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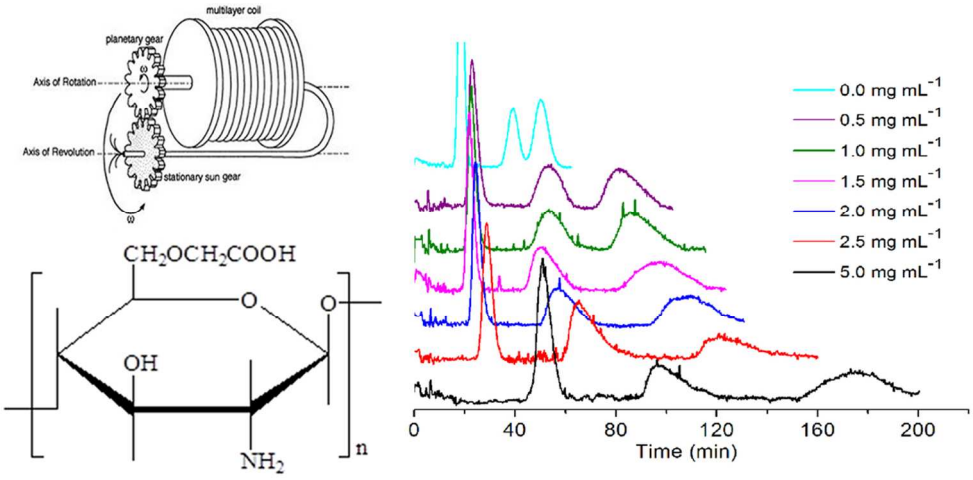

A novel additive, O-carboxymethyl chitosan, of High-speed counter-current chromatography was found and evaluated for the separation and purification of active constituents from natural plants.

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Use of O-carboxymethyl chitosan in High-speed counter-current chromatography: a novel additive of biphasic solvent system

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Abstract

A novel additive, O-carboxymethyl chitosan (O-CMC), of High-speed counter-current chromatography (HSCCC) was found and investigated. It was shown that O-CMC can improve peak resolution (*Rs*). The mechanism of O-CMC as solvent additive to improve resolution was studied using quercetin, luteolin and kaempferol as model analytes. It was indicated that O-CMC improved resolution not by increasing the retention of stationary phase but by introducing intermolecular forces: hydrogen bonding interaction and electrostatic interaction. Steviol glycosides (SGs) were separated by analytical type of HSCCC using biphasic solvent system composed of n-hexane/n-butanol/water at a volume ratio of 1.5:3.5:5 with O-CMC (1.0 mg mL^{-1}) in lower phase. HPLC analysis showed that acceptable separation of SGs was achieved by using O-CMC as solvent additive and the purities of ST, RC and RA were 61.5%, 97.5% and 74.1%, respectively.

1 Introduction

High-speed counter-current chromatography (HSCCC), which possesses a peculiar characteristic that allows no solid support for stationary phase and the separation of solutes in a biphasic solvent system based on different affinities for one or the other phase, eliminates irreversible adsorption in the solid support used in traditional column chromatography and HPLC[1,2]. HSCCC is a powerful preparative technique with its high loading capacity, low cost of stationary phase and low solvent consumption. It has been widely used in preparative separation of active compounds from traditional Chinese herbs and other natural products[3,4]. But it yields low efficiency compared to HPLC due to its relatively low theoretical plates and single separation mechanism. When confronted with two target compounds that have similar partition coefficient resulting in incomplete separation, it is very difficult to obtain good isolation through changing HSCCC chromatography condition[2]. On the other hand, separation of highly polar compounds using HSCCC is hampered by low retention of polar stationary phase[5]. Y. Zeng *et al* introduced a new series of biphasic solvent systems composed of n-butanol/ethanol/saturated ammonium sulfate solution/water at various volume ratios which can be used for separation of extremely polar compounds by HSCCC[6]. However, their novel ultra-polar solvent series were just appropriate for spiral column not multilayer column. Recently, the demand for improving separation efficiency has led to the development of multiple separation mechanisms of HSCCC.

D. Liu *et al* suspended 10 μm average diameter spherical cross-linked 12% agarose gel SuperoseTM 12 into the aqueous phase of an

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organic-aqueous biphasic solvent system, thus creating a liquid–liquid/solid three-phase system. In this way, liquid–liquid partition chromatography and liquid–solid adsorption chromatography were combined in HSCCC making it possible to use HSCCC to separate high-polarity polyphenols in one step[5]. The combination of HSCCC and reverse micelle solvent system was successfully applied to separate bromelain from pineapple fruit[7]. R.M. Liu *et al* successfully used ionic liquid as modifier of HSCCC biphasic solvent system for isolation and purification of chemical constitutes from O. indicum. This study clearly suggested that the partition of some polar compounds which distribute almost all in the aqueous phase could be improved effectively by addition of ionic liquid[8]. In addition, a preparative HSCCC method for isolation and purification of neomangiferin and mangiferin from *Rhizoma anemarrhenae* was successfully established by using ionic liquids as modifier of the biphasic solvent system[9].

Chitosan, a natural polymer obtained by alkaline deacetylation of chitin, is one of the most prominent natural macromolecular polysaccharide biopolymers with the advantages of biocompatibility, biodegradability and non-toxicity. However, this polymer was only soluble in an acidic environment in which the pH was less or if the order of the pKa value of chitosan (5.5–6.5), which restricted its application seriously[10]. When $–CH_2COOH$ groups are introduced onto –OH groups along the chitosan molecular chain, O-carboxymethyl chitosan (O-CMC) (Fig. 1) which is an important water soluble derivative of chitosan, can be obtained. O-CMC has been widely used in drug delivery[11,12], textile, food, papermaking and other fields[13]. To the best of our knowledge, O-CMC has not been used in separation and purification science until now. In this study, we used O-CMC as the additive of biphasic solvent system to improve the separation efficiency of HSCCC.

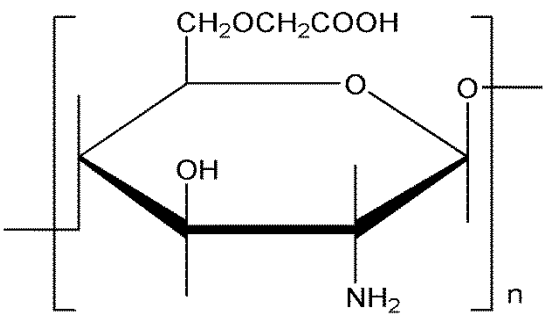
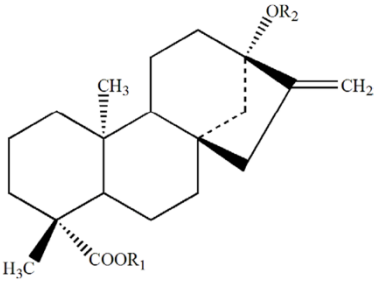


Fig. 1 Chemical structure of O-CMC

Steviol glycosides (SGs), a group of diterpene glycoside, mainly exist in the leaves of Stevia rebaudiana Bertoni. Previous studies suggested that SGs mainly contain about 10 kinds of diterpenoids, among which stevioside (ST), rebaudioside A (RA) and rebaudioside C (RC) are the predominant sweetening ingredients. The chemical structures of the above three compounds are shown in Fig. 2[14]. In recent years, because of their high sweetness (approximately 250-300 times sweeter than sucrose), low calorie, non-nutritive, high stability and many biological effects on human being, SGs have attracted great attention and have been used as non-nutritive high potency and low calorie sweetener in food and beverages in Japan, Korea, China, and South America[15]. SGs possess great advantages as natural sweeteners, especially for diabetic, phenylketonuria patients and obese persons[14,16]. ST and RA have hypotensive effects[17-19], immunologic enhancement[20], antihyperglycaemic, insulinotropic and glucagonostatic effects on diabetic patients[21-23], and as a potential chemopreventive agents for chemical carcinogenesis[24]. In addition, ST and RA did not significantly influence the human intestinal microflora composition[25]. Previous studies also indicated that RA was safe for humans at high dietary intake levels (less than 2 mg kg^{-1} body weight per day)[26-28]. In consideration of the unique bioactivities and the versatilities mentioned above, many technologies of separation and purification of ST, RA and RC have been reported. A method based on the attributes of a given batch of SGs in different solvents to isolate and purify RA was developed[29] and a series of patents of crystallization and recrystallization for RA were authorized by the U.S. Patent office[30]. However, these methods exhibit some shortcomings such as tedious process, time and labor consumption, solvent residue, low efficiency and so on. In our previous research, we systematically studied the preparative separation and purification of RA from SGs by mixed-mode macroporous adsorption resins[30] and successfully isolated and purified ST, RA and RC from the extract of Stevia rebaudiana Bertoni leaves by HSCCC[31]. However, the separation efficiency of these methods were low. Based on these reasons we developed a separation method using HSCCC with O-CMC as the additive of biphasic solvent system to isolate and purify ST, RA and RC.



No.	Compound name	R_1	R_2
1	stevioside (ST)	β-Glc	β-Glc-β-Glc(2→1)
2	rebaudioside A (RA)	β-Glc	β-Glc-β-Glc(2→1) β-Glc(3→1)
3	rebaudioside C (RC)	β-Glc	β-Glc-α-Rha(2→1) β-Glc(3→1)

Fig. 2 Chemical structures of ST, RA and RC

2 Experimental

2.1 Apparatus

A Spectrum HSCCC instrument (DE Spectrum Centrifuge) (Dynamic Extractions Co. Ltd., Slough, UK) equipped with two bobbins was employed in this study. Each bobbin of the HSCCC fits one analytical column and one preparative column made by polytetrafluroethylene (PTFE). The column volumes of analytical column and preparative column are 14.0 mL of 0.8 mm ID and 72.0 mL of 1.6 mm ID, respectively. The β-value is defined as $\beta = r/R$, where r is the coiled tubing radius and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge. In this case, β-values of the multilayer coil varied from 0.64 at the internal terminal to 0.81 at the external terminal. The revolution speed of the instrument is in the range of 0–1600 rpm. The HSCCC system was equipped with two preparative pumps NP7000 (Hanbon Sci. & Tec., Jiangsu, China), a NU3000 UV–Vis detector (Hanbon Sci. & Tec., Jiangsu, China), a DLSB-10/40°C constant temperature circulating instrument (Yarong Instruments Co. Ltd., Zhengzhou, China) and a CBS-A automatic fraction collector (Shanghai Huxi Analysis Instrument Factory Co. Ltd., Shanghai, China) to collect the fraction.

For HPLC analysis, we used an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1322A vacuum degasser, a G1311A quaternary pump, a G1315D diode array detector (DAD) and a G1328B manual injection valve with a 20.0 μL loop. The system was controlled by Agilent Chemstation software (version A.10.02) (Agilent Technologies, Palo Alto, CA, USA). In addition, a SinoChrom ODS-BP analytical column (250 mm × 4.6 mm i.d., 5 μm, Elite, Dalian, China) and Hypersil NH_2 analytical column (150 mm × 4.6 mm i.d., 5 μm, Elite, Dalian, China) were used. The viscosity was determined by a NDJ-8S digital viscometer (Shanghai Sunny Hengping Scientific Instrument Co., Ltd, Shanghai, China).

2.2 Reagents, Standards and samples

N-butanol and n-hexane used for HSCCC separation were of analytical grade and purchased from Zhongqin Chemical Reagent Co., Ltd. (Shanghai, China). Ethyl acetate and methanol used in HSCCC were of analytical grade and purchased from Yantai Shuangshuang Chemical Co., Ltd. (Shandong, China). Acetonitrile and methanol used for HPLC analysis were of chromatographic grade and purchased from Yuwang Group Co., Ltd. (Shandong, China). Ultrapure water, used for preparation of all the samples and solutions, was obtained with a Spring-R10 water purification system (Research Scientific Instrument Co., Ltd, Xiamen, China). O-carboxymethyl chitosan was purchased from Zhejiang Aoxing Biotechnology Co., Ltd. (Zhengjiang, China).

Reference standards luteolin (purity > 98.0%) and quercetin (purity > 95.0%) were from Shanghai Standard Biotech Co., Ltd. (Shanghai, China), and kaempferol (purity > 98.0%) was from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). SGs (purity > 90.0%) were obtained from Jiuquan Wenming Steviol Glycosides Co., Ltd. (Gansu, China). The standards ST, RA and RC (purity > 98.0%) were isolated and purified from SGs by HSCCC in our laboratory[31].

2.3 Measurement of settling time

The settling time was measured as follows[5]: the biphasic solvent system was prepared and completely equilibrated. Three milliliter upper phase and three milliliter lower phase were delivered into a 10.0 mL test tube. The test tube was vigorously shaken to make the two phases thoroughly mix and then allowed to rest in order to measure the time required for the two phases to form clear layers with a distinct interface.

2.4 Measurement of the partition coefficient (*K*)

The suitable biphasic solvent system was selected according to the

partition coefficient ($0.5 < K < 2.0$) of each target compound. The K value of the biphasic solvent system is defined as the concentration of a standard sample in the stationary phase divided by that in the mobile phase[5]. The K values were determined according to previous method[32]. A suitable amount of sample was dissolved in a test tube to which 2.0 mL of each pre-equilibrated biphasic solvent system was added. The test tube was shaken violently for several minutes and left to stand at room temperature until equilibrium was obtained between the two phases; the two phases were separated and dried in vacuum; the upper and lower-phase samples were dissolved in equal volume (1.0 mL) of methanol; then, 20.0 μL of the upper and lower phases were analyzed by HPLC to obtain K values of all target compounds, respectively. In normal-phase HSCCC, K is calculated with the formula: $K = A_L / A_U$ where A_U and A_L were the HPLC peak areas of objective compounds in the upper and lower phase, respectively[32].

2.5 Measurement of the viscosity and density

Viscosity was measured as follows: solvent systems composed of n-hexane/ethyl acetate/methanol/water at a volume ratio of 5:6.5:5:6.5 were prepared in separated funnels; different amount of O-CMC was added to the above solvent systems; each solvent system was shaken vigorously and left to equilibrate at room temperature; 100.0 mL upper and lower phase of each solvent system was put in 150 mL beaker; their viscosity measurements were conducted at room temperature by NDJ-8S digital viscometer equipped with a 0 rotor and 60 rpm shear rate was applied. Phase densities were simply determined by weighing 10.0 mL upper and lower phase of each solvent system and densities were calculated with the formula: $\rho = m/v$ where ρ was density, m and v referred to weight and volume.

2.6 The relationship between O-CMC concentration and *K*

Biphasic solvent systems composed of n-hexane/ethyl acetate/methanol/water at a volume ratio of 5:6.5:5:6.5 were prepared in separated funnels. Different amount of O-CMC was added to the above solvent systems to form a series of solvent systems with different O-CMC concentration in lower phases (0.0 mg mL^{-1}, 1.0 mg mL^{-1}, 1.5 mg mL^{-1}, 2.0 mg mL^{-1}, 2.5 mg mL^{-1} and 5.0 mg mL^{-1}, respectively). The solvent systems were shaken violently and allowed to equilibrate at room temperature. The method of measuring K values is the same as that described in section 2.5.

2.7 HSCCC separation procedure

2.7.1 Preparation of HSCCC solvent systems and sample solutions

Each solvent system consisting of organic solvent, water, and O-CMC was prepared in a separation funnel according to the volume ratio by thoroughly mixing and then completely equilibrated at room temperature. The two phases were divided and degassed by ultrasonication for 30 min shortly before use. The sample solutions were prepared as follows: 2.0 mg of each flavonol aglycon (quercetin, luteolin and kaempferol) and 10.0 mg SGs were dissolved in 1.0 mL of the solvent consisting of equal volume of each phase by sonication for analytical separation.

2.7.2 HSCCC separation

The HSCCC separation was performed in normal-phase mode ("tail-to-head"-mode). The lower phase of the biphasic solvent system was used as stationary phase and the upper phase was used as mobile phase in this separation mode. The multilayer coiled column (28.0 mL) was first entirely filled with the lower phase. Then the upper phase was pumped into the column at a flow rate of 0.8 mL min^{-1} while apparatus was run at a revolution speed of 1600 rpm. After hydrodynamic equilibrium was reached as indicated by a clear mobile phase eluting at the outlet, the sample solution was introduced through the injection valve. All through the experiment, the separation temperature was controlled at 30°C. The effluent in the column was continuously monitored with UV–vis detector. The peak fractions were collected according to the elution profile.

2.8 HPLC analysis and identification of HSCCC peak fractions

Crude sample, reference standards, and fractions separated by HSCCC were analyzed by HPLC. Flavonol aglycons were analyzed with SinoChrom ODS-BP analytical column using a gradient of water (A) and methanol (B). The gradient condition was as follows: 0-25 min, 50%-81% B, at a flow rate of 1.0 mL min^{-1} at a temperature of 30°C. The effluent was monitored at 360 nm. Crude sample of SGs and its fractions obtained from HSCCC separation were analyzed with a Hypersil NH_2 analytical column at 30°C. The

mobile phase was acetonitrile–water (80:20, v/v), the flow rate was kept at 1.2 mL min^{-1} for the over run and the detect wavelength was set at 210 nm. All of the mobile phase was filtered through a 0.45 μm Millipore filter before use.

3 Results and discussion

3.1 O-CMC additive

Previous studies have shown that chitosan could be used as sorbents of natural products, heavy metal ions (Cu^{2+}) and acid dyes. However, chitosan is only soluble in acidic sample solution with pH lower than 6.5[33] which restricts its range of application. O-CMC was chosen as additive of HSCCC in this study because it not only contains the reactive groups of chitosan but also it can dissolve in water well. O-CMC, containing both amino and carboxyl groups in the molecule, is an amphoteric polyelectrolyte[34]. These groups usually could be regarded as active reaction sites for combining other compounds by hydrogen bonds; furthermore, the amino and carboxyl groups can dissociate to form cationic and anionic polyelectrolyte, respectively, which could form electrostatic interaction with negatively or positively charged compounds, respectively.

Table 1 A list of parameters of biphasic solvent system (n-hexane/ethyl acetate/methanol/water at a volume ratio of 5:6.5:5:6.5) with O-CMC at different concentrations in the aqueous phase

O-CMC (mg mL^{-1})	Phase	Viscosity (mpa.s)	Density (g mL^{-1})	ST [c] (s)
0.0	U [a]	1.6	0.742	15
	L [b]	3.4	0.932	
0.5	U	1.6	0.765	16
	L	3.8	0.929	
1.0	U	1.6	0.767	18
	L	4.6	0.932	
1.5	U	1.6	0.762	19
	L	4.9	0.937	
2.0	U	1.6	0.773	21
	L	5.5	0.932	
2.5	U	1.6	0.770	70
	L	6.1	0.928	
5.0	U	1.6	0.772	80
	L	8.3	0.923	

[a] Upper phase of the solvent system; [b] Lower phase of the solvent system; [c] The settling time.

From Table 1 we can know that all the upper phases had the same viscosity and viscosities of lower phases increased with the increase of the additive amount of O-CMC, which told us that O-CMC can be only dissolved in the aqueous phase not in the organic phase. Moreover, a major advantage of O-CMC is its chemical and thermal stability, which is benefit for HSCCC separation.

3.2 Selection of solvent system and other conditions of HSCCC

An appropriate solvent system plays a key role in HSCCC separation. Y. Ito has shown the golden roles in selecting ideal conditions for HSCCC. Biphasic solvent system should satisfy the following requirements: (a) the satisfied K value for HSCCC is usually considered between 0.5 and 2; (b) in order to separate two compounds well by HSCCC, the separation factor ($\alpha = K_1/K_2$, where $K_1 > K_2$) should be greater than 1.5; (c) short settling time (< 20 s) of biphasic solvent system is vital for the retention of stationary phase; (d) the retention of stationary phase has quite direct relation with Rs: the higher retention of stationary phase gives better Rs[35]. As shown in Table 2, the biphasic solvent system n-hexane/ethyl acetate/methanol/water with volume ratio of 5:6.5:5:6.5 was selected for the separation of flavonol aglycons because their K values were acceptable. In our previous research, the partition coefficients of ST, RA and RC were measured in biphasic systems composed of ethyl acetate/n-butanol/water, chloroform/n-butanol/water and n-hexane/n-butanol/water, at last n-hexane/n-butanol/water at a volume ratio of 1.5:3.5:5 which had appropriate K values was used as the biphasic solvent system[31]. This solvent system was still chosen in the present research.

In this study, considering the limitation caused by using a UV detector, O-CMC containing aqueous phase has to be used as stationary phase and that is using a "tail to head" mode. However, this HSCCC mode usually results in poor retention of stationary phase, which is detrimental to the resolution. In this study, the settling time of biphasic solvent was measured to forecast the retention of stationary phase. As shown in Table 1, difference in settling time among the selected biphasic solvent systems composed of n-hexane/ethyl acetate/methanol/water (5:6.5:5:6.5, v/v/v/v) was insignificant when the concentration of O-CMC was less than 2.0 mg mL^{-1} in the aqueous phase and the settling time was less than 20

s. All other solvent systems have long settling time (more than 66 s) due to high viscosity of O-CMC. Considering settling time and separation effect, 1.0 mg mL^{-1} was chosen as the additive amount of O-CMC in the aqueous phase.

Table 2 The partition coefficient (*K*) of flavonol aglycons and steviol glycosides in different solvent systems

No	Organic-aqueous solvent system					*K* values					
	HEX	BuOH	EtOAc	MeOH	Water	Quercetin	Luteolin	Kaempferol	ST	RA	RC
1	2	0	3	2	3	0.24	0.37	0.075	_	_	_
2	5	0	6.5	5	6.5	0.70	1.12	0.40	_	_	_
3	5	0	6	5	6	1.48	2.14	0.54	_	_	_
4	1	0	1	1	1	4.79	6.81	1.29	_	_	_
5 [31]	1.5	3.5	0	0	5	_	_	_	1.311	0.564	0.889

3.3 Separation of standards by HSCCC using O-CMC as additive in stationary phase

Quercetin, luteolin and kaempferol were used as model analytes to investigate mechanisms of O-CMC as the additive of HSCCC solvent system. As shown in Fig. 3, the flavonol aglycons display the same elution order (the order of peaks is kaempferol at first, then quercetin and luteolin at last) in the biphasic solvent system composed of n-hexane/ethyl acetate/methanol/water at a volume ratio of 5:6.5:5:6.5 with/without O-CMC in the stationary aqueous phase with organic phase as mobile phase. This phenomenon may be interpreted as that it was still the partition in the biphasic solvent system that dominated the retardation.

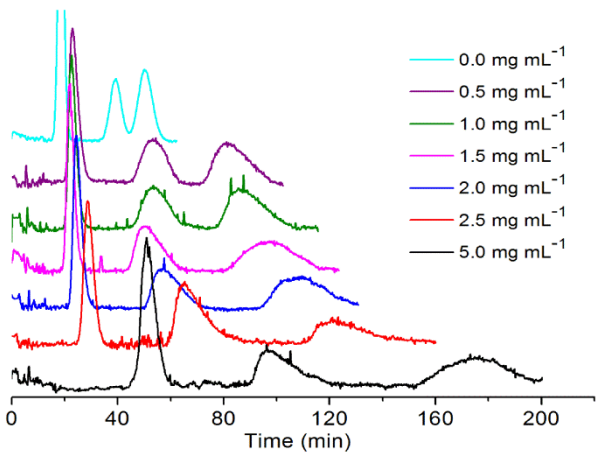


Fig. 3 Separation of flavonol aglycons by analytical HSCCC mode. Experimental conditions: Apparatus: Type-J coil planet centrifuge (DE Spectrum, 28.0 mL column volume with two coils of 0.8 mm ID tubing); solvent system: n-hexane/ethyl acetate/methanol/water 5:6.5:5:6.5 v/v with different concentrations of O-carboxymethyl chitosan in lower phase; sample: 2.0 mg of each quercetin, luteolin and kaempferol in 1.0 mL of equal volume of upper and lower phase; elution mode: upper organic phase was the mobile phase in a "tail-to-head" or "ascending" HSCCC mode; flow rate: 0.8 mL min^{-1}; revolution speed: 1600 rpm; S_f: 66.1% (no additive and 0.5 mg mL^{-1}), 62.5% (1.0 mg mL^{-1}), 60.7% (1.5 mg mL^{-1} and 2.0 mg mL^{-1}), 57.1% (2.5 mg mL^{-1}) and 35.7% (5.0 mg mL^{-1}); UV detection at 360 nm.

As shown in Fig. 3, as the additive amount of O-CMC increased, the gap between retention time became wider. As we all know, *Rs* quite depends on the retention of stationary phase (S_f). It was demonstrated that S_f is linearly decreasing with the square root of mobile phase flow rate[36].

$$S_f = A - B\sqrt{F} \quad (1)$$

the constant *A* was demonstrated to be related to the HSCCC column full of stationary phase (S_f= 100%) minus the dead volume, V_d, ratio (V_d/V_c, V_c refers to the column volume)[36]. The constant *B* is linked to the experimental conditions as:

$$B = \frac{800}{\pi d_c^2}\sqrt{\frac{\mu_M}{\omega^2 R|\rho_L - \rho_U|}} \quad (2)$$

where d_c is the internal diameter of the coiled tube, μ_M is the viscosity of mobile phase, ω is the rotational speed, *R* is the revolution radius or the distance between the spool axis and central axis, and ρ is the density of the liquid phase in which the subscripts U and L refer to the upper and lower phase, respectively[36].

As shown in Table 1, the viscosity of mobile phase (μ_M) which was the upper phase in this research did not vary with the additive amount of O-CMC. At the same time, the densities of the upper and

lower phase listed in Table 1 almost did not vary because the additive amount of O-CMC was little which was not enough to make the density vary. In this study, the same rotor rotation speed (1600 rpm) was used in each separation. From the above we can know that *B* in equation 2 was invariable when the additive amount of O-CMC was increased. Connecting dead volume (V_d) was unaltered, and *A* was constant in each separation. So, from equation 1 we can know that O-CMC had no impact on the stationary phase retention (S_f). Therefore, it can be speculated that O-CMC improved resolution by introducing intermolecular forces. The chemical structure of O-CMC was shown in Fig. 1. Hydrogen bonds can easily generate between hydroxyl, amino, carboxyl groups of O-CMC and hydroxyls of flavonol aglycons. In addition, electrostatic interaction may generate because some amino groups contained in O-CMC can be protonated to form cationic polyelectrolyte, meanwhile quercetin, luteolin and kaempferol were negatively charged due to slight dissociation of some hydroxyl groups contained in these compounds. Fig. 3 showed that as the O-CMC additive amount increased, the retardation of flavonol aglycons became stronger and the tailing of solute peaks became seriously. Tentatively, this can be interpreted as stronger intermolecular forces between O-CMC and solutes generated due to more O-CMC was added.

3.4 Effect of O-CMC concentration on *K* and *Rs*

$K = C_S / C_M$ and in the "tail to head" mode, it can be calculated as $K = A_L / A_U$ where A_U and A_L were the HPLC peak areas of objective compounds in the upper and lower phase. As shown in Fig. 4, *K* values of quercetin, luteolin and kaempferol increased with the increase of O-CMC concentration in stationary phase basically. Tentatively this can be interpreted such that intermolecular forces generated between O-CMC and flavonoids made more quercetin, luteolin and kaempferol retain in stationary phase. The more O-CMC was added in lower phase, the stronger intermolecular forces generated and thus the *K* values became bigger.

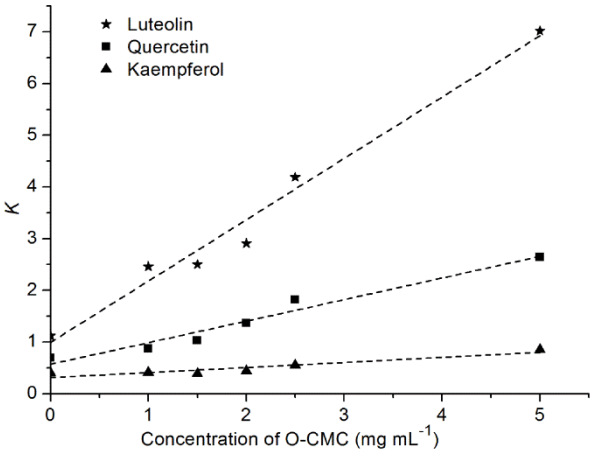


Fig. 4 Effect of O-CMC concentration on partition coefficient (*K*)

Peak resolution (*Rs*) is a parameter which is used to measure the separation effect of two adjacent chromatographic peaks. *Rs* is calculated with the formula:

$$Rs = 2(t_{R2} - t_{R1}) / (W_1 + W_2) \quad (3)$$

where t_{R2} and t_{R1} are the retention time of the two adjacent chromatographic peaks; W_1 and W_2 are the peak width at base.

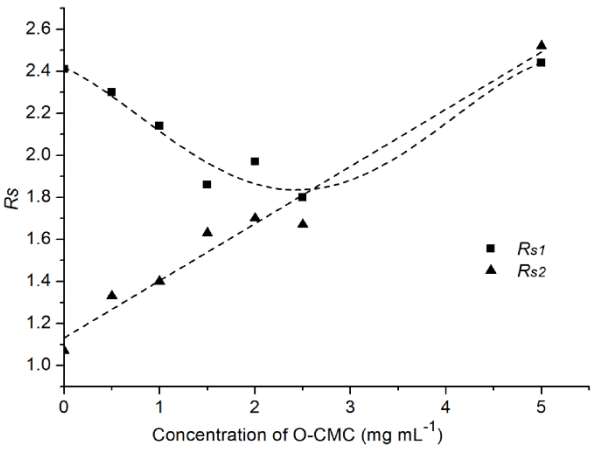


Fig. 5 Effect of O-CMC concentration on resolution (*Rs*). Rs_1: the resolution of peak 1 and peak 2; Rs_2: the resolution of peak 2 and peak 3 (peaks are shown in Fig. 3)

As shown in Fig. 5, Rs_1, the resolution of peak 1 and peak 2, decreased firstly and then increased with the increase of O-CMC concentration in lower phase. However, Rs_2 (the resolution of peak 2 and peak 3) was increased all along. Fig. 3 showed that peak 1 and peak 2 were separated well with the solvent system containing no O-CMC. The addition of O-CMC to the solvent system caused tailing of peak 2, so Rs_1 decreased at first. When the O-CMC concentration was greater than 2.5 mg mL^{-1}, the change of t_R was bigger than that

of peak tailing, so Rs_1 started to increase. As shown in Fig. 3, peak 2 and peak 3 were not baseline separated with the solvent system containing no O-CMC. After O-CMC was added in the lower phase, the retention time differences of quercetin and luteolin became wider due to different intermolecular forces generated between O-CMC and solutes. Although peak 2 and peak 3 broadened with the increase of O-CMC concentration, the change of retention time was more significant, so Rs_2 increased all along.

3.5 Separation of crude extract of SGs using HSCCC with O-CMC as additive

The HPLC chromatogram of SGs used in this study was shown in Fig. 6. In our previous study, "head-to-tail" HSCCC mode was used to separate SGs using a biphasic solvent system composed of n-hexane/n-butanol/water at a volume ratio of 1.5:3.5:5[31]. However, the resolution and recovery were not acceptable. In the present study, crude extract of SGs was separated using the same solvent system with/without O-CMC in a "tail-to-head" HSCCC mode. Firstly, the coiled column of HSCCC (28.0 mL) was first entirely filled with the lower phase as stationary phase. Then, the upper phase, used as mobile phase, was pumped into the coiled column using a peristaltic pump at a speed of 0.8 mL min^{-1}. After hydrodynamic equilibrium was established in the column, l.0 mL of sample solution containing 10.0 mg of crude sample was added and the eluent was monitored at 210 nm.

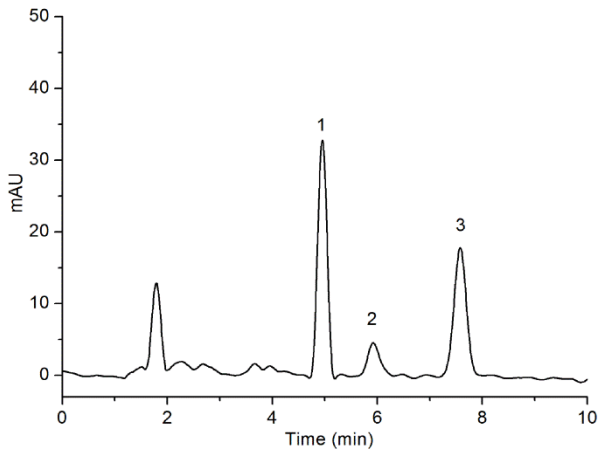


Fig. 6 HPLC chromatogram of crude sample. Experimental conditions: column: Hypersil NH_2 analytical column (150 mm × 4.6 mm i.d., 5 μm); mobile phase: acetonitrile–water (80:20, v/v); flow rate: 1.2 ml min^{-1}; detection wavelength: 210 nm; injection volume: 20.0 μL. Peaks 1, 2 and 3 correspond to ST, RC and RA, respectively.

As shown in Fig. 7, there was only one chromatographic peak in the HSCCC chromatogram when SGs was separated without O-CMC. However, it was surprising to find that several chromatographic peaks appeared when O-CMC was added to the lower phase and its HSCCC chromatography was shown in Fig. 8. Each peak was analyzed on HPLC, and peaks 1, 2 and 4 in Fig. 8 were found to be ST, RC and RA , respectively. Other peak fractions had lower purity and was not further analyzed.

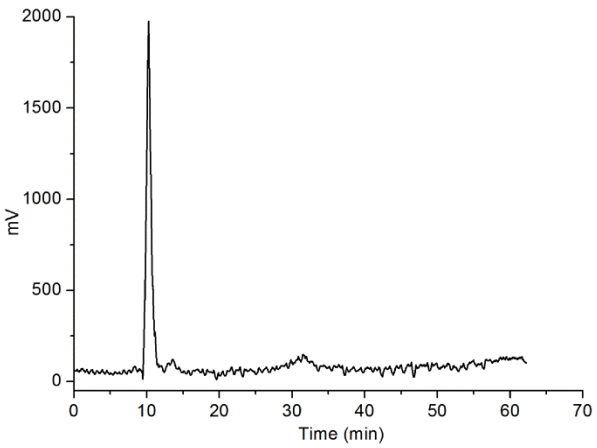


Fig. 7 Separation of SGs by analytical HSCCC mode. Experimental conditions: Apparatus: Type-J coil planet centrifuge (DE Spectrum, 28.0 mL column volume with two coils of 0.8 mm ID tubing); solvent system: n-hexane/n-butanol/water 1.5:3.5:5 v/v; sample: 10.0 mg of SGs in 1.0 mL of equal volume of upper and lower phase; elution mode: upper organic phase was the mobile phase in a "tail-to-head" or "ascending" HSCCC mode; flow rate: 0.8 mL min^{-1}; revolution speed: 1600 rpm; S_f: 45.5%; UV detection at 210 nm.

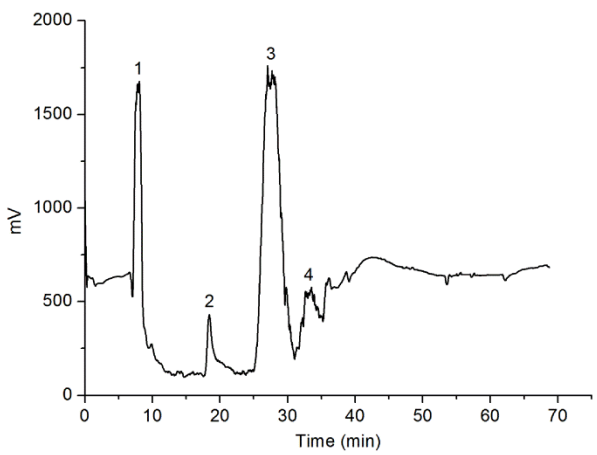


Fig. 8 Separation of SGs by analytical HSCCC mode. Experimental conditions: Apparatus: Type-J coil planet centrifuge (DE Spectrum, 28.0 mL column volume with two coils of 0.8 mm ID tubing); solvent system: n-hexane/n-butanol/water 1.5:3.5:5 v/v with 1.0 mg

mL^{-1} O-carboxymethyl chitosan in the lower phase; sample: 10.0 mg of SGs in 1.0 mL of equal volume of upper and lower phase; elution mode: upper organic phase was the mobile phase in a "tail-to-head" or "ascending" HSCCC mode; flow rate: 0.8 mL min^{-1}; revolution speed: 1600 rpm; S_f: 46.4%; UV detection at 210 nm. Fractions 1, 2 and 4 correspond to ST, RC and RA, respectively.

Conclusions

A new additive of HSCCC solvent system was found and successfully used to separate SGs with acceptable purity, which indicates the potential of O-CMC as the additive of HSCCC biphasic solvent system. O-CMC was deemed to separate compounds with similar structures. The present study also demonstrated that the mechanism of O-CMC to improve separation effect is introducing intermolecular forces: hydrogen bond and electrostatic interaction. We are undertaking further studies on the use of O-CMC additive in preparative HSCCC and intend to report on the outcome of the studies in due course.

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