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Short communication

One-step purification of epigallocatechin gallate from crude green tea extracts by isocratic hydrogen bond adsorption chromatography on β-cyclodextrin substituted agarose gel media

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Abstract

An oligomerized β-cyclodextrin ligand coupled to brominated allyl-group substituted Sepharose HP has been used for the one-step purification of polyphenolic epigallocatechin gallate (EGCG), an important antioxidant, by isocratic hydrogen bond adsorption chromatography. With a sample load of 1.33 mg crude green tea polyphenolic extract per ml column packing and with water/ethanol/acetonitrile (57/30/13, v/v) as the optimum mobile phase, an EGCG purity of about 98% with a recovery of approximate 73% could be achieved by proper peak cutting. After about 10 sample applications, the column performance started to deteriorate but could be regenerated to its original function by cleaning with 0.35 M NaOH.

Keywords: β-Cyclodextrin; Tea polyphenols; EGCG; Catechin

1. Introduction

Green tea is a popular beverage particularly in East Asia. Recent studies have shown that green tea contains components that possess antioxidant, antifatigue and anticarcinogenic activities [1]. The effects are ascribed to an abundance of polyphenols, mainly catechins, of which epigallocatechin gallate (EGCG) is the main effective component. Other investigations have shown that EGCG can inhibit cell adhesion of murine melanoma [2], increase the mitogenic effect on mouse splenic B cell [3] and increase the prostacyclin production of endothelial cells [4]. A mixture of tea polyphenols such as epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) is obtained by organic solvent extraction [5], and their separation is usually achieved by reverse phase chromatography [6,7]. Recently, the separations of catechins were reported using preparative HPLC [8,9] as well as high-speed countercurrent chromatography (HSCCC) [10].

In the present work, a new chromatographic method is introduced for the single step separation and purification of EGCG from a crude extract of green tea polyphenols using oligomerized β-cyclodextrin coupled to an agarose gel via a brominated allyl-group. In a previous report, this adsorbent was shown to be useful also for the purification of puerarin [11]. The major advantages of the agarose based gel are its high chemical stability, allowing regeneration using alkali and a low column back pressure allowing easy scaling-up for preparative applications.

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2. Experimental

2.1. Reagents

Standard EGCG with a purity higher than 99% was purchased from Sigma. Crude green tea polyphenol extract was obtained from Shenlong Bioproduct Company, Guangxi, China. Double distilled water was used in all chromatographic separations and HPLC analyses. All other reagents were of analytical grade and obtained from Beijing Chemical Reagent Company, Beijing.

2.2. Equipment

The pump LP-20C was obtained from Beijing Xinda Company; the UV detector 8823A from Beijing New Technology Application Institute and the data system N2000 from Zhejiang University, Hangzhou, China. The motor valve MV-7, the glass chromatography columns and Sepharose HP (base matrix) were obtained from GE Healthcare Bio-Science, Uppsala, Sweden. Allyl-substituted Sepharose HP and oligo-β-cyclodextrin coupled Sepharose HP were prepared in our own laboratory and packed in 10 mm i.d. columns with lengths 300 and 380 mm, respectively. HPLC analysis of the green tea polyphenols was carried out using a chromatography system from Alltech Company, USA.

2.3. Synthesis of oligo-β-cyclodextrin coupled Sepharose HP

The synthesis of the oligo-β-cyclodextrin ligand and its coupling to brominated allyl-group substituted Sepharose HP base matrix was described in detail in reference [11].

2.4. Sample preparation

For optimization of the separation conditions, 400 mg crude green tea polyphenol extract was dissolved in 200 ml mobile phase. One milliliter samples were injected into the columns. For the study of column loading capacity, sample concentrations up to 40 mg/ml were used. Prior to injection, all sample solutions were clarified by microfiltration using a 0.45 μm syringe filter supplied by Xinya Equipment Company, Shanghai, China.

2.5. Separation of green tea polyphenols

Separation of green tea polyphenols was investigated in isocratic adsorption chromatographic mode at a mobile phase flow-rate of 1 ml/min unless specified otherwise. The column effluents were monitored at 280 nm and the chromatograms were recorded by the N2000 data system from Zhejiang University, Hangzhou, China.

2.6. HPLC analysis of green tea polyphenols

The crude green tea polyphenols extract and the eluted peaks from the chromatography experiments were analyzed using the Alltech HPLC system equipped with a 250 mm × 4.6 mm RP C18 column from Beijing Analytical Instrument Apparatus Factory, Beijing, China. The mobile phase was composed of water/acetonitrile/ethylacetate (86/12/2, v/v) adjusted to pH 3–4 with concentrated sulfuric acid. The flow-rate was 1 ml/min and the UV absorbance of the column effluents was monitored at 280 nm.

2.7. MS analysis of the separated components

ESI/APCI–MS (LCQ Deca XP, Thermo Finnigan, US San Jose) was performed with the positive or negative ion measurement mode with a spray voltage of 3.5 kV, a Sheath gas of 35 arb, an aux gas of 0 arb and a capillary temperature of 275 °C. Full scan spectra were obtained by scanning masses between *m*/*z* 200 and 1000 at 4 s/scan.

3. Results and discussion

3.1. Purification of EGCG on oligo-β-cyclodextrin coupled Sepharose HP

Fig. 1 shows the main constituents of green tea polyphenols of which EGCG represents approximately 13%. The composition of the mobile phase strongly affects the resolution of EGCG on the oligo-β-cyclodextrin coupled Sepharose HP column. Different concentrations of ethanol and acetic acid and mixtures of these solvents were tested, but no satisfactory separation was obtained (data not shown). However, the addition of acetonitrile to the water/ethanol mixture both decreased the retention time and increased the EGCG resolution. The optimum water/ethanol/acetonitrile composition is 57/30/13 (v/v) and in Fig. 2 is shown the corresponding chromatographic profile in which peak 2 contains the EGCG.

In Table 1 is shown the effect of sample loading on the resolution between EGCG and its closest neighbour (peak 1 in Fig. 2) using the optimum mobile phase composition. An increase in the sample loading to 1.33 mg/ml column packing (i.e. 40 mg on a 30 ml column) resulted in the chromatogram shown in Fig. 3. After appropriate peak cutting (ranging from

Table 1
Effect of the green tea polyphenolic extract sample load on the resolution between EGCG and the closest neighbouring peak (see Figs. 2 and 3, respectively) using the oligo-β-CD coupled Sepharose HP column

Sample load (mg/ml gel)	Resolution
0.27	1.14
0.89	0.76
1.33	0.65

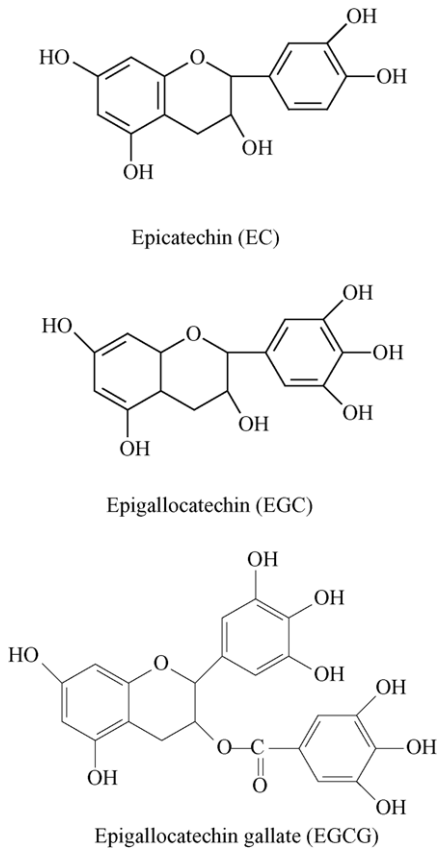

Fig. 1. The molecular structures of epicatechin (EC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG).

"A" to "B"), an EGCG fraction with a purity of 98% and with a recovery of 73% (3.8 mg) could be collected.

3.2. Column cleaning and regeneration

After about 10 cycles, the separation efficiency starts to decline due to column fouling. Different solvents and reagents such as acetone, methanol, ethanol, acetic acid and NaOH were investigated for the regeneration of the gel. The best result was obtained using 0.35 M NaOH followed by distilled water, 30% acetic acid and 20% ethanol (one column volume of each solution at a flow-rate of 1 ml/min). The column is stored in 20% ethanol.

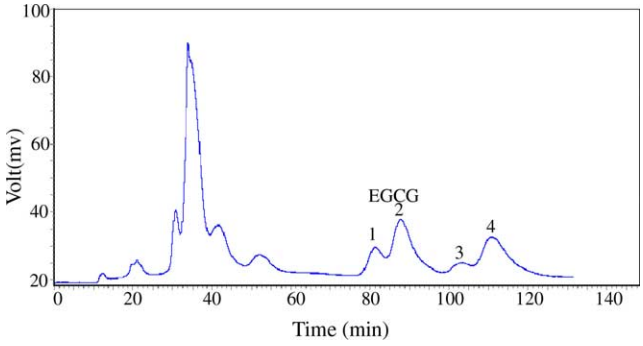

Fig. 2. Isocratic hydrogen bond adsorption chromatography of 2 mg green tea polyphenols on a 30 ml (380 mm × 10 mm) oligo-β-cyclodextrin coupled Sepharose HP column. Mobile phase: water/ethanol/acetonitrile (57/30/13, v/v). Sample volume: 1 ml.

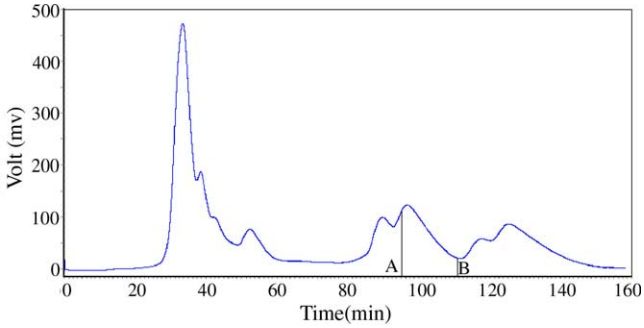

Fig. 3. Isocratic hydrogen bond adsorption chromatography of 40 mg green tea polyphenol extract on a 30 ml (380 mm × 10 mm) oligo-β-cyclodextrin coupled Sepharose HP equilibrated in water/ethanol/acetonitrile (57/30/13, v/v). Sample volume: 1 ml.

3.3. Mass spectrometry analysis of the separated fractions

Mass spectra were obtained of standard EGCG (458 Da) and of samples from peaks 1, 2, 3 and 4 in Fig. 2 (data not shown). The mass spectra were obtained in positive mode. Reference EGCG gave a pseudo-molecular ion $[M+H]^+$ at *m*/*z* 459 and so did the component of peak 2. The two samples also displayed identical UV absorbance spectra (data not shown). As also the retention times for peak 2 and reference EGCG were identical (87.5 min), it is tentatively safe to conclude that the component in peak 2 is EGCG.

The components in peaks 1, 3 and 4 in Fig. 2 were analyzed by ESI-MS in negative mode (data not shown). Their molecular weights are tentatively ascribed 478, 478 and 494 Da, respectively, according to the molecular ions $[M-H]^-$ obtained at 477, 477 and 493.

3.4. Interpretation of the retention behavior of EGCG on oligo-β-cyclodextrin coupled Sepharose HP and on related media

The starting material and intermediate used in the synthesis of oligo-β-cyclodextrin coupled Sepharose HP are Sepharose HP (base matrix) and allyl-substituted Sepharose HP, respectively. The retention behavior of EGCG on these media was tested using 30–50% ethanol, with and without the addition of various concentrations of acetonitrile, as mobile phase. No useful separation of EGCG was obtained on either column (data not shown).

β-Cyclodextrins are α-1–4-linked seven member cyclic oligomers of D-glucopyranose units with the shape of a hollow truncated cone in which the interior surface is relatively

Table 2
Effect of temperature on the retention time of the EGCG peak on the oligo-β-CD coupled Sepharose HP column

Temperature (°C)	Retention time (min)
0	261
10	172
15	127
20	104
25	88.2
30	65
35	53.5
40	47

Table 3
Effect of urea concentration on the retention time of the EGCG peak on the oligo-β-CD coupled Sepharose HP column

Concentration of urea (M)	Retention time (min)
0	53.5
1	44.5
3	43.0
5	38.5
8	29.0

The retention time analysis was performed at 35 °C in order to reduce the viscosity of the urea solutions and thus the pressure drop over the oligo-β-CD coupled Sepharose HP column.

hydrophobic compared to the hydrophilic hydroxyl group-containing rims. This unique geometry allows them to form inclusion complexes with guest molecules of appropriate size and shape [12,13]. By oligomerization using epichlorohydrin before coupling to the agarose gel, the ligand concentration of the β-cyclodextrin can be increased significantly.

In Tables 2 and 3 are shown the effect of temperature and urea concentration, respectively, on the retention of EGCG on oligo-β-cyclodextrin coupled Sepharose HP. The effect of both these parameters support the tentative interpretation that it is hydrogen bonding between the hydroxyl groups of the β-cyclodextrin moieties, and/or the ether bonds formed as a consequence of the cross-linking reaction between these, and the polyphenol groups of the EGCG that are responsible for the retention rather than hydrophobic interaction with the interior surface of the β-cyclodextrin. It is textbook knowledge that as the temperature decreases the strength of hydrophobic interaction decreases, a phenomenon opposite to that valid for hydrogen bonds. It is also well known that urea at high concentrations has the capacity to suppress hydrogen bond formation.

4. Conclusions

Oligo-β-cyclodextrin coupled to Sepharose HP was successfully used for the separation and purification of epigallocatechin gallate (EGCG) from crude green tea polyphenolic extract (GTP) with the solvent mixture water/ethanol/acetonitrile (57/30/13, v/v) as mobile phase at a flow-rate of 1 ml/min. In a preparative run with the optimal sample load 1.33 mg/ml gel, 98% EGCG could be obtained with the recovery of 73% in single operation step. The column could be restored to its original performance using a regeneration procedure encompassing regeneration with 0.35 M NaOH followed by water, 30% (v%) acetic acid and 20% (v%) ethanol.

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