Highlights

- A sample injection strategy in CCC
- Injection process as two separate stages: injection and post-injection
- “The best solvent” approach to sample solution
- Loading increase by 1.8 times from 0.66 g/100mL V_c to 1.2 g/100mL V_c.
- Throughput increase of 46.5% from 3.1 g/h to 4.5 g/h and in yield from 82.0% to 85.5% with honokiol purity of >99% and magnolol purity of <0.1%.

Sample injection strategy to increase throughput in counter-current chromatography: case study of Honokiol purification

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Abstract

Counter-current chromatography (CCC) has been widely used as a preparative separation method to purify natural products from plant extracts and fermentation broths. Traditionally, throughput optimization in CCC has focused on sample concentration and sample volume. In this paper sample injection was considered as consisting of three variables: injection flow rate, post-injection flow rate and sample solvent. The effects of these parameters were studied using a honokiol purification from a *Magnolia officinalis* bark extract as a case study aiming to achieve the highest throughput/yield ratio for greater than 99% purity of this potential anti-cancer drug obtained for submission to the Chinese FDA. An injection method was established that increased the throughput of honokiol by 46.5% (from 3.05 g/h to 4.47 g/h), and decreased the solvent consumption of mobile phase and stationary phase per gram of honokiol by 40.0% (from 0.68 L/g to 0.41 L/g) and 48.4% (from 0.40 L/g to 0.21 L/g) respectively. These results show the importance of understanding the whole injection process when optimizing a given CCC separation.

Key words: Counter-current chromatography, CCC, sample injection, honokiol, magnolol, sample loading, throughput

1. Introduction

Magnolia officinalis Rehd. et Wils. (Houpu in Chinese) bark extract is a traditional Chinese medicine, which has been widely used to treat many diseases such as thrombotic stroke, typhoid fever, anxiety and nervous disturbance [1]. Recent research has found that the extract of *Magnolia officinalis* bark has other bioactive effects such as prevention and treatment of Alzheimer's disease [2]; pneumonia [3] and its anti-neuroinflammatory & antiamyloidogenic effects [4]. Honokiol (HK) is one of the major bioactive ingredients in *Magnolia officinalis* bark extract. Previous research demonstrated diverse bioactivities of honokiol, including inducing apoptosis and inhibiting growth of several tumor cell lines [5-7], crossing the blood-brain barrier and inhibiting brain tumours [8], anti-angiogenesis activities in vitro and in vivo [9]. Due to its significant anti-tumour activity, our team has performed a pre-clinical research study and as a result, submitted a new drug application to the China Food and Drug Administration. In preparing for clinical trials, it is important to establish the most efficient and economical process for production of the required amount of HK at a minimum of 80%

yield and at a minimum of 99% purity with the magnolol (MG, honokiol's isomer) content <0.1%.

To deliver enough material for the pre-clinical HK research study, different methods to produce HK were considered. Firstly, the synthetic route has been tried as the most common approach in the pharmaceutical industry. The procedure proposed in [10] required the use of a heavy metal based catalyst and additional purification steps with low final yield. Therefore, the next approach was based on the use of a traditional extraction of HK from Magnolia bark followed by a single purification step. For the latter preparative HPLC, normal phase preparative middle pressure column chromatography and counter-current chromatography (CCC) were tested. It was found that CCC was the most efficient method for our sample (data not shown). CCC is a liquid-liquid partition chromatography, which was introduced by Ito [11] and has been widely used for natural product purification [12-15]. The advantage of having a high ratio of liquid stationary phase retained in the column makes CCC an excellent preparative separation method [13] because of its high sample loading capacity. This paper will be focusing on CCC as an alternative separation method for HK because of its high throughput and good repeatability characteristics as well as being more environmentally friendly with lower solvent consumption. For any preparative separation method, throughput is an important evaluation parameter at a set purity and yield, which has been mentioned only in a few papers [1, 16, 17]. It has been demonstrated before by the authors [1] that CCC can successfully purify HK with a hexane-ethyl acetate-methanol-water (5:2:5:2) two-phase system at preparative and pilot scales. However, in this study, to reduce the toxicity of the solvent system, methanol was replaced with ethanol, which consequently changed the physico-chemical properties of both liquid phases including the polarity difference between them.

There have been few publications focussing on optimising sample loading. Walter Conway in his book on Countercurrent Chromatography mentioned that "the upper limit of sample size is determined primarily by solubility" and hinted that better resolution might be obtained by injecting the sample dissolved equally in each phase [18], but was very much working in the linear range. Berthod [19] was perhaps the first to explore beyond the linear range when scaling up separations and pushed his sample loading so much that the mix became very viscous and resulted in plug flow with total loss of stationary phase. He over came this by varying the flow regime around sample injection – in his case 17 minutes at 4 mL/min for the sample in MP followed by 30 mins of MP from T>H. The flow was then stopped and reversed (H>T) for 10 mins "to dissolved the plug of injected phase" before once more reversing the flow after a short 5 minute period of no flow and re-equilibration. Berthod therefore demonstrated in a one-off experiment the effectiveness of modifying flow regimes when injecting and also demonstrated the loss of stationary phase with high sample loading. Much later, Zhao and He [20] showed that there was a good correlation between predictions of peak height and width for varying sample loads using the Van Deemter theory, but they were working in the linear range.

Finally, there have been excellent studies on optimising the injection step when scaling up for production using centrifugal partition chromatography (CPC) [21] and then putting it into practice [22]. It should be noted that CPC is considered overall as a hydrostatic process (despite hydrodynamic cascade mixing in each chamber) as when the flow stops the stationary phase remains trapped in each chamber. The high performance countercurrent chromatography process (HPCCC) we are describing in this paper is a hydrodynamic process where if the flow stops the upper phase moves to the head end of the column and the lower phase moves to the tail (countercurrent). Therefore as Luc Marchal [21] says "The flow

pattern, mass transfer and solute resident time distribution in CPC is fundamentally different from CCC". Nevertheless flooding (loss of stationary phase) and possibly viscous fingering can occur in both but will require different approaches to overcome them as Berthod [19] demonstrates.

Therefore, in the present work, a complete stationary phase retention study and the effect of different CCC operational parameters on throughput, purity and yield was systematically studied. Also various scenarios for sample injection have been investigated, building on these previous studies, aiming for much higher sample loading on a 1 L lab scale CCC instrument.

2. Experiments

2.1 Reagents

Solvents used for CCC were of analytical grade and for HPLC analysis were HPLC grade from Fisher Chemicals (Loughborough, UK). HPLC grade water was purified by a Purite Select Fusion pure water system (Thame, UK).

2.2 Apparatus

A Midi-DE CCC centrifuge (Dynamic ExtractionsTredegar, UK) fitted with 4 mm I.D. preparative columns made of polyfluoroalkoxy tubing (PFA) with volumes of 459 and 453 mL was used to perform the counter-current extractions. The distance between the column axis and central axis of the planetary centrifuge for these columns was 11 cm with a β value range of 0.52-0.86. A Knauer K-1800 HPLC pump (Berlin, Germany) was used to pump solvent into columns. A Knauer K-2501 spectrophotometer with a preparative flow cell was operated at 254 nm to monitor the elution.

HPLC was performed on a Waters Alliance 2695 separation module (with Empower software) connected to a Waters 2996 photodiode array (PDA) detector (210-400 nm) using a Sunfire C18 column (150 mm × 4.6 mm I.D., 5 μm) (Waters, Milford, MA, USA).

2.3 *Crude preparation*

The dry bark of *Magnolia officinalis* (10 Kg, obtained from Xinhehua Traditional Chinese Medicine Ltd.) was mixed with 1 Kg calcium oxide and 200L water in a multi-functional extracting tank. After 24 hours, the extract was filtered and the pH of the solvent was adjusted to 1.5 with 10% hydrochloric acid to precipitate honokiol and magnolol. After filtration, the residue was dissolved in ethanol, then the solution was filtered again and evaporated at 30 ºC under reduced pressure. The residue was kept in a vacuum for 24 hours to produce a dry crude extract of 258g. The content of honokiol in the crude extract was 60.5% by HPLC (Fig.1).

[Insert Fig. 1]

2.4 CCC separation procedure

The solvent system *n*-hexane-ethyl acetate-ethanol-water (5:2:5:2, v:v:v:v) was developed as a part of a Chinese FDA submission report for honokiol production required for clinical research. In this study the upper and lower phases were made separately using solvent ratios determined by GC analysis (see Table S1 in Supplementary material). The column was filled

with lower (stationary) phase, then the rotor speed was set at 1250 rpm (192*g*), and the upper (mobile) phase was pumped into the column to establish hydrodynamic equilibrium at 50 mL/min in normal phase (NP) mode. Then the sample solution was injected and elution started, which was monitored with an UV detector at 254 nm and 50 mL volume fractions were collected. The volume of stationary phase in each fraction was recorded to establish a stationary phase stripping characteristic against time. For each fraction, 100 μL of upper and lower phases were pipetted into separate tubes and 900 μL acetonitrile was added to dilute them for further HPLC analysis. The honokiol yield was calculated by the equation:

Yield=Peak area of combined fractions/ peak area of all fractions

For each CCC separation, the cycle time was calculated taking into account that the filling/equilibrating stage takes 15 min so that the throughput could be calculated accurately. In all results the throughput and yield is for a honokiol purity of >99% and of a magnolol content of <0.1%.

3. Results and discussion

3.1 Initial stationary phase retention

It is well known that successful separation in CCC is directly related to stationary phase retention (S_f) [24]. The higher the S_f value the better chance for compounds to be well separated at high sample loading. In turn, the former is dependent on rotational speed (or *g*-field level) of the column and mobile phase flow rate. The higher the rotational speed and the lower the flow rate the higher stationary phase retention. Therefore, the balance between the two allows the optimum S_f to be achieved. The rotational speed range at which a CCC instrument can be operated is generally defined by the CCC manufacturer, while mobile phase choice has no technical restrictions. Du et al [24] demonstrated a linear relationship between the square root of mobile phase flow rate ($\sqrt{F}$) and retention of stationary phase for a variety of two phase solvent systems run with the lower phase mobile (the lower phase was not aqueous for all the systems therefore, the term “reversed phase mode” has not been used). However, linearity is not always the case, especially when upper phase is used as the mobile phase [25]. It can be seen from Fig. 2 that the Du plot for HEEWat system used in this study is not linear after 50 mL/min ($\sqrt{F}$~7). Therefore, a 30-50 mL/min range of flow rate was chosen for further experiments.

[Insert Fig. 2]

3.2 Effect of injection flow rate

Working at its best, as a preparative technology, CCC is generally used with high sample loadings, which is achieved by a combination of high concentration and high volume sample solutions. As a consequence of this, it often leads to the additional loss of stationary phase after injecting the sample solution because the latter has very different physico-chemical properties (density & viscosity) to the solvent system itself. When a highly concentrated sample is injected into a column, it is seen as a third phase [21] leading to a loss of stationary phase until the sample is sufficiently diluted by the solvent system. To help this dilution process the injection flow rate can be lowered and maintained low for some time after injection has been completed.

The effect of injection flow rate (F_{inj}) on the retention of stationary phase and the separation at fixed sample volume are shown in Fig. 3 and Fig. 4. When the mobile phase flow rate of 50 mL/min was kept throughout the run the 50 mL (5.5% of column volume) sample injection led to a 57.4% drop in S_f value from the initial 83.4% (after column equilibration) down to 26.0%. As a consequence, the resolution between HK and MG was only 1.1, in other words, they co-eluted. When injection flow rate decreases from 50 to 10 mL/min, the retention of stationary phase and separation is improved. The 18.7% drop in S_f value at 10 mL/min gave peak resolution of 1.3. Complete peak resolution of 1.6 can be achieved at F_{inj} of 1.0 mL/min. Further decrease in F_{inj} did not make any difference in Sf value or resolution but the separation time becomes impractical. While lowing injection flow rate reduces loss of stationary phase and increases resolution and yield, it will also reduce throughput. For example, as injection flow rate changes from 50, 10, 1.0 to 0.1 mL/min, throughput in g/h (yield%) in each case changes respectively as follows: 3.05 (82.1%), 2.85 (98.7%), 1.64 (99.8%) and 0.32 (99.9%). This suggested that injection flow rates either side of 10 ml/min (5, 10 and 20 ml/min) would be optimal for further experiments.

[Insert Fig. 3]

[Insert Fig. 4]

3.3 Optimization of mobile phase and injection flow rates and sample concentration and volume

Based on the above results, the affects of mobile phase flow rate (F - 30, 40, 50 mL/min), sample injection flow rate (F_{inj} - 5, 10, 20 ml/min), sample concentration (SC – 100, 120, 140 mg/mL) and sample volume (SV – 50, 75, 100 mL) on throughput (g/h) were studied to give a purity of Honokiol of >99% with less than 0.1% of magnolol . Sample temperature was excluded because its increase in 30-60 °C range made the separation marginally worse (results are not shown). The parameters, levels and results are given in Table 1.

[Insert Table 1]

Experiments 3, 4, 7 and 8 had the highest sample loading of 1.1-1.5 g/100 mL column volume at 100-140 mg/mL concentration injected in 8-11% of column volume (V_c). In all these runs stationary phase was completely lost after sample injection even when the injection flow rate was as low as 5 mL/min. Experiments 6 & 9 gave the best results with a throughput of 3.11 and 3.09 g/h respectively, but this was not much better than our original experiment (Table 1) which had a throughput of 3.05 g/h. Yield levels from these experiments were 97.4%, 95.9% and 82% respectively. As we were looking for a step change we explored further injection optimisation by changing the injection solution as discussed in the next section.

3.4 Further optimization of the injection procedure

CCC technology is well known for high loading due to higher solubility in a mixture of solvents composing a two-phase system. To maintain reproducibility for preparative and pilot scale separations it is better to make a sample solution in one phase only (even if it gives a suspension) rather than in a mixture of upper and lower phases. Another approach developed by the authors was to dissolve the sample in the best solvent from the solvent system used and then carefully add the rest of the solvents in ratios proportional to the phase composition. All these approaches have been used in this study. Yet the highest throughput achieved was 3.1 g/h.

Traditionally any solvent system is built around the "best" solvent from a sample solubility point of view. Therefore, the idea to push back the bounds of expectation was to apply this approach directly to the sample solution and make it in the best solvent only. From hexane, ethyl acetate, ethanol and water of the HEEWat solvent system used in this study, ethanol provides the highest solubility for Honokiol crude extract at a concentration of up to 600 mg/mL in comparison with 140 mg/mL in the case of using lower phase of the HEEWat while sample solution remains homogeneous.

The results shown in Table 1 indicate that loadings above 9 g in various combinations of sample concentration and volume destroys the solvent system in the column and causes a complete loss of stationary phase. Therefore, 9 g loading at 600 mg/mL sample concentration was chosen for the following experiments aiming to keep the volume of ethanol injected into the column to a minimum. Injecting 9 g of sample dissolved in 15 mL of ethanol led to a 67.4% drop in Sf value from the initial 96% to a final 28.6% providing partial separation. While injecting 9 g made in the HEEWat lower phase (giving a suspension) caused a complete stripping of stationary phase (from the initial 94% to a final 5.1%) with no separation occurring (the graphical data are given in the Supplementary materials – Figure S1). These results confirmed that there is a possibility of further increasing throughput by adjusting the injection procedure of sample solution in ethanol.

It was considered to split injection into two stages and study each one of them separately. The first stage is the loading/injecting of a sample onto the column; the second is the post-loading/injection time and flow rate allowing sample to be diluted even more.

[Insert Fig. 5]

The importance of the second stage can be seen from Fig. 5. When the injection flow rate was kept at 1 mL/min for 5 more minutes after the sample had been loaded onto the column, the final Sf value improved from 29% to 43% providing a better separation. Therefore, various combinations of duration/flow rate were tested but the volume of the injected sample was kept the same.

The duration of the first (injection) stage was cut to the minimum while the second (post-injection) stage was gradually extended (see Table 2). Interestingly, injecting the ethanol sample for 18 seconds at the same flow rate as for equilibrating (50 mL/min) did not affect the stationary phase (SP) retention because the flow rate was dropped afterwards down to 1.0 mL/min for 10 min. This allowed the highly concentrated sample to get diluted in the column without causing too much loss of SP resulting in a 91.4% yield and 3.81 g/h throughput. Further extending post-injection time improved peak resolution and also allowed an increase in the sample loading. The latter was done via injecting a larger volume because 600 mg/mL was the solubility limit for the honokiol extract solution in ethanol. When sample mass was increased to 11 g, the throughput of honokiol reached 4.17 g/h. Whereas a 12 g injection led to a reduced yield of 72.2% even with the extended post-loading stage.

A final improvement of throughput can be obtained by collecting only the central part of peaks, in other words, by cutting "the peak tail" which contains a low concentration of the target. As shown in Fig. 6 stopping separation at 49.4 min. will give the highest throughput of 4.47 g/h with yield of 85.5%.

It should be noted that the fractogram in Fig. 6 obtained for an 11 g loading under the optimized conditions caused the honokiol peak to be non-Gaussian in comparison with the earlier fractogram shown in Fig. 4 for a 6 g loading. This demonstrates that the solvent

system/column were running in non-equilibrium conditions while still providing a good throughput & yield. Also the solvent consumption of mobile phase and stationary phase per gram of honokiol was decreased dramatically by 40.2% (from 0.68 L/g to 0.41 L/g) and 48.4% (from 0.40 L/g to 0.21 L/g) respectively.

[Insert Table 2]

[Insert Fig. 6]

4. Conclusions

This research is based on optimizing an injection procedure as a separate process by splitting it into injection and post-injection stages, and applying "the best solvent" approach to the sample solution. This allowed the sample loading to be increased by 1.83 times (from 0.66 g/100mL V_c to 1.21 g/100mL V_c) and reach non-equilibrium conditions for the target peak. The Honokiol crude extract was dissolved in ethanol at 600 mg/mL concentration and successfully separated with a hexane-ethyl acetate-ethanol water (5:2:5:2) system in normal phase. Developing a separate flow rate "programme" for the injection stage led to an increase in throughput from 3.05 g/h to 4.47 g/h and in yield from 82.0% to 85.5% while maintaining a Honokiol purity of >99% and Magnolol purity of <0.1%. Further work is required to understand the hydrodynamics of this type of separation process including prediction of operating parameters.

5. Acknowledgments

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Legends

Figure 1: HPLC chromatogram of *Magnolia officinalis* bark extract and structure of honokiol and magnolol. HPLC conditions—column: Sunfire C18 column (150mm×4.6mm I.D., 5 μm); mobile phase: acetonitrile-0.1% formic acid aqueous solution (65:35, v:v); flow rate: 1ml/min; temperature: 30 °C; detection wavelength:254 nm.

Figure 2: Du Plot. Conditions: column volume 912 ml; phase system: *n*-hexane-ethyl acetate-ethanol-water (5:2:5:2, v:v:v:v); NP mode; rotation speed:1250 rpm.

Figure 3: Effect of different sample injection flow rate on stationary phase retention. For conditions see section 2.4. Sample: 50 mL, 120 mg/mL in LP HEEWat, NP mode.

Figure 4: Effect of different sample injection flow rates on separation. For conditions see section 2.4. Sample: 50 mL, 120 mg/mL in LP HEEWat, NP mode.

Figure 5: Effect of different injection procedure. For conditions see section 2.4. Sample 15 mL, 600 mg/mL in ethanol, NP mode.

Figure 6: Fractogram after optimization, showing throughput and yield for a honokiol purity >99% and magnolol purity <0.1%. Sample volume: 18.3 mL; sample concentration: 600 mg/mL; flow rate of mobile phase: 50 mL/min; injection procedure: 50 mL/min for 0.4min then 1.0 mL/min for 15 min.

Table 1. Parameters, levels and results in orthogonal experimental design for honokiol and magnolol purities of >99% and <0.1% respectively.

Table 2. Final Sf, yield and throughput with different injection procedure and sample mass for honokiol and magnolol purities of >99% and <0.1% respectively.

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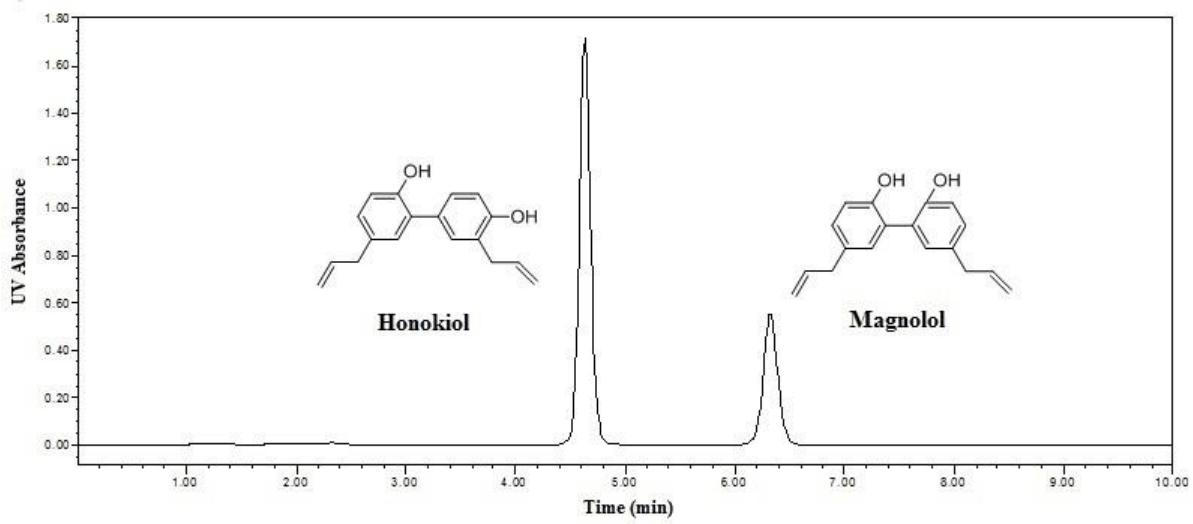

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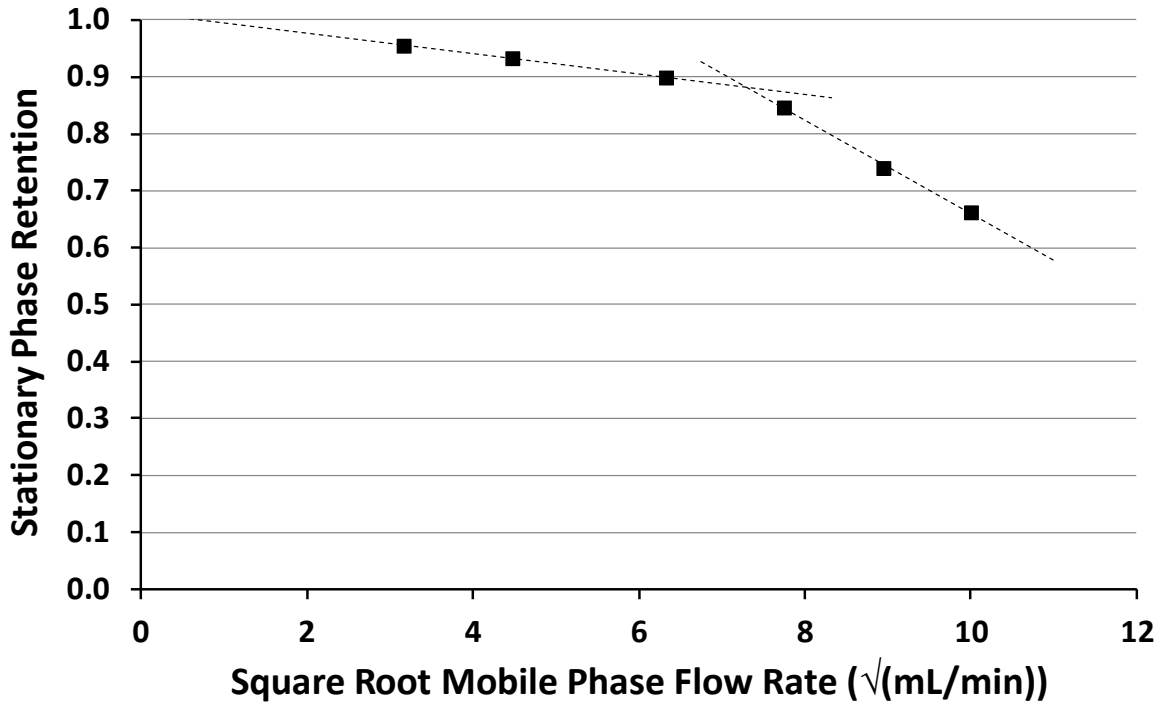

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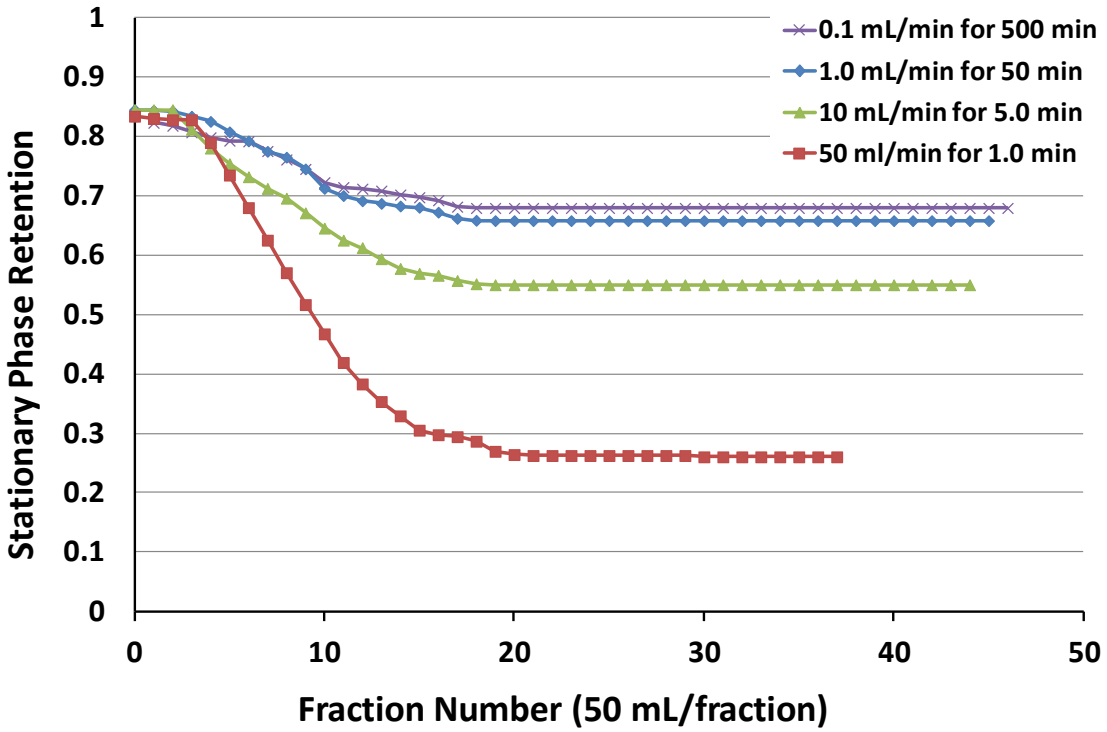
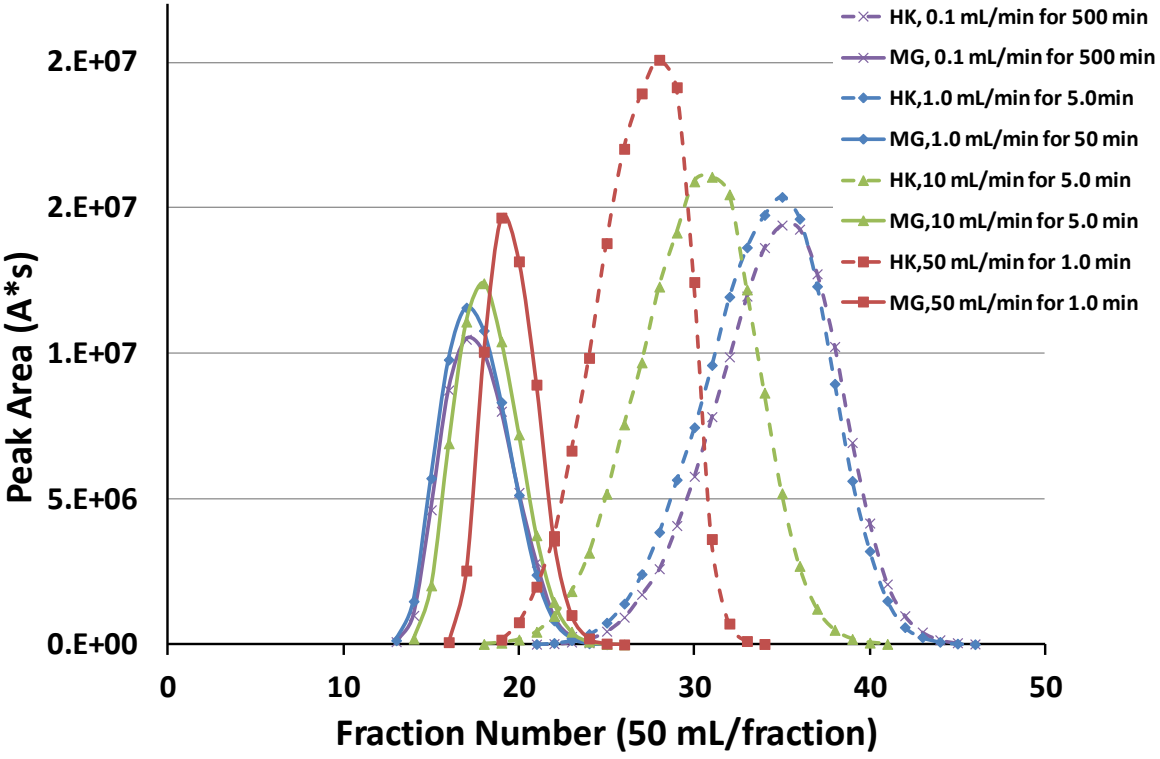
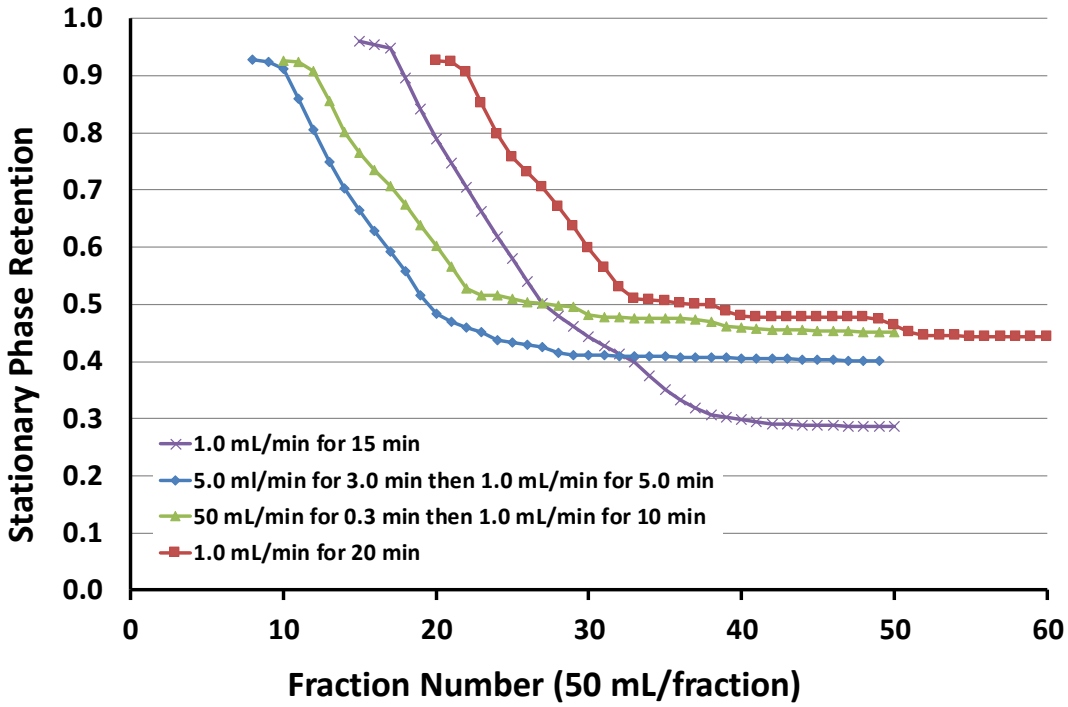


Figure 4: Effect of different sample injection flow rates on separation. For conditions see section 2.4. Sample: 50 mL, 120 mg/mL in LP HEEWat, NP mode.



406 Figure 5: Effect of different injection procedure. For conditions see section 2.4. Sample 15
407 mL, 600 mg/mL in ethanol, NP mode.



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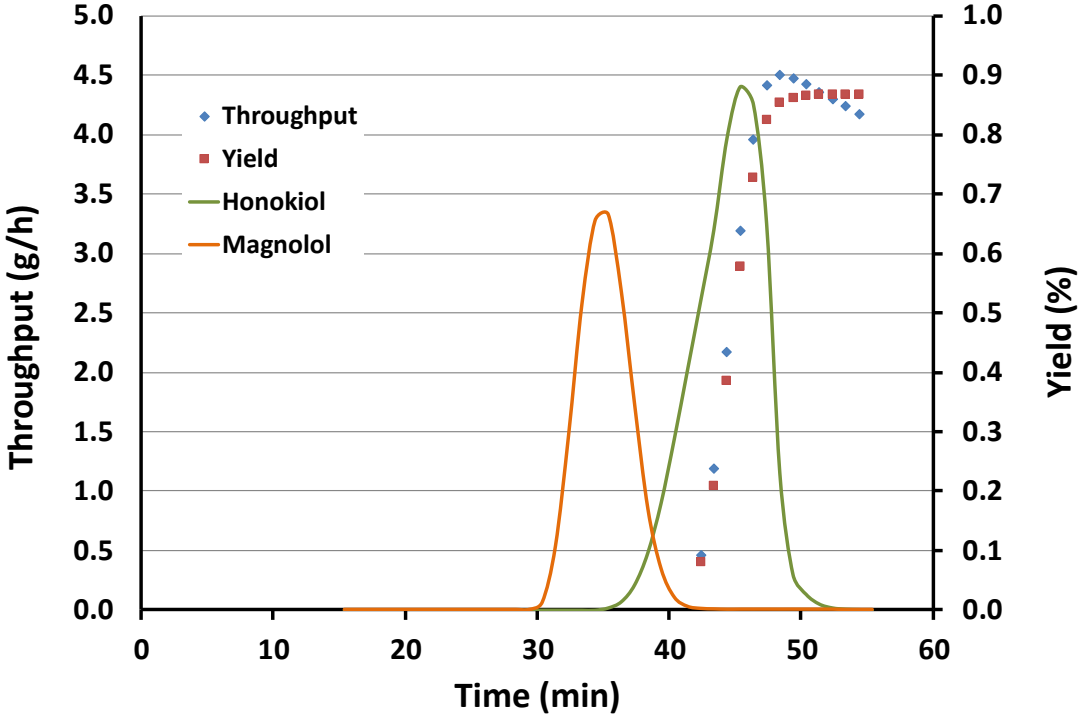
411 purity >99% and manolol purity <0.1%. Sample volume: 18.3 mL; sample concentration:
412 600 mg/mL; flow rate of mobile phase: 50 mL/min; injection procedure: 50 mL/min for
413 0.4min then 1.0 mL/min for 15 min.



414

415 Table 1. Parameters, levels and results in orthogonal experimental design for honokiol and
416 magnolol purities of >99% and <0.01% respectively.

Experiment No.	Mobile phase F (mL/min)	Injection flow rate (mL/min)	Sample concentration (mg/mL)	Sample volume (mL)	Sample mass (g)	Final Sf (%)	Throughput (g/h)
1	30	5	100	50	5	90	1.30
2	30	10	120	75	9	46	2.68
3	30	20	140	100	14	<5%	0.00
4	40	5	120	100	12	<5%	0.00
5	40	10	140	50	7	44	2.84
6	40	20	100	75	7.5	50	3.11
7	50	5	140	75	10.5	<5%	0.00
8	50	10	100	100	10	<5%	0.00
9	50	20	120	50	6	45	3.09
Original	50	50	120	50	6	26	3.05

417

418 Table 2: Final Sf, yield and throughput with different injection procedure and sample mass
419 for honokiol and magnolol purities of >99% and <0.01% respectively.

Sample mass (g)	F_1 (mL/min)	T_1 (min)	F_2 (mL/min)	T_2 (min)	Final Sf (%)	Yield (%)	Throughput (g/h)
9	1	15	/	0	23.6	57.0	2.43
9	1	20	/	0	41.7	88.1	3.17
9	5	3	1	5	35.6	71.9	3.32
9	50	0.3	1	10	40.8	91.4	3.82
11	50	0.4	1	15	35.1	86.8	4.17
12	50	0.4	1	15	29.6	69.4	3.92
12	50	0.4	1	20	27.4	72.2	3.73

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Supplementary materials

Table S1. Constituent of different solvents in upper phase and lower phase in HEEWat (5:2:5:2, v/v) solvent system as measured by GC analysis.

	composition of upper phase (%)	composition of lower phase (%)
n-Hexane	81	6.5
Ethyl acetate	12.9	18.9
Ethanol	6.1	50.5
Water	0	24.2

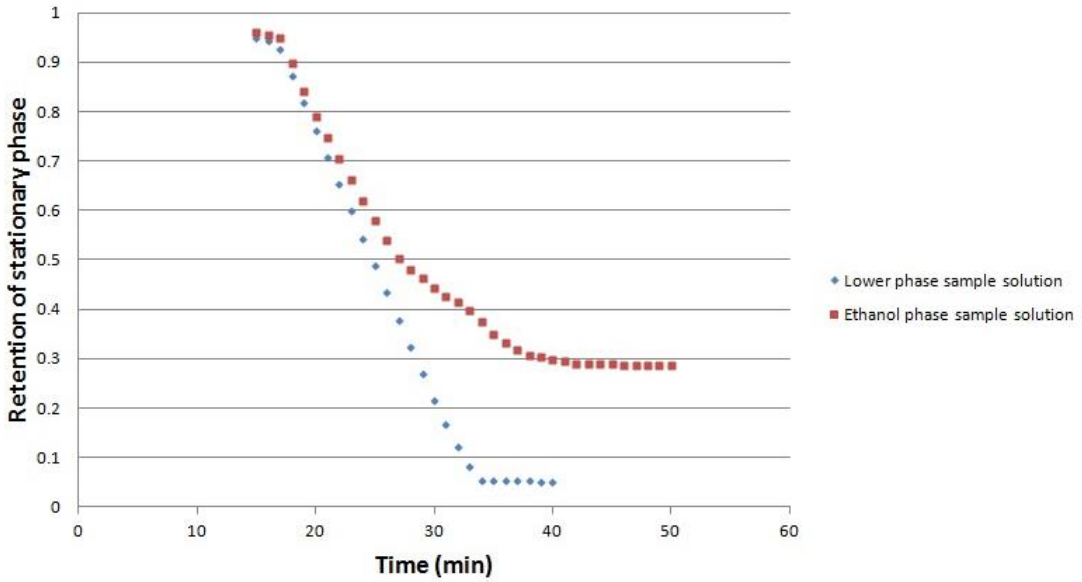

Fig. S1. The effect of different sample solvent to stationary phase stripping. Conditions: column volume: 912 mL; phase system: *n*-hexane-ethyl acetate-ethanol-water (5:2:5:2, v:v:v:v); stationary phase: lower aqueous phase; rotation speed:1250 rpm; detection wavelength: 254 nm; sample volume: 15 mL; sample concentration: 600 mg/mL; flow rate of mobile phase: 50 mL/min; sample injection flow rate 1.0 mL/min for 15 minutes.