HHP has been successfully used for extraction of ginsenoside from Korean red ginseng (Kim et al., 2007), anthocyanins from grape skins (Corrales et al., 2008, 2009). According to the mass transfer theories, the pressurized cells increase permeability (Yan, 2002). Based on the phase behaviour theories, the solubility is stronger while pressure increases (Noble, 1988; Richard, 1992). HHP can also cause deprotonation of charged groups and disruption of salt bridges and hydrophobic bonds, resulting in conformational changes and denaturation of proteins, which lead to the cellular membranes

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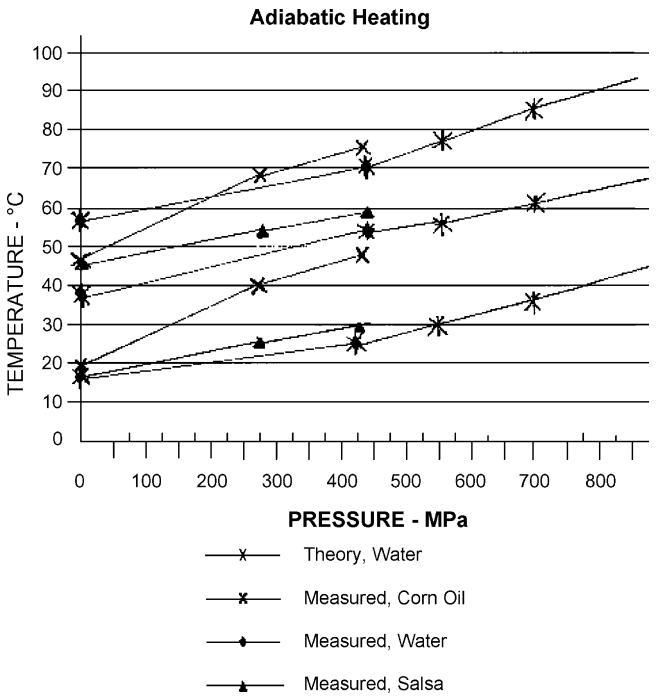

Fig. 1. Increase in temperature of water, corn oil and salsa as a result of adiabatic compression. Note that the increase in temperature upon compression is also a function of the initial temperature.

to be less and less selective. Thereby the compounds are more accessible to extraction up to equilibrium (Barbosa-Canovas et al., 1998).

There are various methods for extracting polyphenols from green tea leaves, such as heat reflux extraction (Ge and Jin, 1994; Hu et al., 1997), ultrasonic extraction (Hu et al., 1997), extraction at room temperature (Lu, 1995), supercritical carbon dioxide extraction (Li and Feng, 1996; Jacques et al., 2007; Esmelindro et al., 2005; Chang et al., 2001) and microwave extraction (Pan et al., 2003). Besides long extracting time, most of these methods have to employ heating which could easily lead to some thermo-sensitive ingredients to lose their biological activities or to transform into other substances. On the contrary, HHP extraction can be operated at room temperature. Therefore, the purpose of this work is to develop a HHP extraction method and to evaluate both HHP extraction and conventional extraction methods for the extraction of polyphenols from green tea leaves.

2. Materials and methods

2.1. Plant materials and chemicals

Dry green tea leaves (*T. sinensis* L.) (place of origin: Hangzhou, China) were purchased from a local market.

Ethanol, methanol and acetone used in the experimental work was all of analytical reagent grade chemicals (Beijing Chemical Reagents Company, Beijing, China). Folin-Ciocalteau reagent and other chemicals for analysis of tea polyphenols were also from Beijing Chemical Reagents Company (analytical grade, Beijing, China). Gallic acid, pharmaceutical grade standard, was purchased from the National Institute for Control of Pharmaceutical and Biological Products (China). The spectrophotometer (751-GW) was from Shanghai Analytical Instrument Overall Factory (Shanghai, China).

The high hydrostatic pressure isostatic apparatus (DL700—0.55 × 1.5) was purchased from Shanghai Dalong Superhigh Pressure Machine Co. Ltd. (Shanghai, China). Effective volume of vessel: 0.35 L; maximal working pressure: 700 MPa; inner diameter: 55 mm; pressure transmitting media: mixture of water and glycol (20:80, v/v).

2.2. HHP extraction method

The green tea leaves were dried in vacuum at 60 °C for 6 h, then pulverized and sieved. Five-gram sample of green tea leaves powder through 40 mesh screen were mixed with an appropriate solvent and placed into a sterile polyethylene bag. The bag was sealed after eliminating air from the inside and placed into a hydrostatic pressure vessel. After processed in the high hydrostatic pressure isostatic apparatus for several minutes (high pressure level: 100, 200, 300, 400, 500, 600 MPa; holding time: 1, 4, 7, 10 min) at room temperature, the mixture was filtered through filter paper. The extraction solution was centrifuged at a speed of 4000 rpm for 10 min, and the supernatant was collected and stored at 4 °C in refrigerator for subsequent determination.

2.3. Conventional extraction methods

Except HHP extraction, other extraction methods are traditional in the references, such as heat reflux extraction (Ge and Jin, 1994; Hu et al., 1997), ultrasonic extraction (Hu et al., 1997) and extraction at room temperature (Lu, 1995).

2.3.1. Heat reflux extraction

Green tea leaves ethanol extracts were boiled (5 g of green tea leaves powder, mixed with 100 mL of 50% ethanol in water) at boiling point, about 85 °C for 45 min (super-boiling of the solution did not occur). Then, the extracts were filtered through filter paper. The extraction solution was centrifuged at a speed of 4000 rpm for 10 min, and the supernatant was collected and stored at 4 °C in refrigerator for subsequent determination.

2.3.2. Extraction at room temperature

Green tea leaves powder ethanol extracts was prepared (5 g of green tea leaves powders, making up the volume to 100 mL with 50% ethanol in water (mL/mL)) in the absence of bright light, with moderate shaking, at room temperature (20 °C). After 20 h, the extracts were filtered. The extraction solution was centrifuged at a speed of 4000 rpm for 10 min, and the supernatant was collected and stored at 4 °C in refrigerator for subsequent determination.

2.3.3. Ultrasonic extraction

Five-gram sample of green tea leaves powder were put into 200 mL conical flask. After 100 mL of 50% ethanol was added in water solution (mL/mL), the flask was sonicated for 90 min in an ultrasonic bath (frequency 50 Hz, power 250 W) at 20–40 °C. Then, the extracts were filtered. The extraction solution was centrifuged at a speed of 4000 rpm for 10 min, and the supernatant was collected and stored at 4 °C in refrigerator for subsequent determination.

2.4. Determination of polyphenol content

The amount of polyphenol was reference measured by a photometric Folin-Ciocalteu assay according to a proposed international standard method (ISO, 1994; Chen et al., 2008; Turkmen et al., 2006). The method is based on the reduction of phosphotungstic acid ($H_3P[W_3O_{10}]_4$) in alkaline solution to phosphotungstic blue. The absorbance of formed phosphotungstic blue is proportional to the number of aromatic phenolic groups and is used for their quantification with gallic acid as the standard (Daniela et al., 2009). Briefly, a calibration curve of gallic acid (ranging from 0.005 to

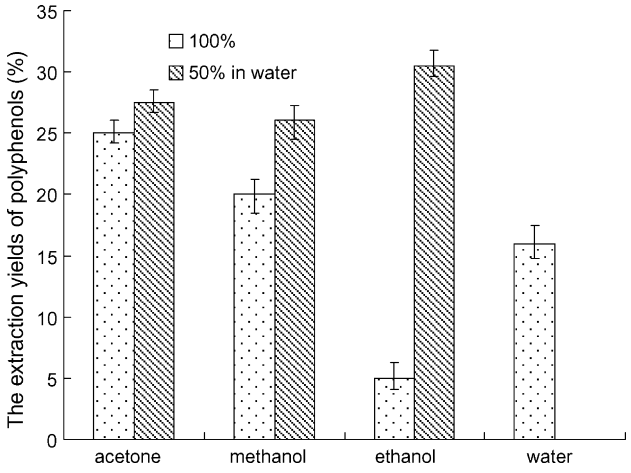

Fig. 2. The effect of difference solvents on the extraction yields of polyphenols. HHP extraction: 500 MPa pressure, HHP for 5 min, 20:1 (mL/g) liquid/solid ratio, 5 g dry green tea leaves. Bars are means ± standard deviations of triplicate measurement ($P < 0.05$, Student's t-test).

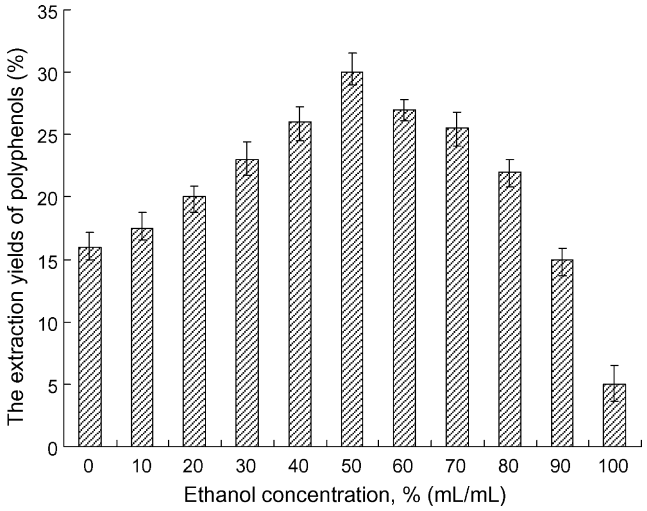

Fig. 3. The effect of ethanol concentration in water on the extraction yields of polyphenols. HHP extraction: 500 MPa pressure, HHP for 5 min, 20:1 (mL/g) liquid/solid ratio, 5 g dry green tea leaves. Values are means ± standard deviations of triplicate measurement ($P < 0.05$, Student's t-test).

0.05 mg/mL) was prepared and the results, determined by regression equation of the calibration curve ($y = 60.56x - 0.72$, $R^2 = 0.9996$), were expressed as mg gallic acid equivalents per gram of the sample. In this method, 1 mL of tea extract diluted 10–75 times with deionized water (to obtain absorbance in the range of the prepared calibration curve) was mixed with 1 mL of 3-fold-diluted Folin-Ciocalteu phenol reagent. Two milliliter of 35% sodium carbonate solution is added to the mixture, which was then shaken thoroughly and diluted to 6 mL by adding 2 mL of water. The mixture is allowed to stand for 30 min and blue color formed is measured at 700 nm using a spectrophotometer.

2.5. Statistical analysis

All experiments were done in triplicate and data in tables and figures represent mean values ± standard deviation ($n = 3$). Results were evaluated for statistical significance using one-way ANOVA by the SPSS Statistic Method (Version 11.5). The confidence level for statistical significance was set at a probability value of 0.05.

3. Results and discussion

3.1. The effect of different solvents on the extraction yields of polyphenols

Fig. 2 shows that acetone can be used to obtain higher extraction yields of polyphenols than methanol, water and ethanol can, respectively. If water was added to ethanol, the ethanol/water (1:1 mL/mL) solution gave higher extraction yields of polyphenols than the other solvents tested. The ethanol is non-toxic, easy to be recycled and mixed with water in different ratios, so it was chosen to extract polyphenols from green tea leaves.

3.2. The effect of ethanol concentration on the extraction yields of polyphenols

Fig. 3 shows that the extraction yields of polyphenols in green tea leaves were greatly influenced by the ethanol concentration. When the ethanol volume percentage in the solvent was lower than 50% (mL/mL), the extraction yields increased with the increase of ethanol concentration. When the ethanol volume percentage in the solvent was higher than 50% (mL/mL), the extraction yields decreased with the further increase of ethanol concentration. The reason may be related to solvent polarity and the solubility of polyphenols. Taking into account of cost, moderate concentration ethanol solution should be chosen as extracting solvent. So 50% ethanol concentration in water (mL/mL) was used in the following experiments.

3.3. The effect of pressure on the extraction yields of polyphenols

Fig. 4 shows that the extraction yields of polyphenols were influenced by pressure. When the pressure increased from 100 to 600 MPa, the extraction yields of polyphenols increased from 15 ± 1.4 to 30 ± 1.3%. It is obvious that pressure is useful for improving the extraction yields of polyphenols. According to Le Chatelier's theory (Chen et al., 2005), the volume of system tends to be reduced during the pressure promoting period. In this process, the extracting solvent comes into cells to integrate with bioactive components. Besides, the pressurized cells show increased permeability (Yan, 2002). The higher the hydrostatic pressure is, the more solvent

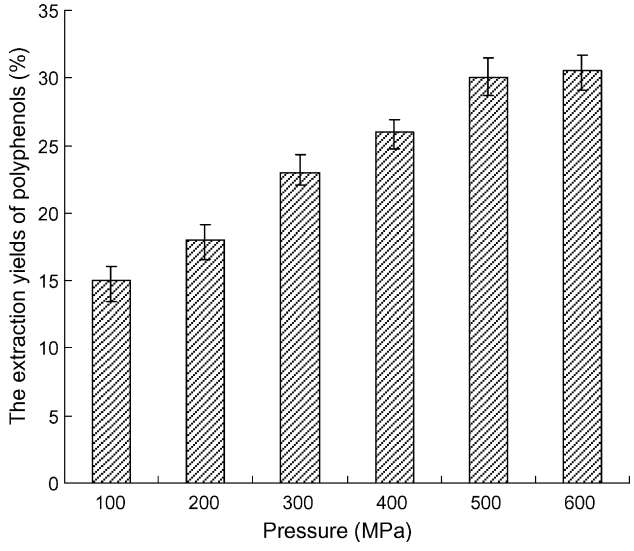

Fig. 4. The effect of pressure on the extraction yields of polyphenols. HHP extraction: 50% ethanol concentration, HHP for 5 min, 20:1 (mL/g) liquid/solid ratio, 5 g dry green tea leaves. Bars are means ± standard deviations of triplicate measurement ($P < 0.05$, Student's t-test).

Table 1
The effect of pressure holding time on the extraction yields of polyphenols.

Pressure holding time (min)	The extraction yields of polyphenols (%)
1	29.5 ± 1.4 a
4	30.7 ± 0.8 a
7	31.2 ± 1.5 a
10	30.6 ± 1.3 a

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$, Student's t-test). HHPE: 500 MPa pressure, 50% ethanol concentration, 20:1 (mL/g) liquid/solid ratio, 5 g dry green tea leaves.

can enter cells and the more compounds can permeate out to the solvent. The equilibrium of solvent concentration between inner and outer of cells would be established during the pressure holding period. When the HHP is suddenly released, the cell wall is disrupted to release the cytoplasm which contains a high concentration of target material (Fernandez Garcia et al., 2001), Butz et al. (1994) reported that pressures of 100 MPa were enough to cause rupture of intracellular vacuoles and plant cell walls in onions. Furthermore, in the extraction process with high pressure, the solubility of extracts is improved as the pressure increases (Noble, 1988; Richard, 1992). So increasing pressure could increase the extraction yields of polyphenols. These results are in accordance with studies of Sanchez-Moreno et al. (2004).

However, the higher the pressure, the more expensive the equipment, the more energy would be consumed and the safety factor would decrease. Thus, 500 MPa pressure was chosen to extract polyphenols from green tea leaves.

3.4. The effect of pressure holding time on the extraction yields of polyphenols

The effect of pressure holding time was investigated in the range of 1–10 min. Based on above analysis, the main function of pressure holding time is to form the equilibrium of solvent concentration between inner and outer of cells and to get in full touch with bioactive components and solvent. The different pressure between inner and outer cell membrane is so large that it will lead to instant permeation. Table 1 shows that the extraction yields of polyphenols (29.5 ± 1.4, 30.7 ± 0.8, 31.2 ± 1.5, 30.6 ± 1.3) had no significantly increase ($P < 0.05$) when the pressure holding time was beyond 1 min. Therefore, 1 min was enough to complete the equilibrium. According to Pascal theory (Chen et al., 2005), during the HHP treatment process, the pressure could transfer to the whole material uniformly and instantaneously. So the rate of pressure transfers rapidly with no stress gradients, which make the extraction process easy and effective.

3.5. The effect of liquid/solid ratio on the extraction yields of polyphenols

The effect of liquid/solid ratio was investigated in the range of 10:1 to 25:1 (mL/g). Fig. 5 shows that the extraction yields of polyphenols increased with the increase in the liquid/solid ratio. When the liquid/solid ratio increased from 10:1 to 25:1 (mL/g), the extraction yields of polyphenols increased from 17 ± 1.4 to 30 ± 1.3%. It is obvious that the liquid/solid ratio is useful for improving the extraction yields of polyphenols. The dissolving process of bioactive components into the solvent was a physical process. When the amount of solvent increased, the chance of bioactive components coming into contact with extracting solvent expanded, which lead to higher leaching-out rates. But if the extraction was carried out under a higher solid/liquid ratio, the concentration of polyphenols in the extraction solution was very low. The liquid/solid ratio of 20:1 (mL/g) was sufficient to reach the high extraction yields and it was used afterwards.

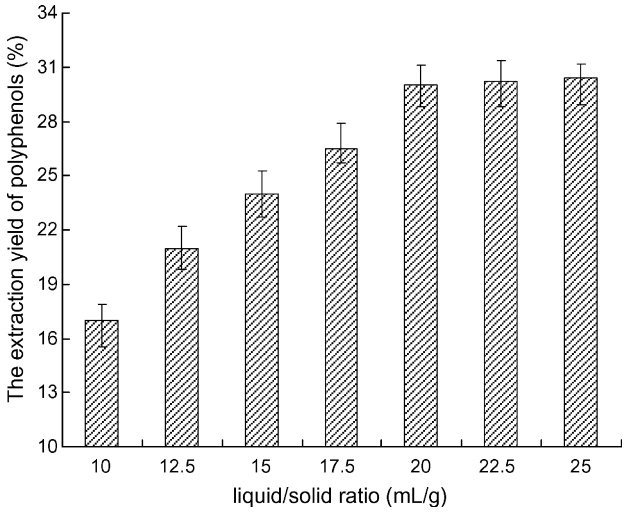

Fig. 5. The effect of liquid/solid ratio on the extraction yields of polyphenols. HHP extraction: 500 MPa pressure, HHP for 1 min, 50% ethanol concentration, 5 g dry green tea leaves. Values are means ± standard deviations of triplicate measurement ($P < 0.05$, Student's t-test).

3.6. The optimal conditions of HHP extraction

Therefore, the optimal conditions of HHP extraction were as follows: 50% (mL/mL) of ethanol concentration, 20:1 (mL/g) of liquid/solid ratio, and 500 MPa of high hydrostatic pressure for 1 min. Under such conditions the extraction yields of tea polyphenols was up to 30 ± 1.3%.

3.7. Comparison of HHP extraction and conventional extraction methods

Green tea leaves composition varies with climate, season, tea variety and age of the leaf (Tanizawa et al., 1984). In order to compare the results of HHP extraction with other traditional extraction methods, we performed all experiments using dry green tea leaves from the same batch, and the technique of extraction methods

Table 2
Comparison of the results of HHPE and conventional extraction methods.

Extraction method	Extraction time	The extraction yields of polyphenols (%)	The percentage of polyphenols in extracts (%)
Extraction at room temperature	20 h	30.5 ± 1.4 a	76.6 ± 1.87 a
Ultrasonic extraction	90 min	29 ± 0.8 a	76 ± 2.3 a
Heat reflux extraction	45 min	31 ± 1.5 a	78 ± 1.8 a
HHPE	1 min	30 ± 1.3 a	77 ± 2.0 a

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$, Student's t-test). Extraction at room temperature: 50% ethanol concentration, 20:1 (mL/g) liquid/solid ratio, at about 20 °C for 20 h. Ultrasonic extraction: frequency 50 Hz, power 250 W, 50% ethanol concentration, 20:1 (mL/g) liquid/solid ratio, at 20–40 °C for 90 min. Heat reflux extraction: 50% ethanol concentration, 20:1 (mL/g) liquid/solid ratio, at boiling point about 85 °C for 45 min. HHPE: 500 MPa pressure, HHP for 1 min, 50% ethanol concentration, 20:1 (mL/g) liquid/solid ratio.

(extraction at room temperature, ultrasonic extraction, heat reflux extraction) were exactly the same as those given in the literature.

Table 2 shows that the extraction yields of tea polyphenols with HHP for only 1 min were given the same as those of heat reflux extraction for 45 min, ultrasonic extraction for 90 min and extraction at room temperature for 20 h, respectively. The results show that the time of heat reflux extraction, ultrasonic extraction and extraction at room temperature was respectively about 45, 90, 1200 times than that of HHP extraction. Thus HHP extraction can greatly reduce the extraction time for the same extraction yields.

Table 2 also shows that HHP extraction gave the same percentages of polyphenols in extracts as those by extraction at room temperature, ultrasonic extraction and heat reflux extraction, respectively. Thus the HHP extraction can give the higher extraction selectivity.

4. Conclusions

Conditions for HHP extraction of polyphenols from green tea leaves have been studied. The optimal conditions of HHP extraction were as follows: 50% (mL/mL) of ethanol concentration, 20:1 (mL/g) of liquid/solid ratio, and 500 MPa of high hydrostatic pressure for 1 min. Under such conditions, the extraction yields of tea polyphenols was up to $30 \pm 1.3\%$. HHP extraction has been shown to be an efficient method for extraction of polyphenols from green tea leaves. Compared with the conventional extraction methods, the HHP procedure provided higher extraction yields, higher extraction selectivity, requiring shorter time, and less labor intensive.

Therefore, HHP extraction is suitable for fast extraction of polyphenols from green tea leaves. Food and medicinal industries will benefit from this emerging technology, which is fast, safe and more eco-friendly than conventional extraction methods.

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