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Full Length Research Paper

Determination of cyclic oligomers residues in tea catechins isolated by polyamide-6 column

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Cyclic oligomers ranging from tetramer to octamer and a tetramer plus $-NH(CH_2)_2CH_3$ in ethanol extract of polyamide-6 (PA) were identified by HPLC/MS and MS/MS. An HPLC method was developed to detect the residues of the cyclic oligomers in tea catechins isolated by PA column. It showed that catechins had interference on HPLC analysis of the cyclic oligomers, but the interference could be eliminated using polyvinylpolypyrrolidone as an adsorbent to remove catechins in the sample. It is found that pretreatment of PA using 95% (v/v) ethanol was an effective method to eliminate cyclic oligomers residues in the final products isolated by PA column.

Key words: Tea catechins, polyamide-6, cyclic oligomer, residue, high-performance liquid chromatography (HPLC), mass spectrometry (MS).

INTRODUCTION

Tea catechins, a group of polyphenols in tea leaf, are a promising raw material for functional foods and medicines owning their health benefits and functionalities such as antioxidation and radical scavenging activity (Liang et al., 2007). Polyamide-6 (PA), an artificial polymer, is increasingly used as an adsorbent in production of tea catechins owing to its great affinity to polyphenols and excellent physical properties (Aharoni, 1997; Weidner et al., 2004; He et al., 2006; Wang et al., 2009). As a polycondensate of caprolactam, linear and cyclic oligomers are inevitably mixed into PA in the process of PA manufacture (Mengerink et al., 2000) though many measures are exerted to remove oligomers, such as hot-water extraction and vacuum heating (Chen et al., 1997). It is reported that caprolactam acts as clastogenic agents and possibly as aneuploidogenic agents, increasing the frequency of genotoxic bioindicators and inducing cell death via apoptosis (Ochoa-Olmos et al., 2009).

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The broader use of PA in separation and purification of polyphenols may present a potential risk to consumers if PA oligomers residues enter the final product. There is still no report on this topic, which makes the products safety problems in suspense. It is necessary to develop a method to detect oligomers residues in the final products isolated by PA column.

Many methods have been used determine PA oligomers, including thin layer chromatography (Belenky et al., 1976), gas chromatography (Mori et al., 1970), size exclusion chromatography (SEC) (Zhang et al., 2007) and high-performance liquid chromatography (HPLC) (Mengerink et al., 2002; Tran and Doucette, 2006). HPLC is regarded as a routine method to determine trace chemicals qualitatively and quantitatively. Mengerink et al. (2000) developed a sandwich injection HPLC method to detect linear and cyclic oligomers of PA. However, its sample preparation was time consuming and sophisticated, which makes it not suitable for determination of PA oligomers in natural products.

In this paper, an HPLC method was developed to detect cyclic oligomers of PA in tea catechins and a pretreatment procedure of PA was investigated so as to eliminate the residues of cyclic oligomers of PA in final products.

MATERIALS AND METHODS

Polyamide-6 (PA, 0.25 to 0.60 mm in diameter) was purchased from Luqiao Sijia Biochemical Plastic Company (Taizhou, China). Green tea was supplied by the CinoTea CO., Ltd. (Hangzhou, China). Chemical purity grade ethanol was purchased from Sinopharm Chemical Reagent CO., Ltd. (Beijing, China).

Polyvinylpolypyrrolidone (PVPP) and HPLC reference compounds of catechins and caffeine was Sigma products (Sigma-Aldrich, St. Louis, USA). The other chemical reagents used were of HPLC grade (Jinmei Biotech Corporation, Tianjin, China). The water used in the tests was prepared by an EASYPure II UV-UltraPure Water System (Barnstead International, Dubuque, IA, USA).

Extraction of PA oligomers

To obtain sample of oligomers from PA, 10 g PA was refluxed in a soxhlet apparatus with 150 ml ethanol at 60°C for 10 h. The obtained ethanol solution was evaporated and then dried under reduced pressure at 60°C. The dried solid sample was weighted and dissolved in ethanol at concentration of 1.0 mg ml^{-1}. This solution was used to identify cyclic oligomers by HPLC/MS and MS/MS, respectively.

Identification of cyclic oligomers of PA

In order to find a suitable detection wavelength for HPLC analysis of oligomers, the aforementioned ethanol solution of PA oligomers was scanned from 190 to 1000 nm by a HP 8453 UV-Visible spectrophotometer (Agilent Technologies, CA, USA). It showed that the cyclic oligomers of PA had a strong absorbance peak at 202 nm. Thus, detection wavelength was fixed at 202 nm in the following HPLC tests.

Agilent 1100 Series LC/MS (Agilent Technologies, CA, USA) was used to identify cyclic oligomers of PA under the HPLC conditions as: injection volume 10 µL, phenomenex C_{18} column (4.6×150 mm), column temperature 35°C, linear gradient elution using acetonitrile as mobile phase from 20% (v) acetonitrile to 37% (v) acetonitrile in 25 min at a flow rate 1 ml min^{-1}. MS conditions were as: fragmentation voltage 100 V, scan range from 100 to 1000 atomic mass unit (amu).

An MS/MS triple quadrupole system Quattro Premier XE (Waters Corporation, Milford, USA) was applied to further identify structures of cyclic oligomers. Cyclic oligomers sample was analyzed by an electrospray ionization (ESI) technique in an ESI positive ion mode. Ion source was set up as follows: capillary voltage: 3000 V, ion source temperature: 110°C, extractor: 3.0 V and RF lens: 0 V, desolvation gas nitrogen at a flow rate of 500 L h^{-1} and temperature at 350°C. Argon was used as the collision gas. The cone voltage was 30 V and the collision energy was 20 eV. Triple quadrupole was set-up to daughter ion scan experiment, and the mass of parent ion was set at 453.5 and 510.3 m/z respectively.

Investigation of interference effect of tea catechins on HPLC of cyclic oligomers

Three gram of green tea was extracted with 150 ml boiling water for 10 min. After filtering through a Xinhua no.101 filter paper (Xinhua Paper Corporation, Hangzhou, China), 10 ml of the filtrate was mixed with 10 ml of aforementioned sample of oligomers. The mixture was divided into two portions (10 ml each). 0.8 g PVPP was added into one portion of the mixture and stirred for 30 min so as to remove tea catechins in the solution. The other mixed solution was used as a control. The two solutions were centrifuged at 5478 × g for 30 min at 4°C and the supernatants were injected into Shimadzu LC 2010A HPLC (Shimadzu Corporation, Kyoto, Japan) according to the aforementioned HPLC condition, and monitored by a Shimadzu SPD ultraviolet detector at 202 nm.

Effect of PA pretreatment on cyclic oligomers residues in catechins products

One kg of PA (0.25 to 0.60 mm in diameter) was pretreated by heating in 8 L 95% (v/v) ethanol at 70°C for 40 min. The ethanol solution was decanted and a fresh ethanol solution was replaced and heated at 70°C for another 40 min. The PA was washed with 8 L water to remove the ethanol. The ethanol–pretreated PA was naturally dried at room temperature and then packed into a glass column (6.6 cm i.d. × 55 cm). Another glass column (6.6 cm i.d. × 55 cm) was packed with unpretreated PA and washed with 8 L water. Each of the two columns was used to isolate tea catechins from tea extracts.

One kg of green tea leaf was extracted with 15 L water at 90°C for 40 min. Tea solution was filtered through two layers of gauze and then loaded onto the column packed with the aforementioned pretreated PA or unpretreated PA at a flow rate of 100 ml min^{-1}. The columns were rinsed with 6 L water and then eluted with 6 L of 80% (v/v) ethanol at flow rate of 35 ml min^{-1}. The ethanol eluate was collected and concentrated to 1 L under reduced pressure at 45°C, and finally dried by an OPD-8 laboratory spray dryer (Ohkawara Dryers CO., Ltd., Shanghai, China) at inlet temperature 190°C and outlet temperature 105°C. The catechins sample prepared by the unpretreated PA column was defined as catechins A and that prepared by pretreated PA column was defined as catechins B.

HPLC analysis of cyclic oligomers in catechins samples prepared by PA columns

To eliminate the interference of catechins on HPLC analysis of cyclic oligomers, the aforementioned catechins samples A or B (8 g each) was dissolved in 100 ml water, then mixed with 8 g PVPP and stirred for 30 min. The solution was centrifuged at 5478 × g for 30 min at 4°C, and the supernatant was injected into HPLC under previously described conditions.

Analysis of tea catechins and caffeine

In order to investigate the effect of PA pretreatment on the composition of the prepared catechins, HPLC was used to analysis the aforementioned catechins samples A and B. 0.2 g catechins sample was dissolved in 100 ml water and then injected into HPLC under conditions described by Liang et al. (2007): injection volume 10 µL, phenomenex C_{18} column (4.6 mm i.d.×150 mm), column temperature 28°C, mobile phase A = acetonitrile/acetic acid/water (6:1:193, v), mobile phase B = acetonitrile/acetic acid/ water (60:1:139, v), linear gradient elution from 70% (v) A to 15% (v) A during 0 to 33 min and followed by 15% (v) A from 33 to 38 min at a flow rate 1 ml min^{-1}. The eluate was monitored by a Shimadzu SPD ultraviolet detector at 280 nm (Ye et al., 2009).

Data analysis

All the tests described in this paper were carried out in triplicate and mean values of the triplicate tests were presented in the present paper.

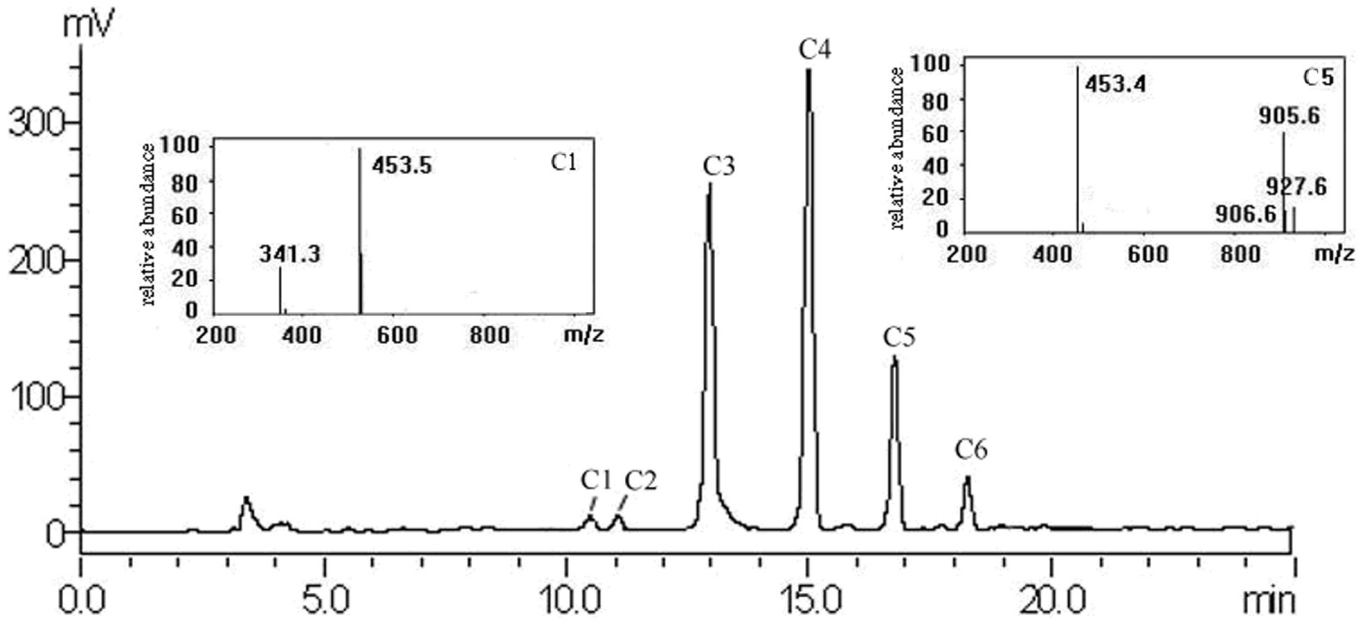

Figure 1. HPLC/MS profile of cyclic oligomers in ethanol extract of PA. C1, tetramer; C2, pentamer; C3, hexamer; C4, heptamer; C5, octamer; C6, a tetramer plus -$NH(CH_2)_2CH_3$.

Table 1. Polyamide adducts with corresponding m/z values.

C_n [a]	m/z	Disassembly	Symbol [b]	Molecular formula
C_1	453. 5	4×113+1	$[M_4+H]^+$	$[C_{24}H_{44}N_4O_4+H]^+$
C_2	566. 4	5×113+1	$[M_5+H]^+$	$[C_{30}H_{55}N_5O_5+H]^+$
C_3	679. 4	6×113+1	$[M_6+H]^+$	$[C_{36}H_{66}N_6O_6+H]^+$
C_4	792. 5	7×113+1	$[M_7+H]^+$	$[C_{42}H_{77}N_7O_7+H]^+$
C_5	905. 6	8×113+1	$[M_8+H]^+$	$[C_{48}H_{88}N_8O_8+H]^+$
C_6	510.3	4.5×113+1	$[M_{4.5}+H]^+$	$[C_{27}H_{53}N_5O_4+H]^+$

[a] C_n, the corresponding peak number in Figure1, [b] M, caprolactam, 113 atomic mass unit (amu).

RESULTS AND DISCUSSION

Identification of cyclic oligomers by HPLC/MS and MS/MS

(Figure 1) shows that a good HPLC resolution was obtained under the HPLC conditions: linear gradient elution from 20% (v) acetonitrile to 37% (v) acetonitrile during 25 min at column temperature 35℃. Six peaks of cyclic oligomers were separated in HPLC profile of ethanol solution of ethanol extract of PA and the adducts with corresponding m/z values were shown in (Table 1). According to MS data and reported data (Mengerink et al., 2000; Tran and Doucette, 2006), these peaks were identified as the protonated cyclic oligomers consisting of caprolactam repeating unit $[M_n+H]^+$. Caprolactam is a cyclic molecule with 113 atomic mass unit (amu). Peak C1 displayed a protonated molecular ion peak at m/z 453.5, which was corresponding to the tetramer of caprolactam plus H^+, viz. $[M_4+H]^+$. C2 was a pentamer plus H^+ at m/z value of 566.4 $[M_5+H]^+$. Accordingly, C3 corresponded to protonated hexamer $[M_6+H]^+$, C4 to protonated heptamer $[M_7+H]^+$ and C5 to protonated octamer $[M_8+H]^+$. Peak C6 had a special m/z value at 508.9, which was considered to be built up with four molecules of caprolactam and a branch of -$NH(CH_2)_2CH_3$. This oligomer type has not been reported and might be a new species of oligomer in PA. To investigate the structure of cyclic oligomers, MS/MS tests were conducted to determine the fragmentation pathways of tetramer and tetramer plus -$NH(CH_2)_2CH_3$, and MS/MS spectra were shown in (Figure 2.) The splitting pathway for tetramer described in (Figure 3) was consistent with the MS/MS spectrum of tetramer (Figure 2 a). Fragmentations observed in MS/MS spectrum of the ion at m/z 510 derived from the fragmentations of the ion at m/z 453 by shifting to a higher mass of 57 amu, the

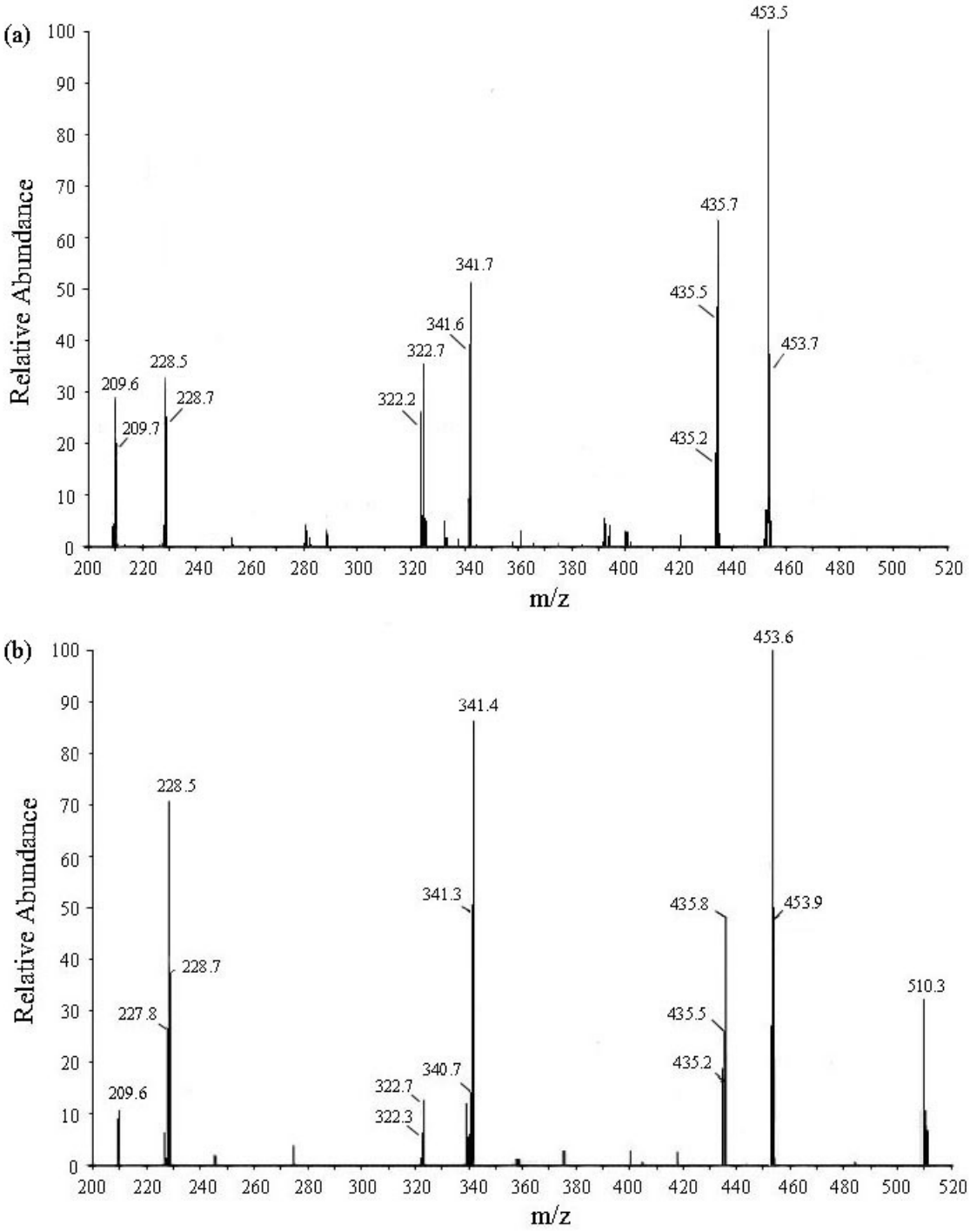

Figure 2. MS/MS spectra. (a) protonated tetramer $[M4+H]^+$ at 453.5 amu, (b) protonated tetramer plus $-NH(CH_2)_2CH_3$ $[M4.5+H]^+$ at 510.3 amu.

mass of $-NH(CH_2)_2CH_3$, suggesting that C6 was made up of four molecules of caprolactam and a branched chain $-NH(CH_2)_2CH_3$. Interpretation of these fragmentation spectra provides evidences to support the identification of these compounds as cyclic oligomers of PA.

Monomer, dimmer and trimer reported in previous researches (Mengerink et al., 2000; Klun et al., 2001) were not detected in the present sample, which might be attributed to the different sources of PA. In our study, PA was purchased from market, and monomer, dimmer and trimer might be removed due to the distillation procedure of industrial PA manufacture, while self-made PA without

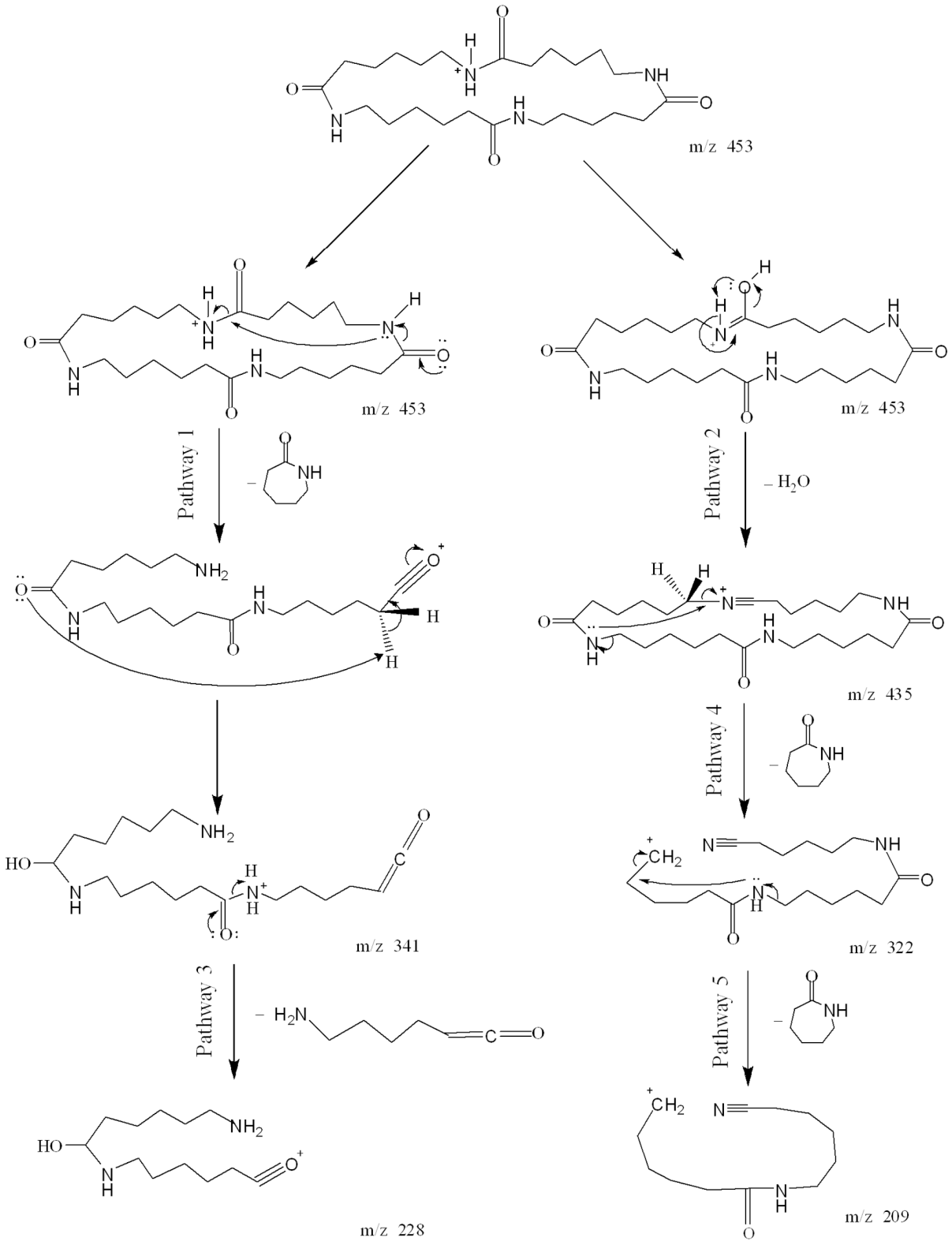

Figure 3. Fragment ions of tetramer.

distillation procedure was used in the reported studies (Mengerink et al., 2000; Klun et al., 2001). Polymerization manner is another factor resulting in the difference between oligomers of commercial PA and self-made PA.

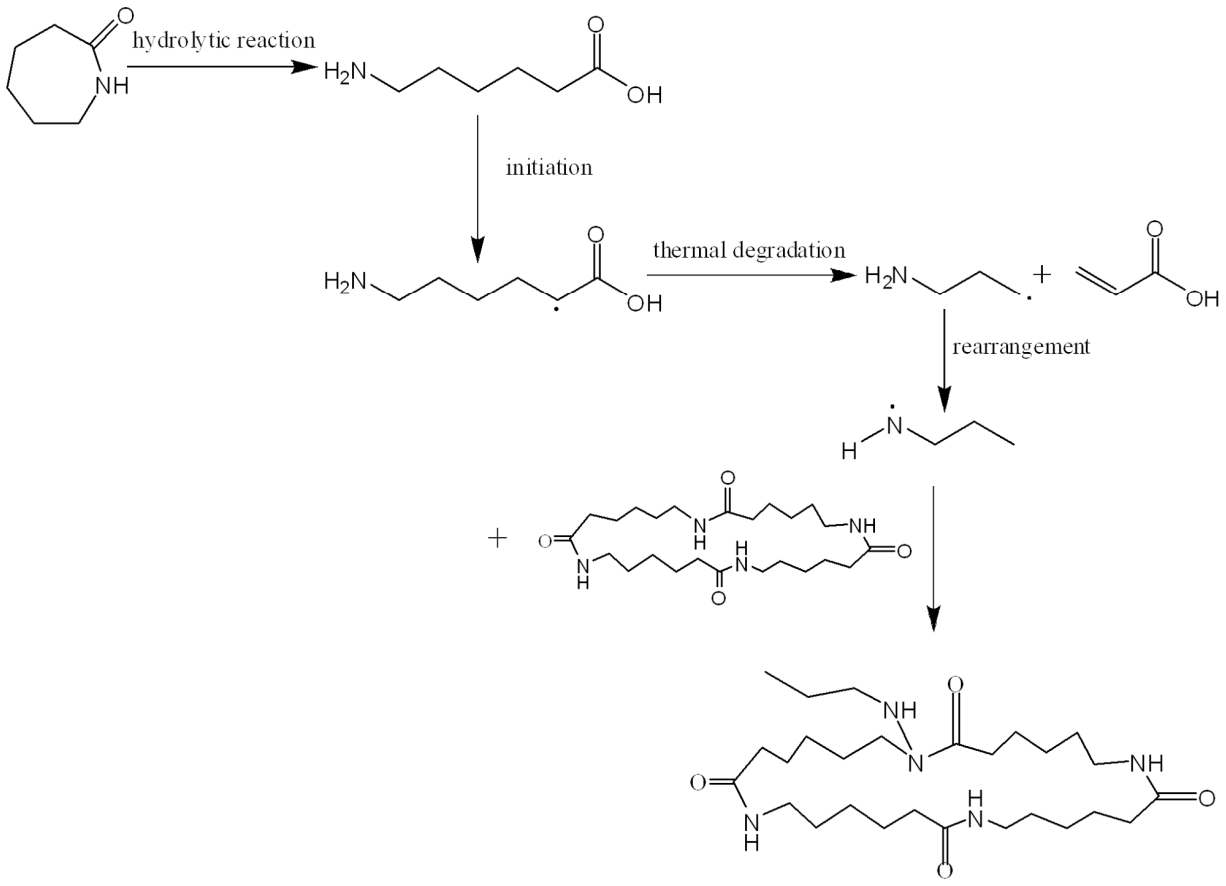

Figure 4. The deduced formation process of tetramer plus -$NH(CH_2)_2CH_3$.

Melt polymerization is the major reaction during commercial PA manufacture which was carried out at 200 to 300°C. This might be the cause of the new oligomer species discovered in commercial PA. The formation process of tetramer plus -$NH(CH_2)_2CH_3$ was deduced in (Figure 4). Firstly, caprolactam was hydrolyzed to 6-aminocaproic acid, and the α-H in 6-aminocaproic acid, viz. the adjacent H of carbonyl group, was initiated under the melt state owing to its great reactivity. Secondly, the initiated 6-aminocaproic acid broke into two parts that is, $CH_2(CH_2)_2NH_2$ and $CH_2CHCOOH$ due to thermal degradation, which was an inevitable side reaction accompanying condensation polymerization of caprolactam under high temperature circumstance. Thirdly, rearrangement of free radical in $\cdot CH_2(CH_2)_2\ NH_2$ was an indispensable process in consideration of the product stability. The retention time of the new oligomer species was later than that of tetramer (Figure 1), suggesting the polarity of tetramer was weakened in the presence of the branch. The $\cdot CH_2(CH_2)_2\ NH_2$ was then transformed into $\cdot NH\ (CH_2)_2CH_3$, since -$NH(CH_2)_2CH_3$ is an alkyl branch which weakened the polarity of tetramer molecule. Finally, the rearranged free radical part $\cdot NH\ (CH_2)_2\ CH_3$ reacted with tetramer, resulting in a stable oligomer tetramer plus -$NH(CH_2)_2CH_3$. The polymerization in solution is a pathway for self-preparing PA in lab. Low temperature in solution polymerization inhibits the initiation of α-H in 6-aminocaproic acid and thermal degradation. This is the reason that tetramer plus -$NH(CH_2)_2CH_3$ corresponding to C6 is only detected in commercial PA.

Interference of tea catechins on HPLC analysis of cyclic oligomers

Figure 5a showed that catechins and caffeine were eluted before 12 min on HPLC profile, while pentamer (C2) had similar retention time with (-)-epicatechin gallate (ECg, peak h) and (+)-catechin gallate (Cg, peak i). It suggests that the catechins interfered the HPLC analysis of cyclic oligomers, and they should be removed before HPLC detection of cyclic oligomers.

Polyvinylpolypyrrolidone (PVPP) is an adsorbent of polyphenols which is commonly used in beer and beverage industry owing to its strong affinity to polyphenols. Tea catechins are a group of plant polyphenols. When PVPP was used to remove catechins

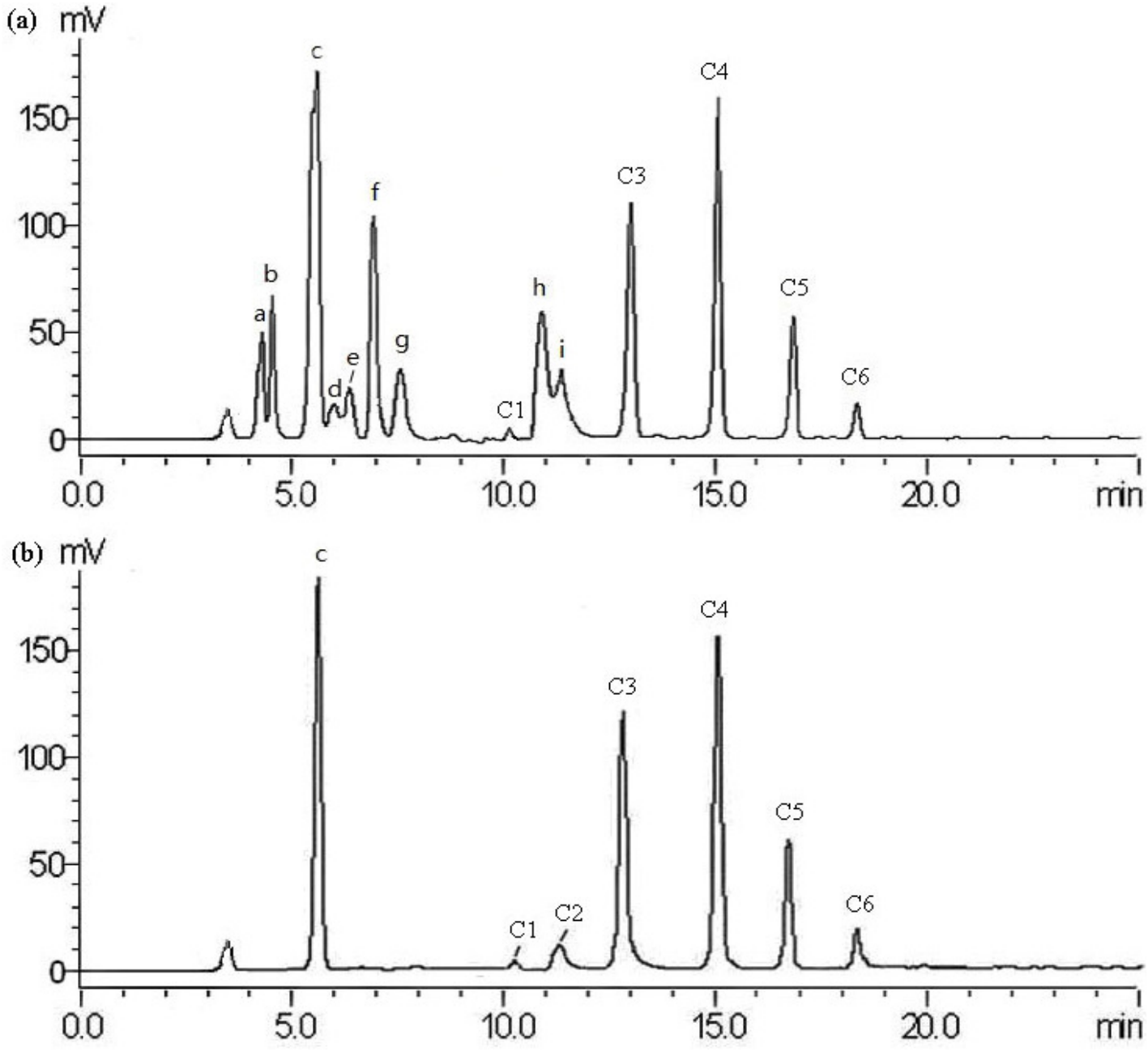

Figure 5. Effect of tea catechins on HPLC profile of cyclic oligomers from PA. a, a mixture of ethanol extract of PA, catechins and caffeine; b, ethanol extract of PA without catechins; C1, tetramer; C2, pentamer; C3, hexamer; C4, heptamer; C5, octamer; C6, a tetramer plus -$NH(CH_2)_2CH_3$. a, (+)-gallocatechin (GC); b, (-)-epigallocatechin (EGC); c, caffeine; d, (+)-catechin (C); e, (-)-epicatechin (EC); f, (-)-epigallocatechin gallate (EGCg); g, (+)-gallocatechin gallate (GCg); h, (-)-epicatechin gallate (ECg); i, (+)-catechin gallate (Cg).

from the mixture of cyclic oligomers and tea extracts, caffeine (peak c in Figure 5) was not affected and it was remained in the HPLC profile. However, caffeine had a different retention time from those of the detected cyclic oligomers and it had no interference on detection of the six species of cyclic oligomers (Figure 5b). Thus PVPP treatment is a effective method to eliminate the interference of catechins on HPLC analysis of residues of the cyclic oligomers in catechins samples.

Effect of PA pretreatment on residues of cyclic oligomers in catechins samples

Cyclic oligomers including hexame, heptamer, octamer and a tetramer plus -$NH(CH_2)_2CH_3$ and caffeine were identified in HPLC profile of catechins sample A isolated by unpretreated PA column (Figure 6a), but the cyclic oligomers were not detected in catechins sample B isolated by ethanol–pretreated PA (Figure 6b). (Table 2)

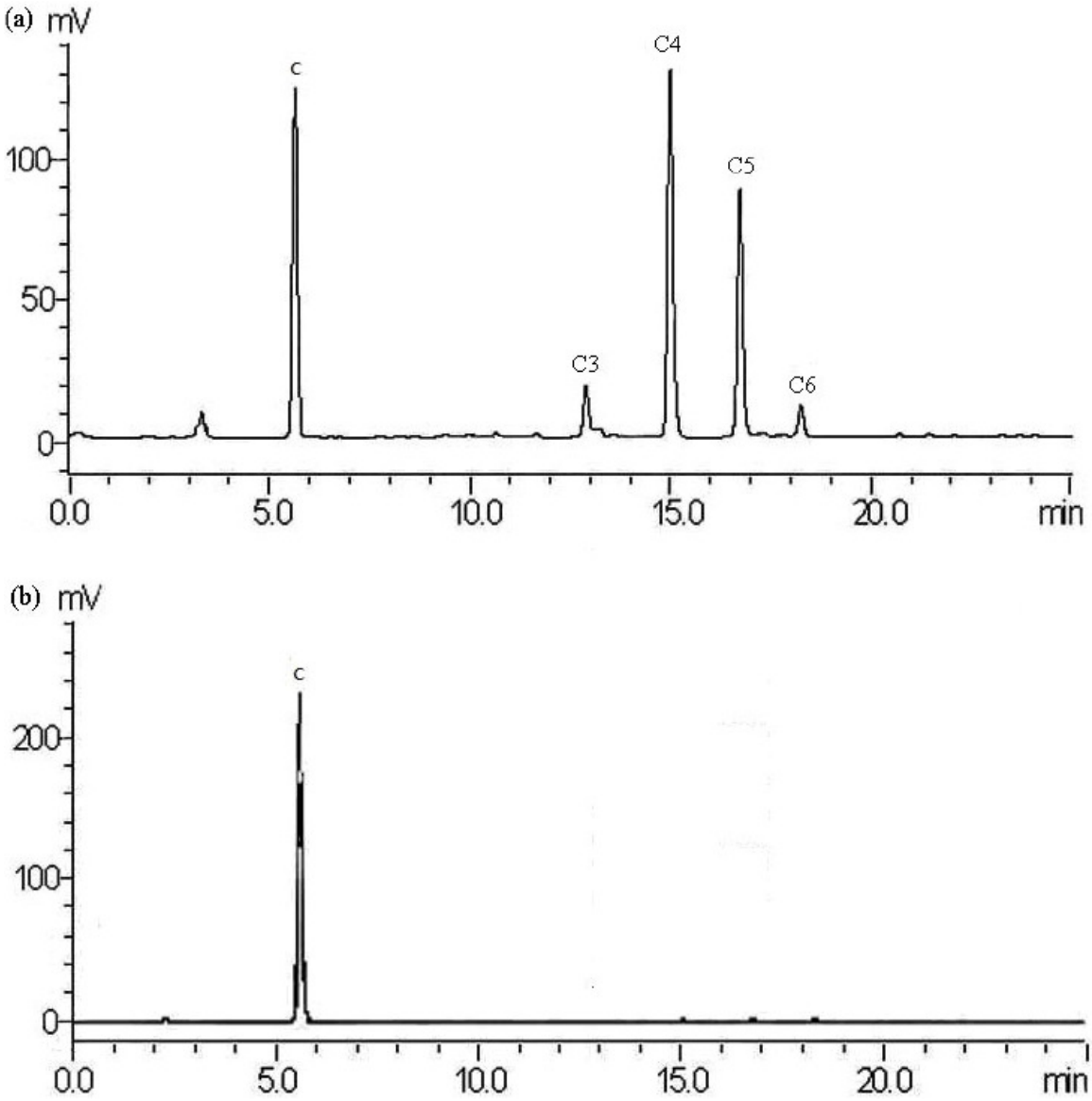

Figure 6. Effect of ethanol pretreatment on cyclic oligomers residues of PA in tea catechins. (a), catechins sample A prepared by unpretreated PA; (b), catechins sample B prepared by ethanol–pretreated PA. c, caffeine; C3, hexame; C4, heptame; C5, octamer; C6, a tetramer plus $-NH(CH_2)_2CH_3$.

showed that there was no significant difference in total catechins levels between catechins samples A and B. However, catechins sample B had higher levels of (-)-epigallocatechin gallate (EGCg), (+)-gallocatechin gallate (GCg) and (+)-catechin gallate (Cg), but lower levels of (+)-gallocatechin (GC), (-)-epigallocatechin (EGC), (+)-catechin (C) and (-)-epicatechin gallate (ECg). EGCg is a major component of physiologically functional compounds in tea (Sharma et al., 2005; Tran and Doucette, 2006; Chen et al., 2010). These results suggest that ethanol pretreatment could not only eliminate the residues of cyclic oligomers from PA in final product, but also increased the concentration of EGCg.

Conclusion

Cyclic oligomer species in ethanol extract of PA were identified by HPLC/MS, among which a new species of oligomer consisting of a tetramer and a branch of $-NH(CH_2)_2CH_3$ was detected. Residues of the cyclic oligomers were detected in catechins sample prepared by unpretreated PA, but the residues could be eliminated when the PA was pretreated by 95% (v/v) ethanol at 70°C for 40 min for twice. Tea catechins had interference on HPLC detection of the cyclic oligomers residues in tea catechins product. The interference was eliminated if the catechins sample was treated by PVPP before HPLC

Table 2. Compositions of tea catechins samples A and B (mg/g) [a].

Sample	GC	EGC	C	EC	EGCg	GCg	ECg	Cg	Total catechins	Caffeine
A	43.57±0.33	124.17±2.43	13.88±0.20	53.92±1.21	295.19±3.01	21.38±0.77	105.40±2.45	4.23±0.43	661.74±10.17	0.62±0.01
B	34.73±0.30	107.65±2.09	12.42±0.19	55.19±1.58	326.22±4.88	27.93±0.76	96.57±2.37	4.93±0.30	665.64±12.47	1.09±0.01

[a] sample A was prepared using PA without ethanol pretreatment and sample B was prepared using PA pretreated by ethanol. GC, (+)-gallocatechin; EGC, (-)-epigallocatechin; C, (+)-catechin; EC, (-)-epicatechin; EGCg, (-)-epigallocatechin gallate; GCg, (+)-gallocatechin gallate; Cg, (+)-catechin gallate.

injection of the sample.

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