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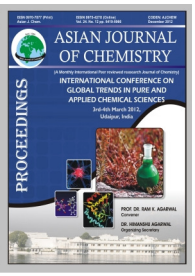

Separation and Preparation Purine Alkaloids from Green Tea Waste†

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A simple, clean and economical method to separate and prepare purine alkaloids from green tea waste in one-step by Sephadex LH-20 column chromatography was developed. From extraction to separation and preparation, distilled water was used. The qualitative analysis of purine alkaloids was performed by time of flight mass spectrometer and the content was quantitatively analyzed by high-performance liquid chromatography. The yield of purine alkaloids was 3.37 % of the dry weight of green tea waste, which contained caffeine (93.01 %) and theobromine (6.92 %) and the recovery of purine alkaloids by column chromatography was 96.84 % and the stability of preparative method is good. The study was considered to be a promising clean method for purine alkaloids preparation without using poisonous organic solvents.

Key Words: Purine alkaloids, Separation, Caffeine, Theobromine, Green tea, Sephadex LH-20

INTRODUCTION

Green tea is one of the most popular beverages in the world due to its sensory properties, stimulating effects and potential health benefits[1-3]. The main bioactive components in green tea are purine alkaloids and tea polyphenols. Green tea contains 2-4.5 % purine alkaloids[4], the most dominant component of the purine alkaloids is caffeine ($C_8H_{10}N_4O_2$; 1, 3, 7-trimethylxanthine) with small amounts of theobromine ($C_7H_8N_4O_2$; 7-dimethylxanthine)[5,6]. Caffeine acts as a stimulant for the heart, respiratory and the central nervous system and an adjunct in numerous analgesic preparations and is a vasodilator as well as a diuretic[7]. Caffeine also has been extensively studied for its physiological effects on human health in terms of behaviour and mood[8,9].

The conventional technique for purine alkaloids extraction was using poisonous organic solvent such as chloroform and dichloromethane, *etc*.[10-13]. Organic solvent extraction method has the shortcomings of environmental pollution and high toxicity of some of these organic solvents and also involves the risk of leaving toxic residues in the extracted products. Recently, some of new methods have been reported: supercritical carbon dioxide extraction caffeine from green tea[14-17], a microwave-assisted extraction (MAE) method for the preparation caffeine from green tea leaves using a solvent mixture of ethanol and water[18], solid liquid extraction of caffeine from tea waste using battery type extractor[19] and caffeine extraction from green tea leaves assisted by high pressure processing[5]. Besides long extracting time, most of these methods are high cost and very complex.

The purpose of this work is to develop a clean and economical method to separate and prepare purine alkaloids from green tea waste by the Sephadex LH-20 column chromatography. A variety of eluting agents have been investigated with the strategy that solvents with cheap and nontoxic.

EXPERIMENTAL

ÄKTA explorer 100 chromatography systems equipped with a P-900 pump, a pH/C-900 detector, a UV-900 detector, a Frac-900 fraction collector, a 2 mL sample loop and a Unicorn 5.11 work station (GE Healthcare, Uppsala, Sweden). The column (600 mm × 10 mm) filled with sephadex LH-20 from GE healthcare. Vacuum freeze drier (PowerDry PL3000) was purchased from Heto Holten A/S (Denmark). Comminuting machine (IKE-WERKE) was purchased from IKE Company (Germany). Centrifuge (5810 R) was purchased from Eppendorf AG (Germany). Time of flight (TOF) mass spectrometer (Agilent 6210, USA).

Distilled water obtained from a Milli-Q (Millipore, Bedford, MA, USA) machine. Caffeine (≥ 99 %), theobromine (≥ 99 %) and acetonitrile (HPLC chromatographic grade) were purchased from Sigma chemical company. Green tea waste

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was obtained during the production of green tea in tea factory of Chongqing, china.

Extraction: The coarse powder of green tea waste was obtained after comminution and filtration (20 mesh) and 15 g powder was extracted with 300 mL water in a water bath at 90 ºC for 20 min. The solution was centrifuged and the supernatant liquid was concentrated by rotary evaporation and metered volume to 50 mL. All samples were pre-filtered through membrane filter (0.45 μm, Membrana, Germany) to remove possible particles before injected to the preparative Sephadex LH-20 column chromatography.

Separation and purification: The injection volume was 2 mL and the flow rate was 30 mL/h. The effluent was monitored at 280 nm on ÄKTA explorer 100 chromatography system. The Sephadex LH-20 column was equilibrated with one column volume of the eluting agent before sample injection. After five times sample injection, the Sephadex LH-20 column was immediately cleaned-in-place with 60 mL 70 % (v/v) and 40 mL 20 % (v/v) ethanol using a reversed flow through the column. The column was stored in 20 % (v/v) ethanol.

LC/MS qualitative analysis: Mass spectral data were obtained on time of flight mass spectrometer, operating in the positive ion mode and equipped with electrospray ion source. Full scan spectra was collected by scanning from *m/z* 50 to 3000. The mass spectrometer operated with a cone voltage of 150 V and a capillary voltage of 3.5 kV. The source temperature was optimized at 350 ºC.

HPLC quantitative analysis: HPLC quantitative analysis was performed on Agilent 1100 series consisted of binary pump, thermostat autosampler, column oven, diode-array detector and HP Chem Station software. Column: Agilent ZORBAX SB-C_{18} (5 μm, 250 mm × 4.6 mm). Injection volume: 10 μL. Flow: 1 mL/min. UV wavelength: 280 nm. Mobile phase: A: 0.2 % formic acid (aqueous); B: acetonitrile. Gradient: Initial 8 % B, keep 5 min; in 35 min to 21 % B, 10 min wash with 100 % B, back to initial conditions.

RESULTS AND DISCUSSION

Investigation of eluting agent: In order to develop a clean and economical way to obtain purine alkaloids from green tea waste, a variety of eluting agent compositions were investigated with the strategy that solvents with as low cost and as low level of pollution as possible should be chosen, *e.g*. distilled water and different concentrations of ethanol aqueous solution were tried. After the sample was loaded onto the chromatographic column, different low concentrations of ethanol aqueous solution (0 %, 10 %, 20 %, 30 %) (v/v) were investigated (Fig. 1). Results indicated that distilled water was a perfect eluting agent for separation of purine alkaloids from other components. All fractions were collected by Frac-900 fraction collector and dried by freeze drying, 41.1 mg purine alkaloids (Fig. 1c) was obtained by carefully weighed.

From our previous studies, we found that the Sephadex LH-20 column need to regenerate per month after use and the procedure was cleaned-in-place with one column volume of 0.1 M NaOH and 20 % (v/v) ethanol, successively.

LC/MS qualitative analysis: The peak C (Fig. 1) was qualitatively analyzed by mass spectrometer. Fig. 2 shows that the result of (+)-ESI time of flight mass spectrometer: 181.07283 *m/z* ($[M+H]^+$) and 195.08889 *m/z* ($[M+H]^+$) and the molecular formulas were concluded to $C_7H_8N_4O_2$ and $C_8H_{10}N_4O_2$ by Agilent mass hunter qualitative analysis software, respectively. In our experience, it was most likely that the two substances were theobromine ($C_7H_8N_4O_2$) and caffeine ($C_8H_{10}N_4O_2$).

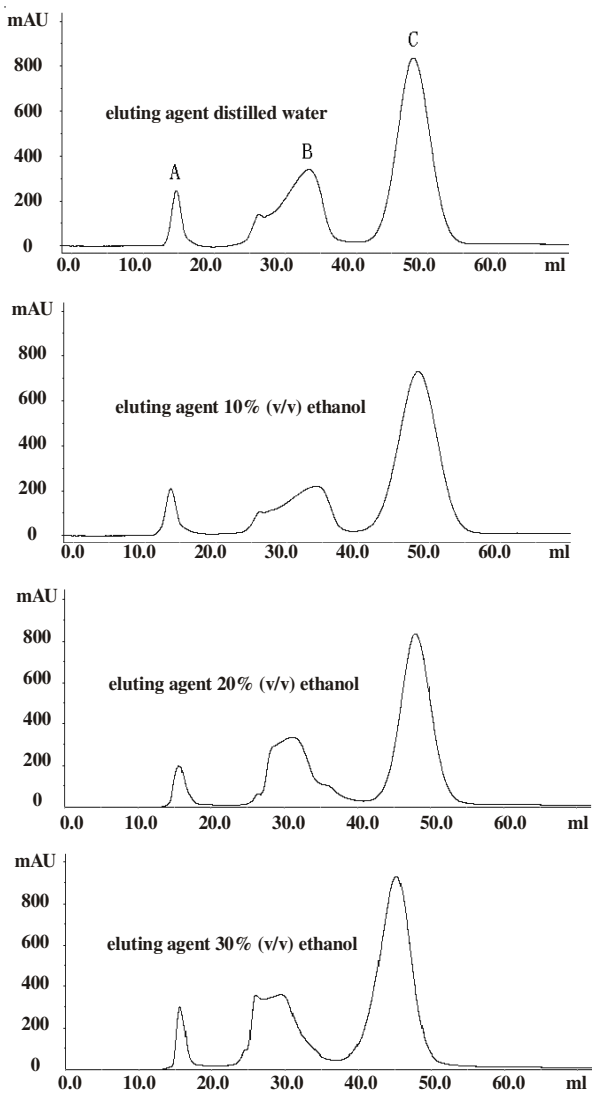

Fig. 1. Chromatogram of one-step elution of extract of green tea waste by Sephadex LH-20 column; Sample: 2 mL; flow rate: 30 mL/h; detector: UV 280 nm

HPLC quantitative analysis: The peak C and extract of green tea waste have been analyzed by HPLC and the HPLC chromatogram was shown in Fig. 3. Through the same retention time of the assumed target peak components and the corresponding reference substance, it was concluded that the most dominant component of the purine alkaloids was caffeine with small amounts of theobromine and it was also in agreement with the conclusion of LC/MS.

The quantitative analysis was carried out using calibration functions of calibration curves obtained with the reference substances of caffeine (10-800 mg/L) and theobromine (1-100 mg/L) and the linear regression of the two calibration curves was obtained with $R^2 = 1$ (Fig. 4).

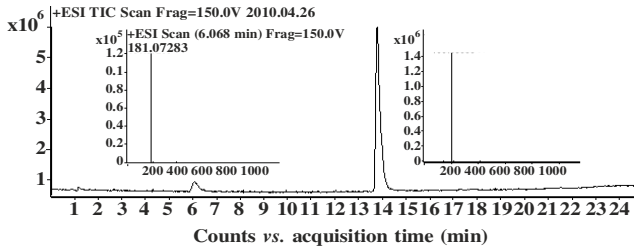

Fig. 2. Time of flight mass spectrometer chromatograms of peak C from green tea waste by sephadex LH-20 column chromatography

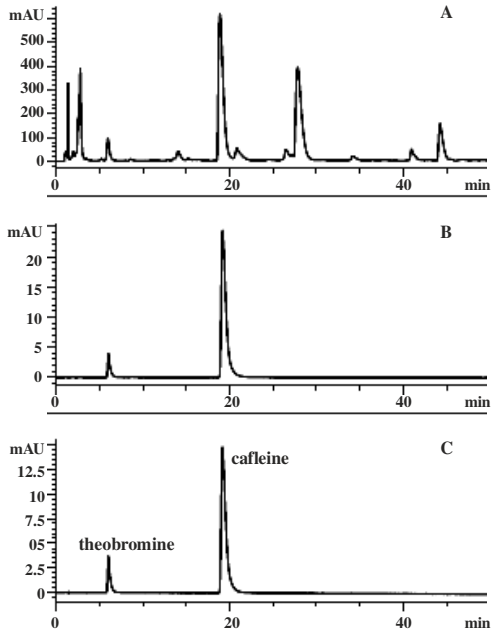

Fig. 3. HPLC chromatograms of: (A) the extract of green tea waste and (B) peak C from green tea waste by sephadex LH-20 column chromatography and (C) reference substances of theobromine and caffeine

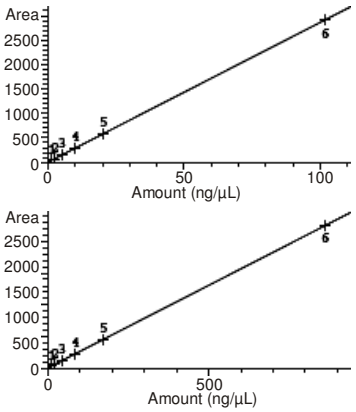

Fig. 4. Linear regression of the two calibration curves of theobromine and caffeine

The yield of purine alkaloids was 3.37 % and the content of caffeine was 93.01 % and theobromine was 6.92 %, respectively. The recovery of sephadex LH-20 column chromatography was 96.84 %; all the results have been calculated from the formula below, respectively.

$$\text{Yield of purine alkaloids}(\%) = \frac{\text{weight of purine alkaloids}}{\text{weight of green tea waste}} \times 100\%$$

$$\text{Recovery of the purine alkaloids}(\%) = \frac{\text{weight of purine alkaloids recovery}}{\text{weight of purine alkaloids load}} \times 100\%$$

$$\text{Theobromine content of purine alkaloids}(\%) = \frac{\text{weight of theobromine}}{\text{weight of purine alkaloids}} \times 100\%$$

$$\text{Caffeine content of purine alkaloids}(\%) = \frac{\text{weight of caffeine}}{\text{weight of purine alkaloids}} \times 100\%$$

Investigation of the stability of preparative method: In order to study the stability of the preparative method of purine alkaloids from green tea waste by Sephadex LH-20 column chromatography, the optimization testing conditions of the method have been investigated in 3 times. The result in Table-1 showed the stability of preparative method was satisfied.

TABLE-1
STABILITY OF PREPARATIVE METHOD

Number of times	1	2	3
Yield of purine alkaloids (%)	3.37	3.25	3.28
Caffeine content in purine alkaloids (%)	93.01	93.58	92.55
Theobromine content in purine alkaloids (%)	6.92	6.50	7.28

Conclusion

A simple and clean method of separation and purification purine alkaloids from green tea waste was established and distilled water was used as the only eluting agent. Compared with conventional separation methods, it has more excellent advantages, such as cheaper raw material, no toxic residues, higher yield, commercially feasible and environment friendly. Our study demonstrated that separation and preparation of purine alkaloids by Sephadex LH-20 column chromatography is a very promising clean method to prepare high-quailty purine alkaloids form green tea waste.

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