

# Sample capacity in preparative high-speed counter-current chromatography

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## Abstract

High-speed counter-current chromatography (HSCCC) is a versatile technique in preparative separation and purification of pure compounds from complex matrices. As a preparative chromatography, there is a need to maximize the column production. Based on the plate theory of Van Deemter, the effect of the sample load on the separation was investigated in a preparative HSCCC with a 1000 ml column capacity. The test samples of hydroquinone, pyrocatechol and phenol were separated using a two-phase solvent system of *n*-hexane–ethyl acetate–ethanol–water (1:1:1:1, v/v/v/v) at different sample loads. The results showed that for the case of HSCCC, the agreement of the effect of sample load on peak height and peak width between the Van Deemter's theory and the experiments is excellent. Furthermore, the factors limiting the mass load, including the resolution between the peaks, the partition isotherm and the solute solubility were also discussed.

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**Keywords:** High-speed counter-current chromatography; Sample load; Peak width; Resolution; Partition isotherm

## 1. Introduction

High-speed counter-current chromatography (HSCCC) is a very effective tool for the preparative separation and purification of natural products and Chinese traditional herbs [1–4]. HSCCC is a form of liquid–liquid chromatography without a solid support, which separates soluble natural product substances on their partition between two immiscible solvents. The principle of separation is the same in both the laboratory and the production plant and is generic in that it can be applied to an extremely broad range of purification problems in many industries. Furthermore, because there is no solid support, there is 100% sample recovery and no need for any pre-purification. Therefore, more and more attention has been paid to the study of HSCCC.

For the separation and collection of a component by HSCCC, the goal is the isolation of a particular compound in a suitable amount, with a certain degree of purity and a certain recovery ratio. The amount of a component that can be separated per unit time (throughput) is also an essential criterion which must be optimized within the limits of the instrumentation. Great progress in HSCCC has been made in recent years. The rela-

tionship between the parameters that affect the chromatography behavior has been thoroughly studied, especially the effect of the flow rate [5–7]. However, how sample load influences the behavior of HSCCC and what are the limitations on the sample loading have not been studied deeply. These problems remain to be investigated.

Van Deemter and Zuiderweg [8] had extended the plate theory to the case where the influence of the feed volume cannot be neglected. This theory has been successfully applied to gas–liquid partition chromatography and the ion-exclusion experiments of Simpson and Wheaton [8]. In this paper, Van Deemter's theory was applied to the HSCCC, and the effects of sample load including the sample volume load and mass load on peak height and width were investigated. Furthermore, the factors limiting the mass load, including the resolution between the peaks, the partition isotherm and the solute solubility were also studied.

## 2. Experimental

### 2.1. Apparatus

Preparative HSCCC was carried out with a Model TBE-1000A high-speed counter-current chromatography (Tauto

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Biotech, Shanghai, China) equipped with a 1000 ml coil column made of PTFE tubing (3.0 mm) and a 100 ml sample loop. The  $\beta$ -value of the preparative column varied from 0.59 at the internal layer to 0.75 at the external layer ( $\beta = r/R$ , where  $r$  is the distance from the coil to the holder shaft, and  $R$  is the rotation radius or the distance between the holder axis and the central axis of centrifuge.). The rotation speed of the apparatus can be regulated with a speed controller in the range between 0 and 600 rpm. The HSCCC system is equipped with a Model SD-9002 constant-flow pump, a HX-2050 water bath, a Model 8823B UV monitor operating at 254 nm and a Model N2010 chromatography workstation.

## 2.2. Two-phase solvent systems and sample systems

All organic solvents used for the HSCCC separation were of analytical grade, and water used was distilled water.

A two-phase solvent system of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio 1:1:1:1 has been used. All tests were conducted with the aqueous phase (heavier phase) as the mobile phase. The chosen test samples were hydroquinone, pyrocatechol and phenol.

## 2.3. Preparative separation by HSCCC

### 2.3.1. Preparation of the two-phase solvent system

In the study, *n*-hexane–ethyl acetate–ethanol–water (1:1:1:1, v/v/v/v) system was prepared by adding all the solvents to a separation funnel according to the volume ratio and thoroughly equilibrated by shaking repeatedly. After settling at room temperature for 12 h, the solvent system was separated into organic and aqueous phases.

### 2.3.2. Preparation of the sample solution

Preparation of sample solution requires some considerations on several factors which would affect the partition efficiency. In order to maintain the normal phase composition, the sample solution was prepared by dissolving the model mixture in the solvent mixture of the lower phase and upper phase (1:1, v/v) of the solvent system.

### 2.3.3. HSCCC separation procedure

In each separation, the column was first filled with organic phase (upper phase) while the column was revolved at a designed speed. Then the lower phase (mobile phase) was pumped into the column in a “head to tail” mode in “forward” direction. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the sample solution was injected through the injection valve. The effluent of the column was continuously monitored by a UV detector at 254 nm. Each peak fraction was manually collected according to the chromatogram. After the separation, the solvents in the column were pushed out.

## 2.4. Measurement and calculation of parameters

The stationary phase retention ( $S_f$ ) at a given flow-rate and rotation speed was determined by the following procedure: the

column was first entirely filled with the stationary phase. Then the mobile phase was pumped into the column at the desired flow rate while the apparatus was rotated at the desired speed. After the mobile phase front emerged and the two phases had established the hydrodynamic equilibrium throughout the column, the volume of the stationary phase eluted from the column was measured. The stationary phase retention is expressed as  $100v_s/v_c$ , where  $v_c$  is the column volume which includes the volume of stationary phase ( $v_s$ ) and the volume of mobile phase ( $v_m$ ).

Peak width ( $W_b$ ) is estimated by drawing tangents to the peak inflection points and extrapolating these to the baseline.

Chromatographic resolution ( $R_s$ ) is defined as the peak separation ( $t_{R2} - t_{R1}$ ) divided by the average peak width ( $(W_{b2} + W_{b1})/2$ ): in this equation,  $t_R$  represents the peak retention time and  $W_b$  represents the peak width.

## 3. Description of the model

In its most simple form, the plate theory in HSCCC has been developed and applied by Kostanian [9,10]. The plate theory for general elution chromatography that includes the case where the influence of the sample load has been described by Van Deemter and Zuiderweg [8] is summarized only briefly here. Its validity in HSCCC was tested by the experiments.

In the plate theory, the column is conceived as consisting of a number of stages or plates in each of which there is equilibrium between the two phases. According to the plate theory, assuming the number of the theoretical plate is  $n$ ,  $v_m$  and  $v_s$  are the volumes of the mobile and stationary phases respectively, in one theoretical plate. In the course of the development of the theory, these volumes will be taken as constant. The feed has a concentration  $C_0$ , and the feed volume is  $A$ . The partial balance for the first plate, if a volume  $dS$  of the mobile phase flows through it, then becomes [8]:

$$v_m dC_{m,1} + v_s dC_{s,1} + C_{m,1} dS = \begin{cases} C_0 dS & \text{for } 0 \leq S \leq A \\ 0 & \text{for } S > A \end{cases} \quad (1)$$

For the other plates,

$$v_m dC_{m,n} + v_s dC_{s,n} + C_{m,n} dS = C_{m,n-1} dS \quad (n > 1) \quad (2)$$

Combining the initial condition, the solution of Eqs. (1) and (2) for all stages including the first is [8]:

$$C_{m,n} = \begin{cases} \frac{C_0}{v} \int_0^S \frac{1}{(n-1)!} \exp\left(\frac{-S'}{v}\right) \left(\frac{S'}{v}\right)^{n-1} dS & \text{for } 0 \leq S \leq A \\ \frac{C_0}{v} \int_{S-A}^S \frac{1}{(n-1)!} \exp\left(\frac{-S'}{v}\right) \left(\frac{S'}{v}\right)^{n-1} dS & \text{for } S > A \end{cases} \quad (3)$$

where  $v$  is the effective plate volume and is defined as

$$v = v_m + K v_s \quad (4)$$

The integrand of Eq. (3) is the Poisson distribution function. This will always be the case when the number of plates is not

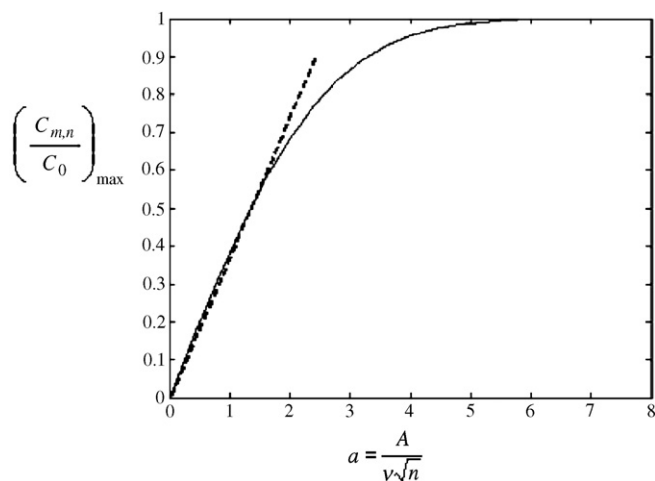


Fig. 1. Height of elution curve.

too small and the argument  $S'/v$  is large, the band has arrived at the end of the column:

$$C_{m,n} = \frac{C_0}{v\sqrt{2\pi n}} \int_{S-A}^S \exp\left\{-\frac{(S'/v-n)^2}{2n}\right\} dS' \quad (5)$$

A straightforward calculation shows that

$$\left(\frac{C_{m,n}}{C_0}\right)_{\max} = \operatorname{erf}\frac{a}{2\sqrt{2}} \quad (6)$$

and

$$\Delta s = a + 2\delta + \sqrt{2\pi}\frac{a+\delta}{\delta} e^{(1/2)\delta^2} \times \left(\operatorname{erf}\frac{a+\delta}{\sqrt{2}} - \operatorname{erf}\frac{\delta}{\sqrt{2}}\right) \quad (7)$$

where

$$s = \frac{S}{v\sqrt{n}} \quad \text{and} \quad a = \frac{A}{v\sqrt{n}} \quad (8)$$

$$\delta e^{-(1/2)\delta^2} = (a+\delta)e^{-(1/2)(a+\delta)^2} \quad (9)$$

It is seen that the peak height  $(C_{m,n}/C_0)_{\max}$  and peak width  $\Delta s = \Delta S/v\sqrt{n}$  are only dependent on the parameter  $a = A/v\sqrt{n}$ . These relations are graphically represented in Figs. 1 and 2.

Fig. 1 shows the relationship between  $(C_{m,n}/C_0)_{\max}$  and  $a$ . It can be easily found that when  $a$  is smaller than 1, the peak height increases with  $a$  linearly; while when  $a$  is larger than 5, the peak height keeps constant. Fig. 2 shows the relationship between the peak width and  $a$ . When  $a$  is smaller than 0.5, the peak width  $\Delta s$  becomes almost independent of the feed volume, and when  $a$  is larger than 3, the peak width increases with  $a$  linearly.

#### 4. Results and discussion

For the preparative chromatography, we are concerned about the preparation ability and the limitations of the maximal sample load. It is well known that the column volume has great influence on the sample capacity and the sample capacity increases sharply

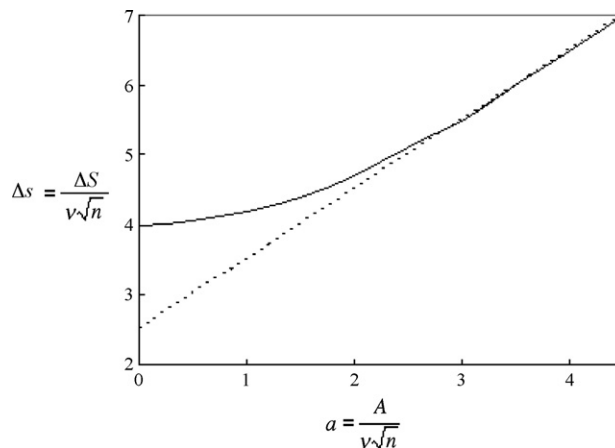


Fig. 2. Width of elution curve.

with the column volume. Wu et al. [11] have separated successfully 120 mg of crude sample with a HSCCC instrument of 260 ml column volume. In our lab, the author [12] has separated atractylon and atractylenolide III from 1000 mg *Atractyloides macrocephala* crude sample with a 1000 ml column volume HSCCC instrument. Du et al. [13,14] successively developed two kinds of semi-industrial scale counter-current chromatography. One is an apparatus equipped with a 10-l capacity column made of 8.5 mm i.d. convoluted tubing. Using this apparatus, 150 g of crude tea extracts were separated. The other is an apparatus equipped with a 40 l capacity column and used for separation of 500 g crude extract. The relationship between the sample load and the column volume is shown in Fig. 3. From Fig. 3, it can be found that the sample load increases with column volume exponentially. Increasing column volume is an effective way to increase the sample capacity.

However, under a certain HSCCC apparatus, the column volume is unchangeable, and the injected sample size can be raised in two ways: (1) increasing the sample concentration with a constant injection volume (mass load conditions), (2) increasing the injection volume of a sample at the constant concentration (volume load conditions). Both methods are utilized in the following study.

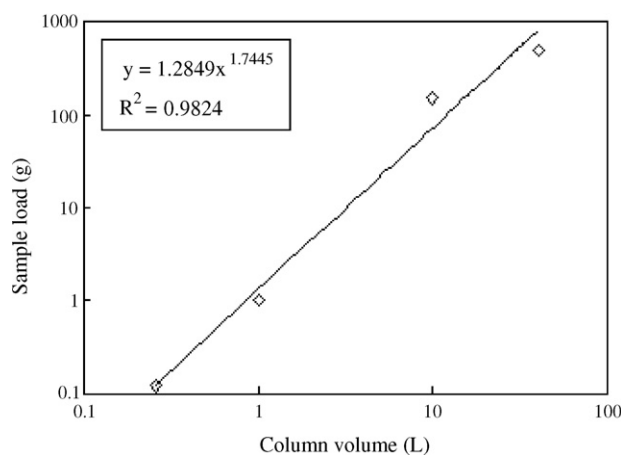


Fig. 3. Relationship between sample load and column volume.

Table 1  
Experimental data on the width and retention time of elution peaks

	A (ml)	C <sub>0</sub> (mg/ml)	M <sub>0</sub> (g)	W <sub>b</sub> (min)	t <sub>R</sub> (min)	n	a
Hydroquinone	10	10	0.1	16	104	676.0	0.50
	20	10	0.2	20	109	484.0	0.80
	40	10	0.4	27	110	260.8	1.19
	10	20	0.2	20	110	492.8	0.40
	20	20	0.4	24	111	336.1	0.67
	40	20	0.8	36	118	171.9	0.89
Pyrocatechol	10	10	0.1	28	160	522.4	0.29
	20	10	0.2	31	159	420.9	0.52
	40	10	0.4	39	158	262.6	0.82
	10	20	0.2	31	160	426.2	0.26
	20	20	0.4	37	158	291.8	0.43
	40	20	0.8	43	162	227.1	0.74
Phenol	10	10	0.1	60	288	368.6	0.13
	20	10	0.2	61	283	344.4	0.26
	40	10	0.4	71	285	257.8	0.45
	10	20	0.2	61	285	349.3	0.13
	20	20	0.4	68	284	279.1	0.24
	40	20	0.8	72	283	247.2	0.44

Where  $M_0$  is the mass load;  $W_b$  is peak width at the baseline;  $t_R$  is the peak retention time; and  $n$  is the theoretical plate number.

#### 4.1. The effects of sample size on peak width and peak height

In our investigations about the effects of sample size on peak width and peak height, the model sample was separated with various combinations of sample concentration and sample volume. Within the operating range of the sample load, we investigated the sample mass load from 0.1 to 0.8 g (each component 0.1 or 0.8 g) at different concentrations. The experimental data are given in Table 1.

It is easily found in Table 1 that the values of  $a$  are smaller than 1 except for the sample load of 0.4 g with 40 ml volume of hydroquinone, so as the model predicted in Fig. 1, and the correlation between the  $(C_{m,n}/C_0)_{\max}$  and  $a$  is expressed as

$$\left(\frac{C_{m,n}}{C_0}\right)_{\max} = 0.4 \frac{A}{v\sqrt{n}} \quad (10)$$

then

$$(C_{m,n})_{\max} = 0.4 \frac{M_0}{v\sqrt{n}} \quad (11)$$

Eq. (11) means that when the value of  $a$  is smaller than 1, the peak height is independent of the sample volume, and the peak height only increases with the mass load linearly. While when the value of  $a$  is larger than 1, the peak height begins to increase slowly.

From Fig. 2, when  $a$  is smaller than 0.5, the peak width is independent of the sample volume and is expressed as

$$\Delta S = 4v\sqrt{n} \quad (12)$$

Eq. (12) means that the peak width is dependent on the effective plate volume and the number of theoretical plates. So in Table 1, there are two kinds of conditions. When  $a$  is larger than 0.5, the peak width increases slightly with  $a$ . On the contrary,

when  $a$  is smaller than 0.5, the sample volume has no effect on the peak width.

As a preparative chromatography, even though the sample solution is introduced into the HSCCC column as short as possible, it is still important to consider the effect of the duration of sample injection on the peak width. The deviation of the real sample injection from an ideal impulse injection can be evaluated by the relative value of sample injection time that is the ratio of injection time (duration of sample injection) to the retention time. In the experiments, the largest duration of sample injection is 40 s, and from Table 1 the mean retention times of three peaks are 110.3, 159.5 and 284.7 min; thus the deviations would be 0.6, 0.4 and 0.2%, respectively. It shows that the effect of the duration of sample injection on the peak width is negligible.

In the cell model of counter-current chromatographic process proposed by Kostanian [9,10], the sample is assumed to be impulsively injected into the first ideally mixed cell. So, for large  $n$ , the distribution becomes Gaussian and the peak height and width can be expressed as follows [9]:

$$\begin{aligned} (C_{m,n})_{\max} &= \frac{M_0}{v_c} \frac{\sqrt{n}}{\sqrt{2\pi}(1 - S_f + S_f K_D)} = \frac{M_0 \sqrt{n}}{nv\sqrt{2\pi}} \\ &= 0.399 \frac{M_0}{v\sqrt{n}} \end{aligned} \quad (13)$$

$$\Delta S = FW_b = \frac{4\bar{\tau}}{\sqrt{n}} F = \frac{4v_c(1 - S_f + K S_f)}{\sqrt{n}} = 4v\sqrt{n} \quad (14)$$

It would be easily found that Eqs. (13) and (14) are identical to Eqs. (11) and (12), respectively. It means that under the operation condition of this paper, the real sample injection can be roughly considered as impulse injection due to the little sample volume (compared with the coil column).

The corresponding chromatograms of the effect of the sample load on the peak height and peak width in HSCCC are shown

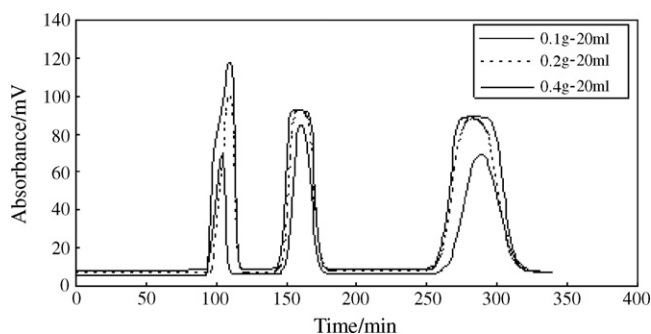


Fig. 4. HSCCC chromatogram with different mass load.

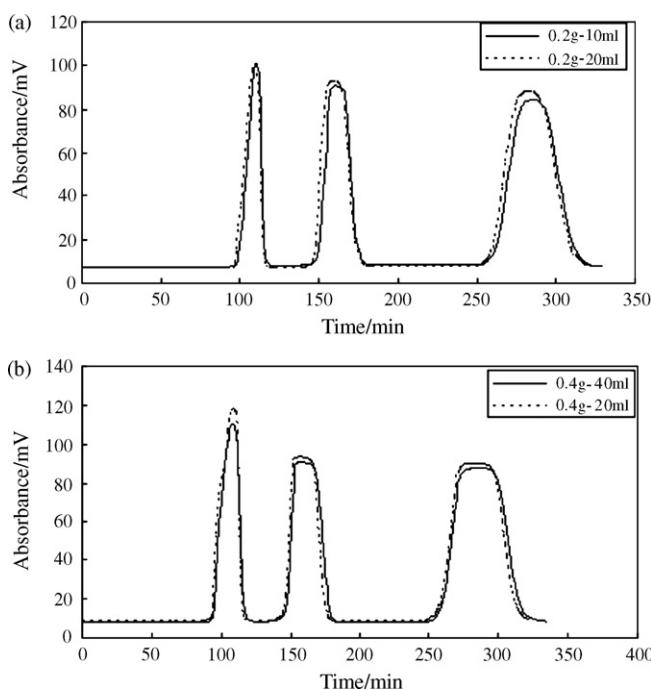


Fig. 5. HSCCC chromatogram with different sample volume. (a) 0.2 g mass load with different sample volume. (b) 0.4 g mass load with different sample volume.

in Figs. 4–6. In Fig. 4, the sample injection is with the same sample volume and different mass loads. The concentrations of the injected solution, corresponding to Fig. 4, were 5, 10, and 20 mg/ml, respectively. It can be seen in Fig. 4 that increasing the sample concentration with the same injection volume, the

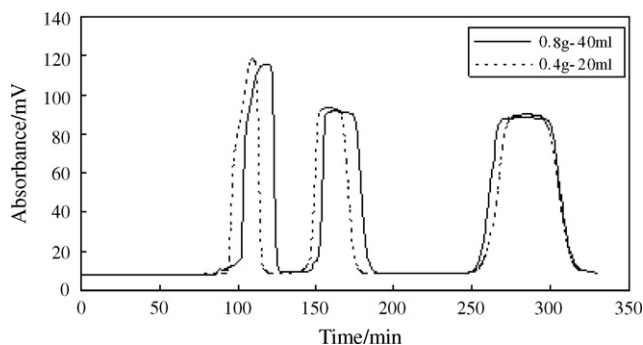


Fig. 6. HSCCC chromatogram with different sample volume with the same concentration.

peak width increases with the mass load increasing. It can also be found that with the increasing mass load, the peak height also increases at first; however, when the mass load arrives at 0.4 g, the peak height nearly keeps constant as a flat top, which is due to the limitation of the absorbance of UV monitor, and also there is a distortion in the first peak, which may be due to the non-linear isotherm.

The chromatogram in Fig. 5 was obtained with the same mass load and different sample volumes. Fig. 5a shows that the increase of sample volume has no obvious influence on the peak's height and peak's width, which increase slightly. The increasing of sample volume makes the peak width to increase slightly, which verify the theory well because the value of  $a$  is larger than 0.5. Therefore, the sample solution should be introduced into the column in as concentrated a form as possible to get sharp separations (narrow elution peaks). Fig. 6 shows the chromatogram of increasing injection volume at a constant concentration. It can be easily found that peak width increased, while the peak height keeps constant as found in Fig. 4 with the mass load of 0.2 and 0.4 g, which are all because of the limitation of the absorption of the UV monitor. The actual maximum concentrations corresponding to the different mass loads are shown in Fig. 7. From Fig. 7, it was easily found that the maximum concentrations increase with the mass load linearly, and the agreement between the theory and experiments is excellent.

Considering the main results of the effect of the sample load on the peak height and peak width in HSCCC compared with the plate theory of Van Deemter, a reasonable agreement is obtained. It would be very helpful to use the theory to predict the result of continuing increase of the sample load.

#### 4.2. The limitation of the sample load

##### 4.2.1. The linear isotherm

The peak shapes most frequently found in the chromatography are of three types which depend on the isotherms. The partition isotherms of hydroquinone, pyrocatechol and phenol are shown in Fig. 8.

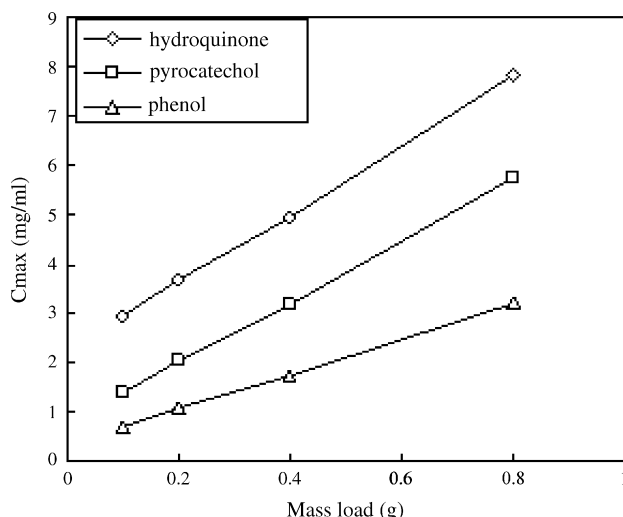


Fig. 7. Correlation between the maximum concentration and the mass load.

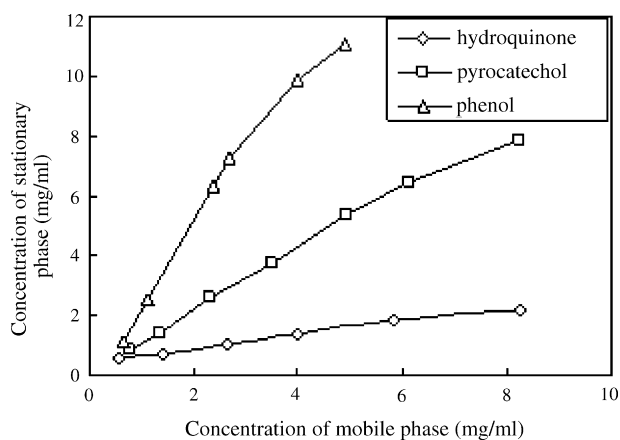


Fig. 8. Partition isotherm in two-phase solvent system *n*-hexane-ethyl acetate-ethanol-water at a volume of 5:5:5:5.

The partition isotherms of hydroquinone between 0 and 6 mg/ml and pyrocatechol between 0 and 5 mg/ml are typical line isotherms in which the distribution ratio is independent of the concentrations. For the partition isotherm of phenol, the distribution ratio after 3 mg/ml decreases with increasing concentration. According to the chromatograms from the experiments, when the maximum concentrations of different mass load in Fig. 6 is compared with the distribution isotherm line, it can be easily found that the maximum concentrations of hydroquinone and pyrocatechol lay in the nonlinearity part of the distribution isotherm, which lead to the screwed peaks and make the resolution decrease sharply. So the linear isotherm is one of the limitations of the sample capacity.

#### 4.2.2. The resolution between the peaks

The resolution is another main limitation of the sample capacity. With the increasing sample load, the peak resolution decreases; the increasing sample volume also decreases the resolution between the peaks. Fig. 9 clearly shows the exponential decreasing relationship between the resolution and the increasing mass load; the resolution decreases sharply from 0.1 to 0.4 g, while from 0.4 to 0.8 g, the resolution decreases slowly. When the sample mass load is up to 0.8 g, the resolution between the hydroquinone and pyrocatechol nearly equals to 1, so the sample mass load cannot be increased any more.

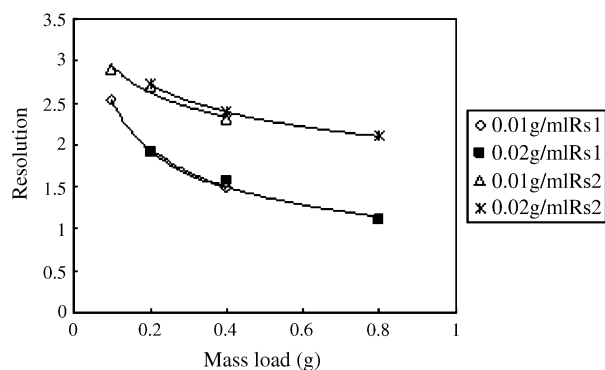


Fig. 9. The relationship between the resolution and mass load  $R_{s1}$ : resolution between hydroquinone and pyrocatechol;  $R_{s2}$ : resolution between pyrocatechol and phenol.

#### 4.2.3. The solubility of the sample in the two-phase system

The solubility of the sample in the two-phase system is the third factor that limits the mass load. Some samples nearly cannot dissolve in the two-phase solvent system, so it is very difficult to increase the sample mass load with limited sample volume. In this paper, the solubility of the test sample in the two-phase solvent system is very large, so it is not the limitation of sample capacity.

#### 4.3. The effect of the sample loading on the stationary phase retention

The effect of the sample loading on the stationary phase retention is not included in the theory of Van Deemer. Usually, with the increasing sample volume, the stationary phase retention decreases slightly. The decrease of the stationary phase retention may result in the decrease of the resolution between the peaks. So the sample volume cannot exceed 5% of the total column volume. In this work, the sample volume is so small (40 ml) that it has no obvious influence on the stationary phase retention, and the slight decrease of the stationary phase retention is mainly due to the sample overloading.

#### 4.4. Recycling the sample loading

Taking into account the limitation of the resolution, the maximum sample mass load can be up to 0.8 g (each component 0.8 g), so the sample mass load cannot be increased anymore in one cycle. However, as in HPLC, it is possible to recycle the mobile phase with the HSCCC apparatus. Preliminary HSCCC experiments were carried out with one injection. A sample can be completely eluted in 330 min, and the start of the first peak is 94 min. So the second sample can be injected at 250 min. In this way, when the elution of the first sample ends, the first peak of the second sample injection begins. The HSCCC chromatogram with two injections is shown in Fig. 10. The separations of the first sample and the second sample were achieved with satisfactory peak resolution.

The recycling sample load mode has some advantages: first it can reduce the preparation time (eliminating the time of pumping the stationary phase, pumping mobile phase to establish the hydrodynamic equilibrium, the dead time of the elution and pushing out the solvents); secondly it can reduce the solvent consumption, especially for the case that the volume of the mobile

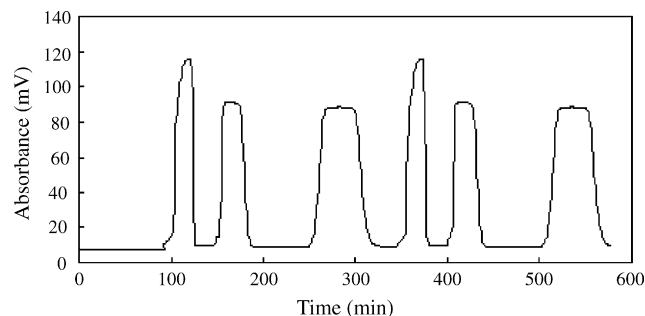


Fig. 10. HSCCC chromatogram with two injections.

phase is much larger than the stationary phase; thirdly, it is also so important that it can increase the throughput of separation.

## 5. Conclusions

Experiments were carried out to prove the effectiveness of the Van Deemter's plate theory to predict the effect of the sample load on the peak height and peak width in HSCCC. It is shown that the plate theory is suitable for describing sample load behavior in HSCCC. Furthermore, the limitations of the sample capacity such as the linear isotherm, the resolution, the solubility and also the stationary phase retention in HSCCC were also discussed. The results showed that the resolution between the peaks is the main limitation of the sample capacity. For increasing the chromatography throughput, the recycling mode provides an alternative method.

There was a need to accommodate much higher loads. However, due to the limitation of the HSCCC apparatus, it was not included here. Therefore, more work was needed to develop a truly preparative instrument.

## 6. Nomenclature

$a$	$(A/v\sqrt{n})$ dimensionless feed volume
$A$	feed volume (ml)
$C$	concentration (g/ml)
$C_0$	concentration in feed (g/ml)
$C_m$	concentration in the mobile phase (g/ml)
$C_s$	concentration in the stationary phase (g/ml)
$M_0$	mass load (g)
$n$	number of theoretical plates
$R_s$	resolution between peaks
$s$	$(S/v\sqrt{n})$

$S$	total volume of mobile phase flowed through (ml)
$\Delta s$	$(\Delta S/v\sqrt{n})$
$\Delta S$	width of elution curve (ml)
$t_R$	peak retention time (min)
$v$	effective plate volume (ml)
$v_m$	volume of the mobile phase in one theoretical plate (ml)
$v_s$	volume of the stationary phase in one theoretical plate (ml)
$W_b$	the peak width at the baseline

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