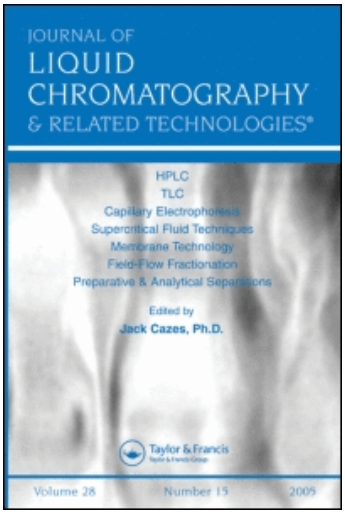

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Separation of Silymarins from Milk Thistle (*Silybum Marianum* L.) Extracted with Pressurized Hot Water using Fast Centrifugal Partition Chromatography

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Abstract: Fast centrifugal partition chromatography was used to separate a class of flavonolignans called silymarins from both a purchased silymarin powder and a crude pressurized hot water extract of milk thistle (*Silybum marianum* L.). Initially, a purchased power of a mixture of the six silymarin compounds was separated with a two-phase solvent system consisting of heptane/ethyl acetate/methanol/water (1:4:3:4 v/v/v/v) in order to verify elution times of the compounds by fast centrifugal partition chromatography. Next, a crude pressurized hot water extract from 10 g of ground seeds of *Silybum marianum* was separated with the same solvent system. The separation from the hot water extract gave yields of silychristin at 70.2% purity, silydianin at 93.7% purity, and a mixture of silybinin and isosilybinin at 96.1% purity.

Keywords: Centrifugal partition chromatography, Flavonolignans, Milk thistle, Pressurized hot water extraction, *Silybum marianum*, Silymarin

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INTRODUCTION

Silymarins are a class of flavonolignans that have medicinal properties and can be found in milk thistle (*Silybum marianum* L.) seeds. Milk thistle is native to the Mediterranean region and grows well in similar climates.[1] Milk thistle, and in particular the silymarins contained in milk thistle seeds, has been used as a medicinal plant to treat diseases of the liver for the past 2000 years.[2] Silymarin has been documented as an effective therapeutic agent for treating liver disease,[2] prostate cancer,[3] and atherosclerosis.[4] The four main compounds that comprise silymarin are: silychristin (SC), silydianin (SD), silybin (SB), and its regioisomer, isosilybin (ISB). Both silybin and isosilybin have stereoisomers A and B and the amalgamation of these four compounds will be denoted as diastereomers throughout this paper. Wallace et al. developed an HPLC method for the detection of all six silymarin components and also a method for the extraction of the silymarins from milk thistle seed using pressurized hot water.[5,6] Pressurized hot water is useful for extracting natural products primarily because of its low environmental impact. In addition, when the temperature of water increases, but remains below the critical point, the dielectric constant also decreases, resulting in a decrease in polarity.[7] When the polarity of water decreases, it is better able to solubilize organic compounds.[8]

Previous work has shown that it is possible to purify gram scale quantities of silymarins from an organic solvent extraction of milk thistle using high speed countercurrent chromatography[9] and that flash chromatography coupled with HPLC can separate gram quantities of the diastereomers from a powdered extract.[10] The present paper describes the bench scale separation, using fast centrifugal partition chromatography (FCPC), of milligram quantities of silymarins from a crude pressurized hot water milk thistle seed extract.

EXPERIMENTAL

Reagents

Analytical grade heptane, ethyl acetate, and methanol were used in FCPC separation. HPLC grade methanol was used for the HPLC analyses. The individual silymarins, silychristin, silydianin, and isosilybin, were purchased from PhytoLab (Hamburg, Germany). Silymarin powder and silybin were purchased from Sigma (St. Louis, MO). Milk thistle seeds were purchased from Horizon Herbs (Williams, OR).

Sample Preparation

Ten grams of milk thistle seeds were ground in a coffee grinder to an average particle diameter of 4 mm according to Standard ASAE S319.3.[11] The seeds were then placed in a Parr reactor (Parr Instrument Company Moline, IL No. 452HC3) and 200 mL of deionized water was added. The reactor was then sealed tightly and pressurized with nitrogen. Silymarin extraction occurred at a temperature of 120°C ($T_{c,water} = 373.9$°C), a pressure of 440 kPa ($P_{c,water} = 22060$ kPa), and an agitation rate of 150 rpm.[5] Extraction was said to begin when the temperature reached 120°C, and lasted 30 minutes; the heat up time was approximately 5 minutes. After extraction, the water was filtered through Miracloth (Calbiochem, Gibbstown, NJ), divided equally between two containers, and then freeze dried. After lyophilization (Model 7753010 Labconco, Kansas City, MO), equal parts of aqueous and organic phase, described below, were added to 1200 mg of freeze dried extract and then placed in at hot water bath at 50°C for 20 minutes to allow the compounds to solubilize in the solvents. Upon removal from the bath, the preparation was filtered with a Whatman No. 4 filter (Whatman, Florham Park, NJ) in order to remove any large particles that may otherwise clog the flow channels in the FCPC rotor.

Solvent System Preparation

The FCPC separation was performed with a two-phase solvent system composed of heptane/ethyl acetate/methanol/water (1:4:3:4, v/v/v/v), and was based on a solvent system proposed by Du et al.[9] The appropriateness of the solvent system was verified by calculating the partition coefficient, K, of four of the compounds present in the purchased silymarin powder. The partition coefficient was determined by adding approximately 50 mg of purchased silymarin powder to 12 mL of the desired solvent system. One milliliter each from the top phase and from the bottom phase were sampled and evaporated to dryness under nitrogen. Each portion was reconstituted with methanol and then analyzed by HPLC. The area under the appropriate peak for each compound was used to calculate K, where K is the area of the peak for the upper phase divided by the area of the peak for the lower phase. The separation factor, $S = Ki/Kj$ when $Kj < Ki$, where Ki is the partition coefficient for component i and Kj is the partition coefficient for component j, should be greater than 1.5 for an acceptable resolution.[12]

The solvent system for FCPC was prepared in a separatory funnel, allowing for full mixing and equilibration. Upon separation, the solvents were promptly used. The organic upper phase was used as the stationary

phase, while the aqueous lower phase was used as the mobile phase, thus, the FCPC was operated in descending mode.

FCPC

FCPC experiments were performed using a bench scale Fast Centrifugal Partition Chromatograph, FCPC (Kromaton, Angers, France). The solvents were pumped into the FCPC with a Waters 510 pump (Waters Milford, MA). While operating in descending mode, the 200 mL column was first filled with stationary phase at 16 mL/min with the rotor set to spin at 200 rpm. Once the rotor was completely filled with stationary phase, the mobile phase was introduced at 4 mL/min with the rotor spinning at 1300 rpm until the column attained equilibrium of both phases. The sample was then placed in the 10 mL sample loop and injected onto the column. The eluent was monitored with a UV detector, set to 290 nm (VUV24 Reflect Scientific, Orem, UT) equipped with a preparatory flow cell. Fractions were collected in a Waters Fraction Collector III (Milford, MA).

HPLC Analysis

The HPLC system consisted of a Waters Alliance 2690 system equipped with a Waters 996 photodiode array detector, controlled by Empower chromatographic software (Milford, MA). The detection wavelength was set to 290 nm. Separation of the silymarins was obtained with a Symmetry (Waters, Milford, MA) C_{18} precolumn placed in series with a Symmetry (Waters, Milford, MA) C_{18} column (150×4.6 mm, 5 mm), set at 40°C. A 10 μL sample volume was injected. Solvent A was 80:20 water:methanol and solvent B was 20:80 water:methanol and followed the gradient described by Wallace et al.[13] The flow rate of solvent was set to 0.75 mL/min.

RESULTS AND DISCUSSION

Du et al.[9] proposed a solvent system for the separation of silymarins, which consisted of 1:4:3:4 (v/v/v/v) hexane, ethyl acetate, methanol, water. This study used heptane in place of hexane, since it has been shown that replacing heptane with hexane has no noticeable effect on separation in FCPC.[14] As a prelude to use in the FCPC, variations in the proposed solvent system were investigated and the results are shown in Table 1. The variations studied showed that the separation factor between silybin A and silybin B decreased when the concentration of heptane in the solvent

Table 1. Partition coefficients and separation factors of silymarins from variations on a solvent system of heptane/ethyl acetate/methanol/water

	Partition coefficient, K			
Solvent system hep: EtOAc: MeOH: H_2O	Silychristin (1)	Silydianin (2)	Silybin A (3)	Silybin B (4)
1:4:3:4	0.231	0.172	0.699	0.825
1.2:4:3:4	0.196	0.263	0.619	0.651
1.5:4:3:4	0.105	0.142	0.336	0.334
2:4:3:4	0.096	0.127	0.347	0.338

	Separation Factor, S		
	S12	S23	S34
1:4:3:4	1.344	4.069	1.180
1.2:4:3:4	1.339	2.347	1.052
1.5:4:3:4	1.356	2.363	1.006
2:4:3:4	1.323	2.745	1.028

Hep = heptane, EtOAc = ethyl acetate, MeOH = methanol

mixture was increased. Table 1 also shows that the ratio of the solvents, within the system, played an important role in improving the separation. The separation factor and the partition coefficients were most favorable for the 1:4:3:4 heptane, ethyl acetate, methanol, water system, and thus the original solvent system ratio proposed by Du et al.,[9] with heptane replacing hexane, was used during separation.

Figure 1 displays the chromatogram of the purchased silymarin powder that was used for initial FCPC trials. The HPLC trace resulted from loading 10 μL of a 15 mg/mL preparation of silymarin in methanol. Peaks for silychrisitin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B were detected at 16.1, 17.8, 24.0, 25.1, 28.6, and 29.6 minutes, respectively. The separation obtained in Figure 1 is similar to the results obtained by Wallace et al.,[13] showing good separation between the diastereomers.

The purchased silymarin power was then separated using FCPC. Exactly 75 mg of the Sigma powder was added to a mixture of 2.5 mL aqueous phase and 2.5 mL of organic phase, resulting in a final concentration of 15 mg/mL, which was of identical concentration to that presented in Figure 1. The mixture was immediately injected after equilibrium of the two phases in the FCPC rotor was attained. Equilibrium was achieved when the stationary phase no longer exited the system. The fraction collector was adjusted such that 4 mL fractions were

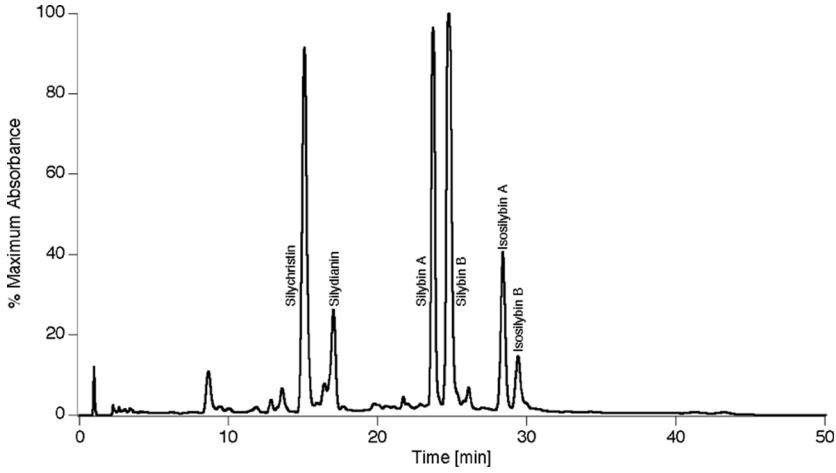

Figure 1. HPLC trace of a solution of 15 mg/mL purchased silymarin in methanol; pertinent peaks are labeled.

obtained. In total, 90 fractions were collected during the 90 minute FCPC run. The fractions were dried, reconstituted in methanol, and analyzed by HPLC. The process was repeated in duplicate, both traces were almost identical, and the trace from the second FCPC separation is shown in Figure 2 with the peaks labeled accordingly. In analyzing the separation,

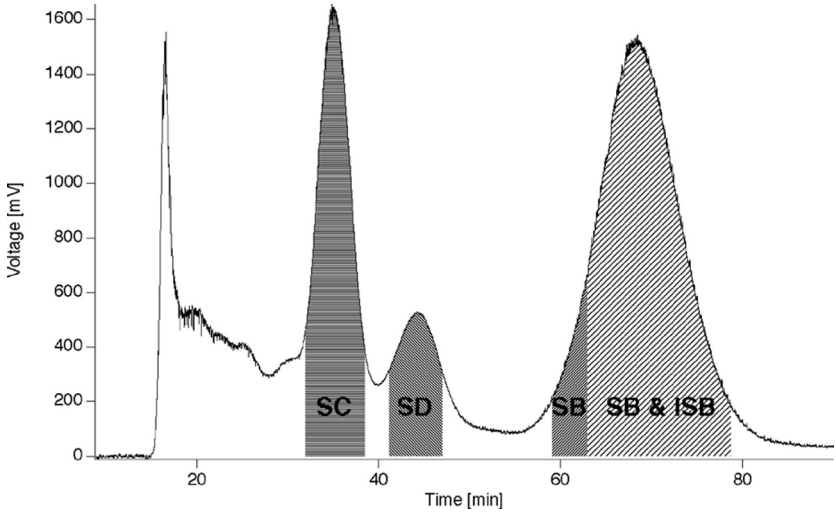

Figure 2. FCPC trace of sigma silymarin powder with peaks highlighted to show compound eluting.

silychristin was the first compound to elute between 33 and 38 minutes; the recovered silychrisitin was 85.7% pure with the remaining 14.3% consisting of a mixture of silydianin and silybin. Silydianin eluted between 43 and 48 minutes and the purity of the collected fraction was calculated to be 62.9%. The remaining 37.1% of this peak was a mixture of the silymarins. The lower purity of silydianin as compared to that of silychristin could be attributed to the lower initial concentration of the silydianin. It appears that a better separation occurs if, upon injection, a higher concentration of the compound to be purified, with respect to the other compounds, is present. Fractions from 58–63 minutes were comprised of silybin B at 78.6% purity, with the remainder consisting mostly of silybin A. The remainder of the final peak, the fractions collected between 64 and 78 minutes, consisted of a mixture of silybin A, silybin B, isosilybin A, and isosilybin B at 96.1% purity. The stationary phase retention was 73.7%.

The pressurized hot water extract of milk thistle seeds was next separated on the FCPC. For this separation, the liquid extract was lyophilized and 1200 mg was reconstituted in 6 mL composed of equal parts aqueous and organic phase. One mL was sacrificed for HPLC analysis. The HPLC trace from the crude hot water extract is shown in Figure 3. Unlike the purchased silymarin powder, the crude hot water extract included additional compounds, and contained less silybin A and B. A difference in the relative concentrations of flavonolignans in the milk thistle seed is not uncommon, as cultivation practices, seed type, rainfall,

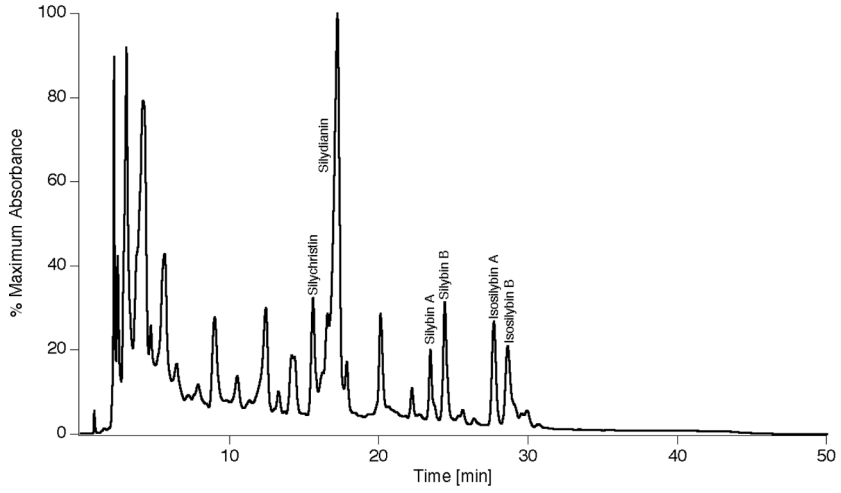

Figure 3. HPLC trace of pressurized hot water extract with relevant peaks labeled.

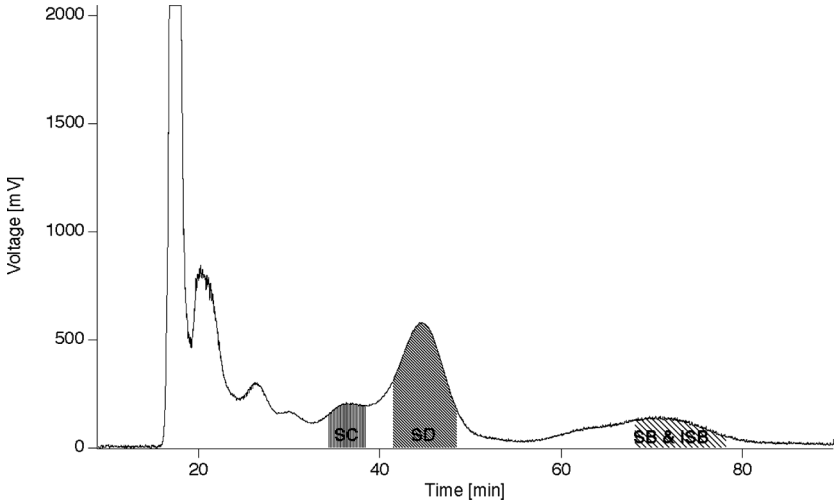

Figure 4. FCPC trace of pressurized hot water milk thistle seed extract with compounds within relevant peaks highlighted.

soil conditions, seed quality, and geographic location impact the phytochemical concentrations.[15] Carrier et al.[15] also reported differences in the concentrations of milk thistle fruits grown in separate years. The most abundant silymarin component was silydianin, which was present at a concentration of 0.778 mg/mL.

The remaining 5 mL of reconstituted extract was injected into the FCPC for separation. Identical FCPC conditions were used as those described for the silymarin powder separation. The 90 fractions were analyzed by HPLC and Figure 4 presents the FCPC trace with the peaks labeled accordingly. The hot water extract was run in duplicate and the FCPC traces for each were almost identical. The compounds in the hot water extract eluted at identical times as those shown in Figure 2. Silychristin, at 70.2% purity, eluted between 33 and 38 minutes. The remainder of this peak was silydianin and silybin. Silydianin eluted between 43 and 48 minutes, which afforded a total of 1.5 mg of 93.7% pure silydianin, with the remaining 6.3% consisting of silychristin and other compounds that are absorbed at 290 nm but are not flavonolignans. Between 68 and 78 minutes a mixture of silybin A, silybin B, isosilybin A, and isosilybin B eluted at a purity of 96.1%.

Unlike the purchased powder, the crude hot water extract separation did not yield fractions containing mostly silybin B, which is likely because the concentration of silybin B injected with the extract, and present in the milk thistle seed, was initially lower than as was present in the powder. The stationary phase retention was 69.7%. Figure 5 displays HPLC traces

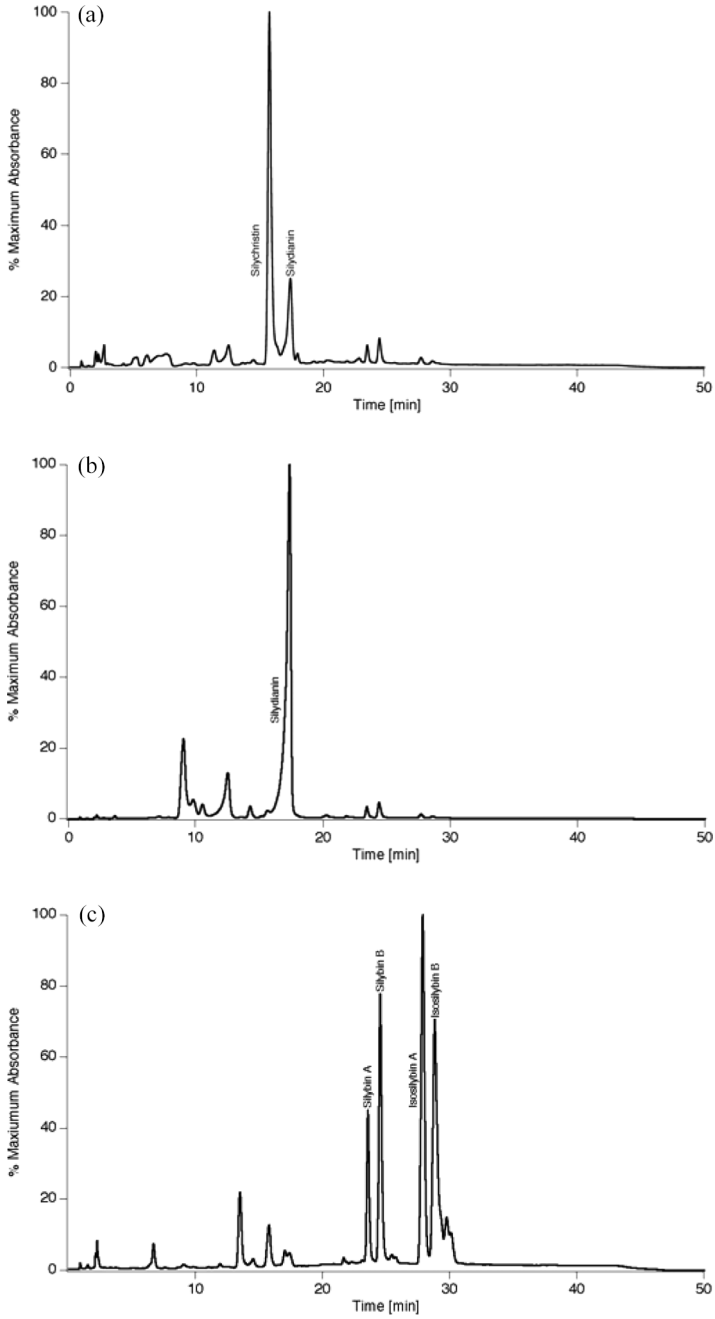

Figure 5. HPLC analyses of the major peaks from the FCPC trace: (**a**) displays minute 36 showing silychristin at 70.2% purity, (**b**) is minute 46 with silydianin at 93.7% purity, (**c**) is minute 73 with a mixute of the silybin and isosilybin at 96.1.0% purity.

for FCPC separated silychristin, silydianin, and a mixture of silybin and isosilybin. Figure 5a shows the silychristin peak at minute 46, Figure 5b is the silydianin peak at minute 46, and Figure 5c displays the silybin/isosilybin peaks at minute 73.

The analyzed fractions demonstrate that FCPC has the capability to separate silychristin and silydianin from the silybin and isosilybin silymarins at a relatively high purity, and is especially useful in separating silydianin from a crude extract. Previous work with preparatory scale silymarin separation by Du et al.[9] using a high speed counter current chromatography unit did not present silydianin as a separate component, but did show separation of silybin A and silbyin B from isosilybin A and isosilybin B at 95.7% and 89.7% purity, respectively.[9] Graf et al.[10] used a hybrid procedure consisting of flash chromatography combined to preparative HPLC in order to purify silybin A (4.1 g), silybin B (4.9 g), isosilybin A (4.4 g), and isosilybin B (3.7 g) at greater than 97% purity for each compound from 10 kg of powdered extract. Achieving such large quantities of pure silybin A and B required a succession of flash chromatography, precipitation, centrifugation, and 154 injections, with 75 mg in each injection, on preparative HPLC; all the while, accumulating and combining the fractions to obtain the 4.1 g of silybin A and 4.9 g of silybin B. Isosilybin A purification required a succession of flash chromatography, precipitation, centrifugation, and 10 injections of 485 mg each on preparative HPLC. Isosilybin B separation required the same initial steps, but required an additional 20 injections of 153 mg on preparative HPLC.[10] In this work, one FCPC purification step isolated a sufficient amount of silydianin from a pressurized hot water extract at high enough purity to be used in an *in vitro* study that determines its ability to quench reactive oxygen species (ROS) production.

Further separation of the diastereomers may be required in order to obtain individual compounds for further study, but one FCPC run was able to afford a high quality mixture of the diastereomers. Two potential methods to obtain higher quantities of the purified silymarins are to: 1) extract more than 10 g of seed during the pressurized hot water extraction or, 2) combine the two containers that were freeze dried and run the entire crude extract at once. Either of these methods may afford higher quantities of silymarins with possibly a higher purity.

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