Simultaneous Separation and Purification of Tea Bioactives from summer Green Tea by Column Chromatography

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Summary: In the present study, feasibility of producing high quality of tea polyphenols, theanine and caffeine from the extract of summer green tea by column chromatography was investigated. Crude extract of summer green tea obtained by hot water extraction was passed through columns of polyamide and NKA-II macroporous resin respectively, resulting in tea polyphenols-enriched fraction (96.27 ± 1.78%), theanine-enriched fraction (99.02 ± 0.43%) and caffeine-enriched fraction (99.25 ± 0.36%). The recovery rates for tea polyphenols, theanine and caffeine were 72.42 ± 1.41%, 66.12 ± 1.66% and 62.07 ± 2.17%, respectively. The separation procedure allowed the production of such products by decreasing impurities and avoiding the use of poisonous organic solvent. The results suggested that it was possible to produce high-quality products of rich-in tea polyphenols, theanine and caffeine by a simple way. From industrial point of view, this novel method has many merits such as high efficiency of separation, low consumption of energy and environment-friendly procedure.

Keywords: Summer green tea; Separation; Tea polyphenols; Theanine; Caffeine; Column chromatography.

Introduction

Nowadays tea is one of the most widely consumed beverages in the world, many people drink it due to its benefits to health. As we know, tea contains many natural bioactives such as polyphenols, theanine and caffeine. Among all tea polyphenols, especially catechins and gallic acid have been considered to be the main players in the beneficial effects for human health. The major tea catechins are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC). A lot of biological functions of tea catechins such as anti-inflammation, anti-oxidation, anti-allergy and anti-obesity have been reported [1, 2], and a large number of evidences for the healthy effect of caffeine consumption have also been reported [3, 4]. Theanine, a non-protein amino acid that was first discovered in tea leaves, is the main free amino acid in tea, representing as much as 50% of the total amino acids in black tea and 1-2% of the dry weight of green tea [5]. It involves in many biological functions, such as promoting relaxation, reducing blood pressure, enhancing anti-tumor activity, and neuroprotection [6, 7].

It has been observed that the most significant effects of green tea on human health are mainly due to its high polyphenol content and other bioactive components [8]. However, the relative higher ratio of tea polyphenols/amino acids for summer green tea makes it bitter than spring green tea and an underutilized natural resource [9-11]. In fact, summer green tea contains a lot of beneficial components as spring green tea [11-13]. Furthermore, we demonstrated that the extract from summer green tea was rich in tea catechins (EGC, EGCG, EC, and ECG), caffeine and theanine [14]. In order to develop these health-promoting compounds as food ingredients or in nutraceutical applications, more research is immediately required for efficient extraction and separation of bioactives from summer green tea. Therefore, we determined recently the optimal conditions for the extraction of tea polyphenols, EGCG and theanine from summer green tea by using response surface methodology [14]. Although several efficient methods including fractionation with organic solvents, precipitation with inorganic ions and so on have been applied to the separation of bioactive compounds from tea, they generally require a large amount of organic solvents or inorganic salts. Recently, resin adsorption technology is widely used for the separation of natural bioactive components due to its unique adsorption property, low operation expense, less solvent consumption and easy regeneration [15-17]. For the production of tea bioactives, it has been reported that polyamide resin exhibits significant selective adsorption of tea polyphenols over caffeine and separates them well [18, 19], and 732 cation exchange resin is suitable for the separation and purification of theanine [20-22]. However, there are few reports about a combined use of resins for the simultaneous separation and purification of tea

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bioactives. Therefore, we report here a simple process in detail for the separation of tea polyphenols, caffeine and theanine from the crude extract of summer green tea by column chromatography, resulting in tea polyphenols, caffeine and theanine with high purity.

Results and Discussion

Static Adsorption and Desorption of Tea Polyphenols, Tea Catechins and Caffeine

The static adsorption capacities and desorption rates of tea polyphenols, tea catechins (EC, ECG, EGC and EGCG) and caffeine of macroporous adsorption resins are listed in Table-1. S-8 resin exhibited the highest adsorption capacity for tea polyphenols and tea catechins, but the rate of desorption was rather low, which made it difficult for practical use. AB-8, HP-20 and NKA-II showed relatively better adsorption and desorption for tea polyphenols, but they could not separate tea polyphenols and caffeine effectively. For polyamide, it showed not only high adsorption capacity for tea polyphenols, but also quite low adsorption capacity for caffeine. It may be due to that tea polyphenols have phenolic hydroxyls which can form strong hydrogen bonding with polyamide [23]. It can provide not only lone pair of electrons, but also an empty orbital. While in caffeine molecule, only nitrogen and oxygen can form hydrogen bond, the ability to provide lone pair of electrons and form hydrogen bond is limited due to the effect of steric hindrance and conjugated π bond of nitrogen and oxygen. So it can be deduced that hydrogen bond may play an important role in the different adsorption of tea polyphenols and caffeine on polyamide resin. The present results are in good agreement with other reported results [18-20, 23]. Therefore, polyamide was selected for the separation of tea polyphenols from summer green tea extract for its satisfied selective adsorption character.

Except tea polyphenols and caffeine, the main components in crude extract of summer green tea obtained by hot water extraction are soluble sugars and amino acids. For polyamide resin, the adsorption capacities for soluble sugars and amino acids were 14.72 ± 1.03 mg/g and 3.61 ± 0.23 mg/g, while the desorption rates for soluble sugars and amino acids were 93.04 ± 2.45% and 96.74 ± 1.13%, respectively. Considering the adsorption selectivity, polyamide is the most suitable resin for the separation of tea polyphenols from the crude extract of summer green tea.

Static Adsorption Kinetics

The adsorption kinetic curve of tea polyphenols on polyamide resin (Fig. 1) was obtained by contacting 30 mL of solution of tea extract with 1 g resin in the shaker bath at 25ºC and monitoring the concentration of tea polyphenols in raffinate at different time intervals till equilibration. It can be seen that the adsorption of tea polyphenols increased rapidly with the increase of adsorption time, and the adsorption equilibrium was almost achieved in 2 h.

Adsorption rate equation:

$$\ln[Q_e/(Q_e-Q_t)] = Kt$$

where Q_t is the adsorption capacity (mg/g) of resin at time t, Q_e is the adsorption capacity (mg/g) of resin in equilibrium, and K is the adsorption equilibrium rate constant.

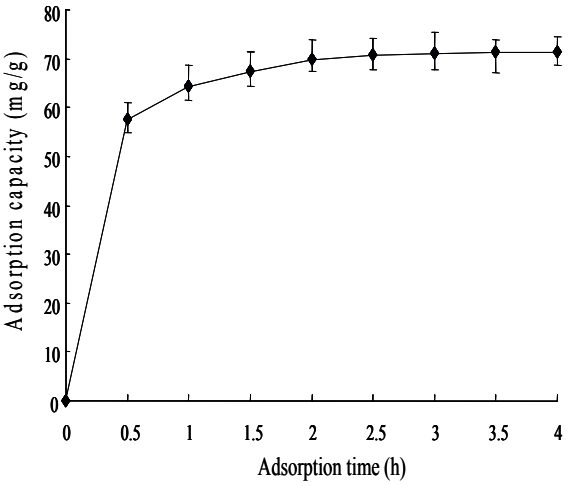

Fig. 1: Static adsorption kinetics of polyamide resin for tea polyphenols.

According to the adsorption rate equation, K for polyamide resin was obtained as 1.753 h^{-1}, which was expressed as monomolecular adsorption. Furthermore, the adsorption rate was high at the beginning, and reached equilibrium in a short time. The character of high adsorption rate and capacity makes polyamide resin great value of industrial application for enriching tea polyphenols from tea.

Table-1: Adsorption quantities and desorption rates of tea polyphenols, tea catechins (EGCG, ECG, EGC and EC) and caffeine of eight resins.

Resins	EGCG		ECG		EGC		EC		Tea polyphenols		Caffeine	
	(mg/g)	(%)	(mg/g)	(%)	(mg/g)	(%)	(mg/g)	(%)	(mg/g)	(%)	(mg/g)	(%)
AB-8	12.35 ± 1.12	72.44 ± 2.88	5.79 ± 0.33	52.78 ± 3.34	3.42 ± 0.27	77.40 ± 4.76	3.50 ± 0.24	88.70 ± 5.79	57.92 ± 3.82	71.67 ± 4.02	13.06 ± 1.28	84.55 ± 3.77
D-101	12.13 ± 1.08	78.93 ± 3.72	6.45 ± 0.43	51.28 ± 4.01	3.08 ± 0.29	70.91 ± 5.79	3.07 ± 0.28	68.82 ± 5.24	56.23 ± 2.44	62.44 ± 2.86	14.09 ± 1.31	99.96 ± 3.02
S-8	16.45 ± 1.41	3.34 ± 0.28	5.76 ± 0.33	12.33 ± 1.08	9.41 ± 0.78	5.23 ± 0.43	5.25 ± 0.47	12.56 ± 1.13	85.02 ± 3.25	11.22 ± 0.45	2.66 ± 0.29	22.07 ± 1.98
HP-20	12.33 ± 1.11	61.13 ± 4.17	6.40 ± 0.54	45.88 ± 3.83	2.47 ± 0.21	65.81 ± 3.42	2.89 ± 0.24	40.79 ± 3.46	57.21 ± 1.29	66.47 ± 3.62	14.44 ± 1.09	81.62 ± 3.22
D-3520	4.35 ± 0.28	65.88 ± 4.34	5.50 ± 0.36	9.01 ± 0.78	5.48 ± 0.43	39.34 ± 2.81	3.89 ± 0.32	2.34 ± 0.18	41.33 ± 3.05	46.59 ± 2.91	7.60 ± 0.68	81.05 ± 4.32
Polyamide	19.04 ± 1.34	56.19 ± 4.12	6.61 ± 0.49	65.37 ± 3.88	3.02 ± 0.17	63.17 ± 2.39	2.26 ±0 .18	74.19 ± 3.18	76.36 ± 1.97	82.67 ± 3.08	1.71 ± 0.12	83.48 ± 3.53
DM-130	10.22 ± 0.88	66.14 ± 2.65	5.36 ± 0.33	17.50 ± 1.34	8.73 ± 0.24	76.64 ± 4.07	1.66 ± 0.12	3.11 ± 0.13	57.14 ± 3.03	69.14 ± 4.03	10.47 ± 0.99	98.72 ± 3.82
NKA-Ⅱ	15.72 ± 1.29	58.42 ± 4.32	5.64 ± 0.41	40.91 ± 3.23	13.41 ± 1.21	70.69 ± 3.92	5.01 ± 0.34	60.85 ± 4.86	43.41 ± 3.56	74.41 ± 3.06	15.84 ± 1.45	82.90 ± 5.71

Static Adsorption Thermodynamics

The static adsorption isotherms of tea polyphenols on polyamide resin, as shown in Fig. 2, was obtained by contacting 1 g resin with 30 mL solution of tea extract in a series of concentrations in the shaker bath. It can be seen that the adsorption increased with the increase of sample concentration, while it decreased with the increase of temperature.

Langmiur equation:

$$c/q = c/q_m + 1/kq_m$$

where q is the adsorption of adsorbent (mg/g), q_m is the maximum adsorption of adsorbent (mg/g), c is the concentration of adsorbate (mg/mL), and k is Langmiur constant. By analysis of the data in Fig. 2, the adsorption isotherm equations at different temperatures can be obtained. It can be seen that the adsorption reached equilibrium gradually with the increase of tea polyphenols content. Therefore, it can be deduced that it was monomolecular adsorption and adsorption isotherms could be described by Langmiur equation. The adsorption isotherms was "L" type, which suggested that tea polyphenols in the solution of tea extract were more easily be adsorbed than solvent molecules. Meanwhile, the adsorption decreased with the increasing of temperature indicated that the adsorption of tea polyphenols was exothermic process. So the adsorption of tea polyphenols would be better at lower temperature. For production cost, room temperature was chosen for the adsorption of tea polyphenols.

Effect of Elution with Water on the Adsorption of Tea Polyphenols

The solution of tea extract was loaded onto the polyamide column, after reaching saturated adsorption; the column was eluted by pure water at a flow rate of 2 BV/h for 2 h. The recoveries of tea polyphenols, caffeine, total amino acids, theanine and soluble sugar were determined to be 6.43 ± 0.42%, 97.32 ± 1.71%, 98.03 ± 1.18%, 98.21 ± 1.33% and 94.42 ± 2.04%, respectively. It can be seen that caffeine, amino acids and soluble sugars could be eluted out thoroughly by pure water, while most of tea polyphenols were still adsorbed by polyamide resin. So in the separation of tea polyphenols by polyamide column chromatography, most interference components (caffeine, amino acids and soluble sugars) could be removed efficiently by only washing the column with pure water. As the different adsorption capacity of tea polyphenols and other components in the solution, hydrogen bonding might also play an important role. Generally speaking, the selectivity and specificity of physical adsorption was far lower than chemical adsorption, so polyamide resin exhibited satisfied selective adsorption character for enriching tea polyphenols from the extract of summer green tea.

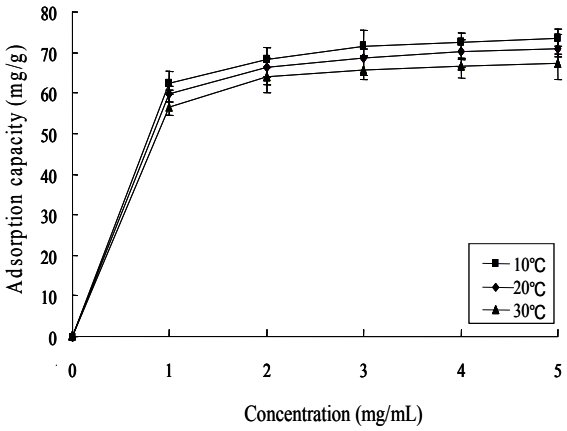

Fig. 2: Adsorption isotherms of polyamide resin for tea polyphenols.

Dynamic Adsorption and Desorption of Tea Polyphenols on Polyamide Resin

The dynamic adsorption and desorption may be influenced by several factors, such as feed rate, initial pH value, initial concentration, flow rate of eluting solvent, temperature and so on. In this study, the feed rate, feed pH value, initial concentration and flow rate of eluting solvent were studied, and all the dynamic experiments were performed at room temperature.

In the dynamic adsorption process, feed rate plays an important role in separation effect, and solid-liquid contact time should be appropriate. When feed rate is too high, the solid-liquid contact time will be reduced and the sample will be leaked in a short time, while too low feed rate may extend the production cycle and lead to higher cost. So we investigated the leakage curve of tea polyphenols in different feed rates under the conditions of pH 3 and sample concentration of 2 mg/mL in the dynamic adsorption. As shown in Fig. 3a, it can be seen that the adsorption of tea polyphenols decreased with the increasing of feed rate, while there was no significant difference in adsorption capacity ($P > 0.05$) when the feed rate was lower than 2 BV/h. So for higher efficiency, feed rate of 2 BV/h was chosen in the dynamic adsorption.

Initial solution pH is an important factor that will affect the adsorption capacity. Since tea polyphenols contain many phenolic hydroxyl groups, the force of hydrogen bonding is closely related to the solution pH value, so the adsorption process should be in acidic condition. In the present study, the leakage curves of tea polyphenols in different pH values under the conditions of feed rate 2 BV/h and sample concentration of 2 mg/mL in the dynamic adsorption were investigated. It can be seen that the adsorption of tea polyphenols increased with the decreasing of pH value (Fig. 3b), while there was no significant difference between the adsorption capacities in experimental conditions ($P > 0.05$). So in the dynamic adsorption, for better absorption of tea polyphenols, initial pH value of 3 was selected.

In the adsorption of tea polyphenols, the initial concentration is also important. The leakage curves of tea polyphenols in different initial concentrations under the conditions of pH value 3 and flow rate of 2 BV/h in the dynamic adsorption are shown in Fig. 3c. It can be seen that the adsorption of tea polyphenols decreased with the increasing of initial concentration, while there was no significant difference between the adsorption capacities in experimental conditions ($P > 0.05$) when the concentration was lower than 2 mg/mL. For higher efficiency in the dynamic adsorption, initial concentration of 2 mg/mL was selected.

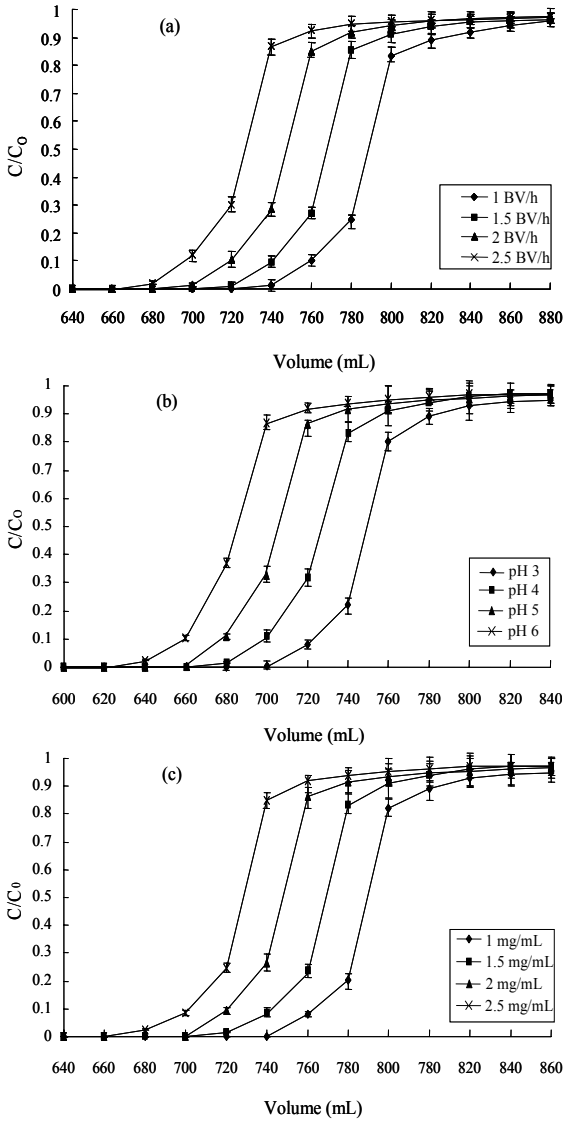

Fig. 3: Effect of feed rate (a), feed pH value (b) and initial concentration (c) on the adsoption of tea polyphenols. C_o: the concentration of tea polyphenols in the sample (mg/mL); C: the concentration of tea polyphenols in the elution (mg/mL).

Eluent should be considered from desorption efficiency, low cost, safety and so on. Ethanol, a kind of non-toxic and efficient organic solvent, is generally used as the eluent in the isolation of natural compounds. In the present study, it was selected as

the eluent, and the effects of flow rates on desorption rates of tea polyphenols are shown in Fig. 4. It can be seen that the desorption rate of tea polyphenols decreased with the increasing of the flow rate, while it had no significance between the flow rate of 1 BV/h and 2 BV/h ($P > 0.05$). For higher production efficiency, a flow rate of 2 BV/h was selected in the dynamic desorption of tea polyphenols.

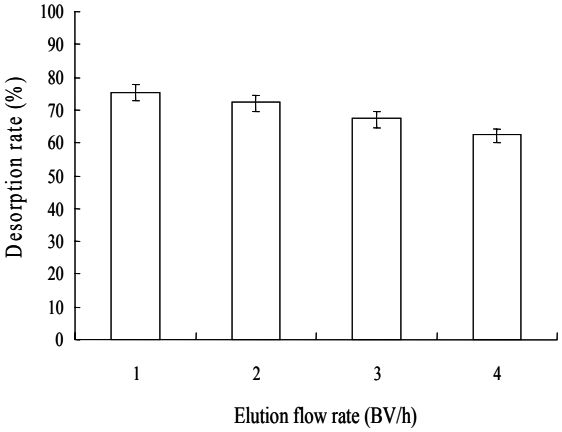

Fig. 4: Effect of flow rate of eluting solvent on the desoption rate of tea polyphenols.

Regeneration of Polyamide

After use of several times, the separation efficiency of polyamide resin decreased and it should be treated for regeneration. Therefore, the column packed polyamide resin was eluted by 5-10% NaOH solution for 2 BV firstly, and then eluted by distilled water until the pH value of the elution neutral. The regenerated polyamide was examined for the separation of tea polyphenols, it can be seen that the purity of tea polyphenols obtained in each time was higher than 95% (Table-2), while the purity or recovery between each time had no significance ($P > 0.05$). The results indicated that the reuse of polyamide resin was convenient, satisfied and provided many advantages such as saving materials, reducing production cost and avoiding the operation of reinstalling column.

Table-2: The recovery of tea polyphenols for regenerated polyamide resin.

Experiment times	Purity of tea polyphenols (%)	Recovery of tea polyphenols (%)
1	96.27 ± 1.78	72.42 ± 1.41
2	96.02 ± 0.66	71.89 ± 1.69
3	95.74 ± 1.89	70.24 ± 2.04
4	95.31 ± 1.64	69.86 ± 2.13
5	95.01 ± 1.07	69.44 ± 2.89

Separation of Theanine and Caffeine by Macroporous Resin Adsorption

The solution of tea extract was loaded onto the column packed with polyamide resin, after saturated adsorption, the column was eluted by water at a flow rate of 2 BV/h for 2 h. In the elution, caffeine, amino acids and soluble sugars were found to be the main compounds. The soluble sugars (mainly polysaccharides) could be removed by alcohol precipitation, while caffeine could be adsorbed by macroporous resin of NKA-II (Table-1). Therefore, NKA-II was investigated whether it was suitable for the separation of caffeine and theanine from the extract of summer green tea.

Dynamic Adsorption and Desorption of Theanine and Caffeine

In the separation process of caffeine and theanine, the elution from polyamide chromatography was loaded onto the column packed with NKA-II (Fig. 5). The column was eluted by pure water, and the concentrations of each component in the elution were determined. In the initial 2 BV washing with water, no caffeine was detected and theanine was eluted out partly, while other amino acids were eluted out thoroughly (Table-3). After that, theanine was eluted out gradually. After theanine was completely eluted out, the column was eluted by aqueous 80% ethanol. It can be seen that caffeine was eluted only partly in the initial 2 BV, after that caffeine was eluted out gradually until the 5th BV (Table-4). The results indicated that caffeine and theanine could be separated well by NKA-II resin chromatography. The method is worth for further study since there are few reports about the separation of caffeine and theanine by macroporous adsorption chromatography [24].

Table-3: The components in water elution of NKA-II column.

Elution volume (BV)	Caffeine (mg)	Soluble sugars (mg)	Theanine (mg)	Amino acids except theanine (mg)
0.5	0	0.75 ± 0.04	0.62 ± 0.03	5.08 ± 0.31
1	0	1.42 ± 0.06	1.44 ± 0.08	17.53 ± 0.44
1.5	0	0.24 ± 0.01	3.85 ± 0.22	7.26 ± 0.06
2	0	0	5.78 ± 0.29	1.32 ± 0.02
2.5	0	0	10.68 ± 0.54	0
3	0	0	7.32 ± 0.42	0
3.5	0	0	3.72 ± 0.18	0
4	0	0	0.87 ± 0.04	0

Table-4: The components in 80% ethanol elution of column NKA-II.

Elution volume (BV)	Tea polyphenols (mg)	Caffeine (mg)
0.5	0.82 ± 0.44	4.76 ± 0.32
1	4.42 ± 0.31	9.04 ± 0.59
1.5	1.67 ± 0.04	29.72 ± 1.53
2	0.43 ± 0.02	42.64 ± 2.89
2.5	0	68.57 ± 3.55
3	0	40.98 ± 2.69
3.5	0	27.72 ± 0.94
4	0	10.68 ± 1.01
4.5	0	3.71 ± 0.11
5	0	0.93 ± 0.03

Regeneration of NKA-II

After several times use for the separation of theanine and caffeine, NKA-II was regenerated by eluted by ethanol for 2 BV and subsequently washed thoroughly by distilled water before its reuse. By use of the regenerated resin, the purity of theanine or caffeine obtained was higher than 98%, and the recovery between each time had no significance ($P > 0.05$, Table-5). The results indicated that the reuse of NKA-II for separation of caffeine and theanine from the extract of summer green tea was satisfied.

Table-5: The recovery rates of theanine and caffeine for regenerated NKA-II

Experiment times	Purity of the anine (%)	Recovery of theanine (%)	Purity of Caffeine (%)	Recovery of Caffeine (%)
1	99.02 ± 0.43	66.12 ± 1.66	99.25 ± 0.36	62.07 ± 2.17
2	98.88 ± 0.66	65.77 ± 2.11	99.00 ± 0.29	61.17 ± 2.24
3	98.82 ± 0.87	65.42 ± 1.98	98.78 ± 0.61	61.02 ± 3.79
4	98.79 ± 0.51	65.21 ± 2.24	98.59 ± 0.46	60.42 ± 3.07
5	98.77 ± 0.36	64.97 ± 1.89	98.51 ± 0.22	60.22 ± 2.55

Preparation of Tea Polyphenols, Caffeine and Theanine by Column Chromatography

As shown in Fig. 5, the solution of tea extract (2 mg/mL, pH 3) was loaded onto the polyamide column at a flow rate of 2 BV/h. After saturated adsorption, the column was eluted by pure water for 2 h, and then eluted by 80% ethanol at a flow rate of 2 BV/h for 4 h. The elution of 80% ethanol (fraction 1) was collected for the preparation of tea polyphenols, while the elution of pure water was collected, concentrated, precipitated by ethanol and loaded onto the NKA-II column. The column was eluted by pure water firstly, and the elution from 2.5th BV to 4th BV (fraction 2) was collected. After eluted by pure water, the column was eluted by 80% ethanol and the elution from 2.5th BV to 5th BV (fraction 3) was collected. The fractions 1-3 which were rich in tea polyphenols, theanine and caffeine, respectively, were concentrated under vacuum in a rotary evaporator and lyophilized, affording the products of tea polyphenols, theanine and caffeine (Fig. 6). The purities of resulting tea polyphenols, theanine and caffeine were 96.27 ± 1.78%, 99.02 ± 0.43% and 99.25 ± 0.36%, respectively.

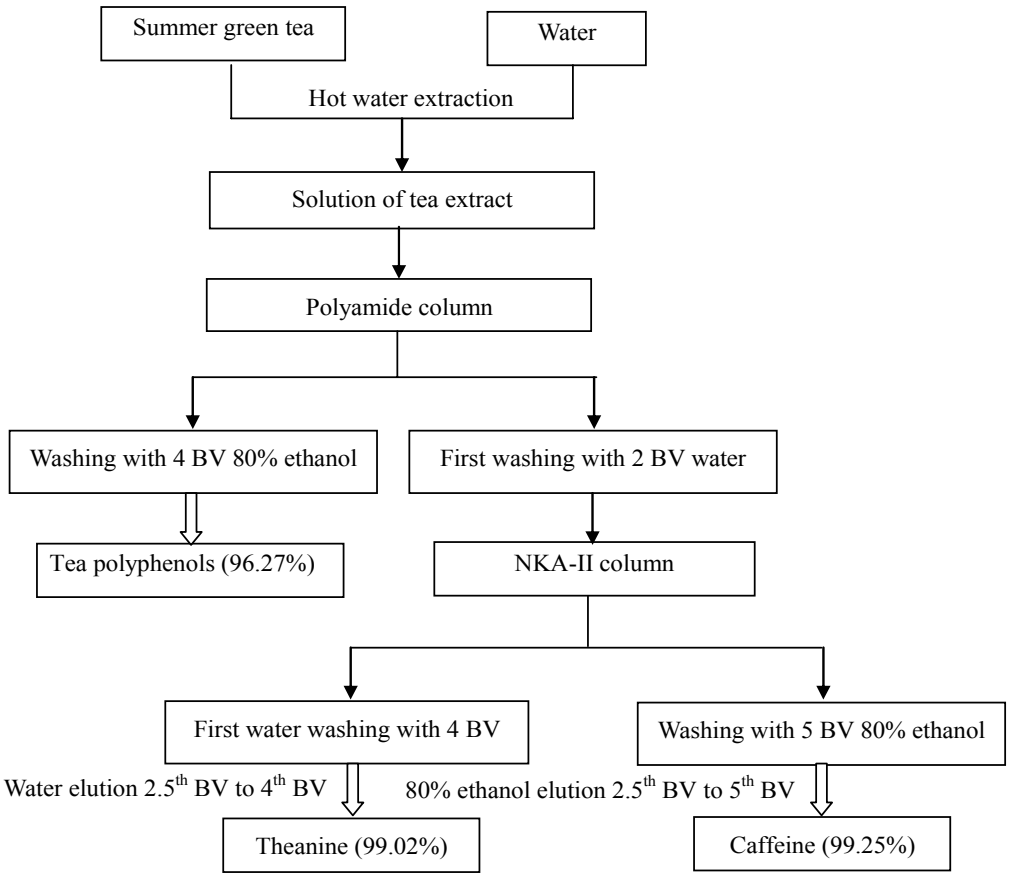

Fig. 5: Diagram of separation of tea polyphenols, theanine and caffeine from summer green tea by column chromatography.

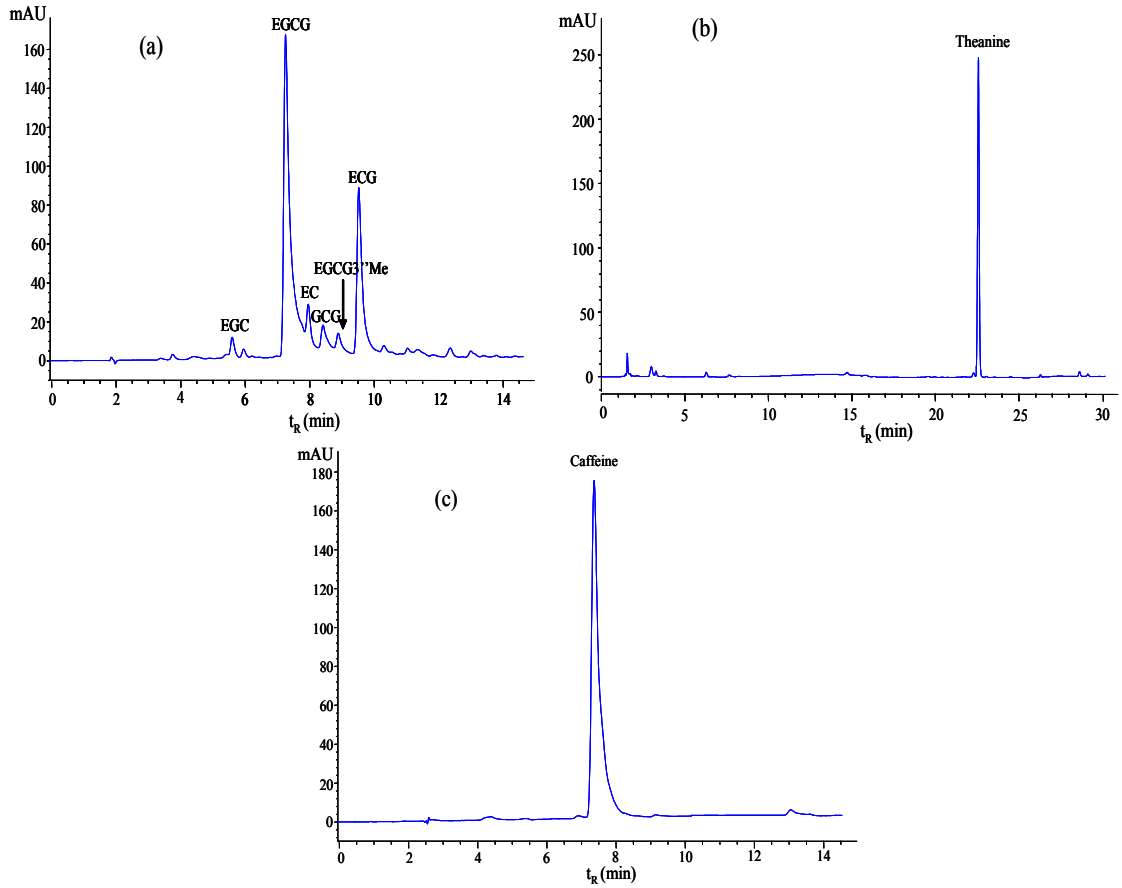

Fig. 6: HPLC chromatograms of products of tea polyphenols (a), theanine (b) and caffeine (c) separated from the extract of summer green tea by column chromatography.

Nowadays, macroporous resin adsorption technology is gaining popularity in pharmaceutical applications and has also been used for the separation of natural active components. However, there are few reports about a combined use of resins for the simultaneous separation and purification of tea bioactives. Although a column-chromatographic extraction followed by sequential adsorption to extract and separate bioactive compounds from green tea was relative convenient [24], it also had some shortcomings. For the separation and purification of three kinds of bioactive compounds, different resins were used, which may led to the loss of raw materials in the purification process; while for theanine, although 732 cation exchange resin had separation effect, it had many disadvantages such as pH value should be adjusted with phosphate buffer for adsorption, and ammonia water was used for desorption, which not only increase operation steps, but also lead to impurities. In our study, only by one special resin, caffeine and theanine could be separated effectively. These results indicated the developed method by sequential use of polyamide chromatography and NKA-II chromatography was simple, convenient and could be used to prepare high-purity of tea polyphenols, caffeine and theanine from the extract of summer green tea.

Experimental

Materials and Reagents

Gallic acid, glycine and caffeine were obtained from Sigma Chemical Co., Ltd (St. Louis, MO, USA). Folin-Ciocalteu reagent was purchased from Fluka (Buchs, Swiltzerland). Standards of EGC, EGCG, EC and ECG were purchased from Funakoshi Co., Ltd (Tokyo, Japan). Theanine was got from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Standards of GCG and (-)-epigallocatechin 3-*O*-(3-*O*-methyl) gallate (EGCG3''Me) were prepared according to our reported method [25, 26]. All solvents used for chromatographic purposes were HPLC grade. Resins of AB-8, D-101, S-8, HP-20, D-

3520, DM-130, NKA-II were purchased from Hefei Sanxing Resin Technology Co., Ltd. (Anhui, China), and polyamide was the product of Wuxi Linjiang Resin Technology Co., Ltd. (Jiangsu, China). The summer green tea, made from the leaves of *Camellia sinensis* [L.] O. Kuntze in August, 2010, was kindly provided by Jingshan Tea Factory (Hangzhou, China). The sample was ground into powder using a milling machine and the material that passed through a 40-mesh sieve was kept in sealed polyethylene bags at -20℃ until use.

Preparation of Tea Infusion

The extract of summer green tea was prepared according to the reported method by using distilled water as extraction solvent [14]. Briefly, 10.0 g of tea powder was placed in an amber glass bottle vial with 160 mL of distilled water, and then placed in a reciprocal shaking water bath at a speed of 100 rpm at 96℃ for 40 min. Upon completion of extraction, the extract was filtered through paper filter, and the insoluble residue was treated again as mentioned above. The extracts were combined and stored at -20℃ until use.

Analysis of Bioactive Components in Tea Infusion

The contents of tea catechins and caffeine were determined by HPLC using an Agilent 1100 series HPLC (Agilent Technologies, USA) consisted of a model G1379A degasser, a model G1311A pump, a model G1316A column oven and model G1315B diode array detector (DAD) [25]. The separation was achieved on a TSKgel ODS-100Z column (150 × 4.6 mm, 5 μm, Tosoh, Japan). The temperature of column oven was set at 40℃. The mobile phase consisted of formic acid solution (pH 2.5, A) and methanol (B). Elution was performed with a linear gradient as follows: 0-15 min, A from 82 to 40%. The flow rate was set at 1.0 mL/min. The injection volume was 20 μL. Calibration plot was constructed with authentic caffeine or tea catechin by plotting peak areas from the DAD absorbance at 280 nm versus standard concentrations.

The content of tea polyphenols was determined by Folin-Ciocalteu method according to the reported procedure [27, 28], and the content of tea polyphenols in extract was standardized against gallic acid and expressed as milligram gallic acid equivalent per gram of sample.

The content of theanine in the extract was determined by HPLC according to the reported method [29]. The separation was achieved on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm, Agilent, USA). The temperature of column oven was set at 40℃. The mobile phase consisted of methanol/acetonitrile/water (45/45/10, A) and phosphate buffer (pH 7.5, B), and elution was performed with a linear gradient as described in the literature [29]. The flow rate was 1.0 mL/min. The injection volume was 20 μL. Calibration plot was constructed with authentic theanine by plotting peak areas from the DAD absorbance at 338 nm versus standard concentrations.

The content of soluble sugars was determined by the anthrone-sulfuric acid assay using glucose as a standard [30]. The amount of total free amino acids was determined with acid ninhydrin reagent, and glycine was used as a standard [31].

Static Adsorption and Desorption Tests for Screening of Resins

All resins were pretreated according to the manufacturer's specifications before use, and they were screened through static adsorption tests which were done as follows: the mixture of resin (1 g) and solution of tea extract (30 mL) was shaken mechanically (120 rpm) at 25□ for 24 h. After adsorption was complete, the resin was desorbed with 80% ethanol (30 mL) by using a mechanical shaker (120 rpm) at 25°C for 24 h. The adsorption capacity of each resin is calculated as follows:

$$Q = (C_o\text{-}C_e)V/W$$

where Q is the adsorption capacity (mg/g), C_o is the initial solution concentration of tea extract (mg/mL), C_e is the solution concentration of tea extract after adsorption equilibrium (mg/mL), V is the volume of adsorption solution (mL), and W is the weight of the resin (g). The rate of desorption resin is calculated as follows:

$$D\ (\%) = [C_dV_d/(C_o\text{-}C_e)V] \times 100$$

where D is the desorption rate, C_d is the concentration of desorption solution, V_d is the volume of desorption solution (mL), V is the volume of adsorption solution (mL), C_o, C_e and V are the same as those mentioned above.

Determination of Static Adsorption Kinetics of the Selected Resin

The mixture of resin (1 g) and solution of tea extract (30 mL) was shaken mechanically (120

rpm) at 25℃. During the shaking, sample (1 mL) was taken out every half an hour and its content of tea polyphenols was determined by Folin-Ciocalteu method.

Determination of Adsorption Isotherm of the Selected Resin

The tests for equilibrium adsorption isotherms on the selected resin were conducted by contacting 30 mL solutions of tea extract at different concentrations with pre-weighed 1 g resin and shaking mechanically (120 rpm) by a reciprocal shaking water bath at different temperatures. After adsorption equilibrium was reached, the content of tea polyphenols was analyzed.

Dynamic Adsorption and Desorption Tests on the Selected Resin

Dynamic adsorption and desorption experiments were carried out in glass columns wet-packed with 60 g of selected resin, and all the dynamic experiments were performed at room temperature. The feed rate was 2 bed volumes (BV) per hour, and the flow rate of elution was also 2 BV/h. After adsorbed, the column was washed with de-ionized water, and then washed with 80% ethanol solution. The content of desired bioactive in the elution was determined.

Statistical Analysis

Data were analyzed by using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Data belongs to the analysis of the content of bioactive components were expressed as mean ± standard deviation (SD). Any significant difference was determined by one-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons considering difference statistically at $P < 0.05$.

Conclusions

In the present study, the separation of tea polyphenols, theanine and caffeine from the crude extract of summer green tea was achieved by the use of polyamide and macroporous NKA-II resin. The purities of tea polyphenols, theanine and caffeine products were 96.27 ± 1.78%, 99.02 ± 0.43% and 99.25 ± 0.36%, respectively. The separation procedure allowed the production of such products by decreasing impurities and avoiding the use of poisonous organic solvent. The results suggested that it was possible to produce high-quality products of rich-in tea polyphenols, theanine and caffeine by a simple way. From industrial point of view, this novel method has many merits such as high efficiency of separation, low consumption of energy and environment-friendly procedure.

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