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A Novel Hybrid Chromatography−Crystallization Process for the Isolation and Purification of a Natural Pharmaceutical Ingredient from a Medicinal Herb

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Abstract:

In this work, a hybrid chromatography−crystallization separation process is suggested and is used to isolate and purify the antimalarial drug artemisinin from the Chinese medicinal herb *Artemisia annua*. Extraction of the aerial parts of *A. annua* by an organic solvent yielded a complex mixture of natural products. A hybrid separation process was therefore proposed to pursue higher recovery and purity of artemisinin. The crude extract was first separated by flash column chromatography, and the fractions containing artemisinin were then separated with a two-step antisolvent crystallization process to isolate artemisinin of high purity. The antisolvent crystallization process was designed on the basis of the solubility of artemisinin in different solvents and the crystallization behaviour of the two polymorphs of artemisinin. The first step in the crystallization process was used to remove impurities from the solution, and the second step was used to isolate the stable orthorhombic form of artemisinin. The results of the present work demonstrate the great potential of combining the advantages of column chromatography and crystallization to isolate and purify a target compound from plant extracts. The separation efficiency of the process can be improved by the synergistic effect of chromatography and crystallization processes.

Introduction

Natural products have been the primary source for drug discovery in the past century. Even in the recent 25 years around 50% of the new chemical entities that came to the market are natural products, natural products derivatives, or synthetic analogues of natural products.[1] Therefore, the traditional medicinal systems of China and India, which are based on thousands of years' experience for natural materials, may serve as a source of inspiration for drug discovery.[2] The potential of this novel way for drug discovery has been demonstrated in the successful development of the antimalarial drug artemisinin from the Chinese medicinal herb *Artemisia annua* L.

Malaria is an age-old disease that has had a large influence on the economies and development of many countries, especially in sub-Sahara Africa. In 1997, nearly 1 million people died of malaria in sub-Sahara Africa and the mortality of this disease has risen in recent years.[3] The reason for the increasing mortality is that the parasites causing malaria have developed resistances to most of the traditional antimalarial drug substances. The discovery of artemisinin in the 1970s[4,5] provided a new class of highly effective antimalarial medicines that are not likely to be affected by resistance in the near future. Nowadays, artemisinin-based combination therapies have been promoted world widely for treating drug-resistant malaria.[6−8]

In addition to the antimalarial effect, the anticancer activity of artemisinin and its activity against other parasite species have also been reported.[9] It has been observed that artemisinin has an effect on coccidian, which is a severe parasitic infection in poultry.[10] The Danish Council for Strategic Research has recently launched a project aiming at developing prototype products based on bioactive compounds from *A. annua* for the treatment of parasitic and bacterial diseases in poultry and livestock. Consequently, it is expected that the worldwide demand of artemisinin will be continuously increasing. On the other hand, the total synthesis of artemisinin that has been discovered in the 1980s[11] consists of at least eight synthetic steps, and the yield of the process is relatively low. Currently, the extraction of artemisinin from *A. annua* is the only source of artemisinin in the market. In order to develop a reliable market supply of artemisinin with a reasonable price, it is of paramount importance to carry out research towards the following goals: first, to increase the concentration of artemisinin in *A. annua* by optimizing the cultivation and harvest conditions, selecting high-yielding cultivars or creating transgenic plants; second, to improve the efficiency of the production process of artemisinin from the plant materials. Several research programs have been set up aimed at cultivating *A. annua* containing more artemisinin, and some high-yield *A. annua* is already available

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on the market.[12] However, little has been done on the optimization of the isolation process to improve artemisinin recovery yields from plant extracts.[13]

A typical isolation and purification process for artemisinin from *A. annua* consists of the extraction of the plant material following isolation and purification of artemisinin from the crude extracts. The isolation of artemisinin in high purity is very challenging due to the fact that the crude extract is a complex mixture of natural products many of which have chemical structures very similar to that of artemisinin, such as artemisinic acid, arteannuin B, artemisitene, deoxyartemisinin, and dihydroartemisinin, and hence chemical and chromatographic characteristics similar to those of artemisinin.[14] Purification of the crude extract by chromatographic methods followed by crystallization of the pure artemisinin is usually carried out in separated steps. In principle, complete separation of the compounds from the crude extracts can be achieved by column chromatography (CC) using a sufficiently long column. However, the operational costs of the separation process can be very high, thus increasing the total manufacturing cost of the product. In practice many of the extraction plants located in the traditional cultivation and processing areas of *A. annua*, e.g., China and Vietnam, have excluded the chromatographic purification step and solely use crystallization for separation. In order to produce a high-purity product, artemisinin has to be recrystallized several times, which leads to significant loss of the product and also large volumes of wastes containing organic solvents. It has been reported from two extraction plants in Vietnam that the yield of artemisinin was approximately 0.2−0.3% based on dry plant. However, the analysis of these *A. annua* plants showed that the concentration of artemisinin in the dry plant was 0.8−1.0%.[15] Obviously, the profitability of this process might become unacceptable in countries where the cost for plant cultivation is much higher than that in Vietnam and the environmental regulations are stricter. The development of an artemisinin production process in such countries requires a well-designed, more efficient and environmental friendly isolation and purification procedure.

As powerful separation techniques, both chromatography and crystallization have their own advantages and limitations. On the one hand, chromatography is capable of fractionating a very complex mixture containing hundreds of compounds; however, the complete separation of the compounds requires a long column and often several separations by CC, thus producing high capital and operational costs. On the other hand, crystallization is appropriate for the isolation of a product with high purity from a multicomponent mixture, but the separation and yield are strongly affected by the presence of other compounds, especially those possessing molecular structures or solubility characteristics similar to those of the crystallizing product. Therefore, the hybrid between chromatography and crystallization can have a synergistic effect, which may lead to an improved recovery and productivity. Regardless of the great potential of this hybrid separation process, little work has been conducted on its application and design. The application of this hybrid process to the enantioseparation of racemic mixtures has been reported by Lim et al.[16] and Lorenz et al.[17] A systematic procedure for synthesizing hybrid chromatography−crystallization separation processes was proposed by Fung et al.[18] However, the mixtures studied in these works contain either two enantiomeric forms or three different compounds. The systematic design procedure proposed by Fung et al.[18] requires some properties of the mixture to be separated, such as the chemical structure and molecular weight of the components and the composition of the feed solution. The separation of the crude extracts normally encountered in the production of natural products is much more complex and challenging. On the one hand, there might be hundreds of components in the extract; on the other hand, most of these components are unknown, and thus no properties of these compounds are available. Therefore, efforts are needed to study the feasibility of applying such a hybrid separation process and, furthermore, to develop it as a robust and efficient separation approach for the isolation of bioactive compounds from a complex multicomponent mixture.

The objective of the present work is to explore the feasibility of combining the advantages of both chromatography and crystallization to generate a hybrid separation process for the isolation and purification of artemisinin from *A. annua*. The solubility of artemisinin in different solvents and solvent mixtures was measured and was then used to design a two-step antisolvent crystallization procedure. On the basis of the solubility and crystallization behavior of artemisinin, a hybrid chromatography−crystallization separation process was proposed. The performance of this hybrid separation process was investigated through a laboratory-scale isolation of artemisinin from an extract of *A. annua*.

Materials and Methods

Chemicals. Artemisinin (purity >99%) was obtained from My Dinh Extraction plant in Vietnam and Xiang Xi Holley Pharmaceutical Co. Ltd. in China. The organic solvents used in all experiments were of HPLC grade from Fisher Scientific (Slangerup, Denmark). Water was purified using a SG Ultra Clear Basic UV system (Holm & Halby, Germany).

Solubility Measurement. The solubility of pure artemisinin in various organic solvents and solvent mixtures was measured at room temperature. The organic solvents used include ethanol (EtOH), methanol (MeOH), dichloromethane (DCM), acetonitrile, acetone, ethyl acetate (EtOAc), hexane, chloroform, 1-butanol, 1-propanol and 2-propanol. The mixed solvents were binary mixtures of hexane−EtOH, EtOH−water, EtOAc−acetonitrile, and acetonitrile−water. Ten milliliters of solvent with excess artemisinin was added to a 25 mL flask. The liquid−solid suspension was then kept under mixing in a water

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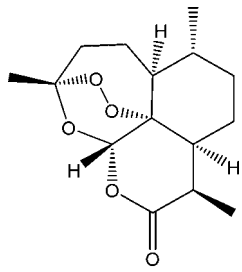

Artemisinin

Figure 1. **Chemical structure of artemisinin.**

bath at 24.5 °C for 2 h. The clear solution was then removed with a syringe filter. One mL clear solution was sampled and after dilution, it was analyzed on an Agilent 1100 series high-performance liquid chromatography (HPLC) system equipped with a photodiode array (PDA) detector (Agilent, Waldbronn, Germany) to determine the solubility of artemisinin. Separations were carried out on a reverse phase Gemini 3 μ C18 110A column (3 μ particle size 100 mm × 3.0 mm i.d., Phenomenex, Torrance, CA, U.S.A.). The column temperature was maintained at 35 °C, and the mobile phase consisted of water−acetonitrile (4:6). Separations were performed by isocratic elution with a flow rate of 0.5 mL/min. Injection volume: 10 μL. Detection: UV at 210 nm.

Plant Material Extraction. *A. annua* was cultivated and harvested at the Department of Horticulture, Research Centre Aarslev, Aarhus University, Aarslev, Denmark, in 2006 and 2007, and was stored at −20 °C until extraction. The aerial parts were cut into small pieces and extracted with DCM at room temperature for 24 h. The ratio of the plant material and the extraction solvent was 150 g aerial parts/800 mL solvent. The plant material was re-extracted once using similar conditions. The extracts were then combined and dried under vacuum.

Hybrid Chromatography−Crystallization Separation Process. The combined extracts were separated using flash CC on silica gel (Merck silica gel 60, particle size ≤0.063 mm) and eluted with DCM. In the first separation, the ratio of adsorbent to solute was 30:1, and the column was eluted with 6 L of DCM. In the second separation, the ratio of adsorbent to solute was decreased to 15:1, and the total volume of the eluted solvent was 4.3 L. The fractions were monitored by thin layer chromatography (TLC). TLC plates were normal-phase silica gel 60 F_{254} 20 cm × 20 cm from Merck (KGaA, Germany), and the mobile phase was DCM. Plates were inspected by UV light followed by visualization with vanillin (30 g of vanillin, 500 mL of EtOH, 5 mL of conc. H_2SO_4). The fractions rich in artemisinin and containing the same compounds other than artemisinin were combined and brought to the crystallization step. The purity of the crystals was analyzed by TLC and HPLC-PDA using similar chromatographic conditions as described above (mobile phase was changed to be water−acetonitrile (6:4)).

Results and Discussion

Solubility of Artemisinin in Pure and Mixed Solvents. Artemisinin is an endoperoxide sesquiterpene lactone (Figure 1). It has been reported that artemisinin is capable of forming

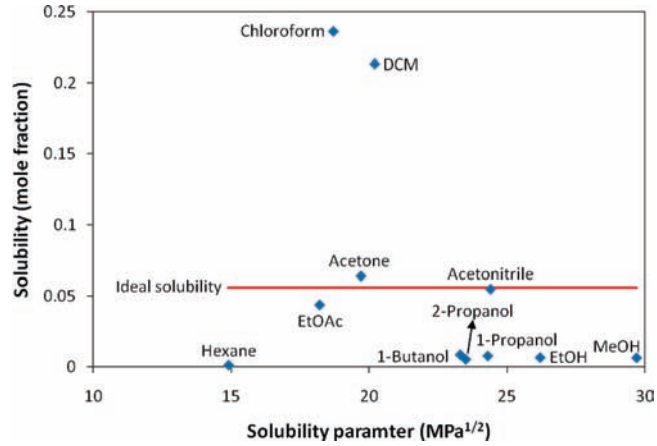

Figure 2. **Solubility of artemisinin in different organic solvents at room temperature (24.5 °C).**

two polymorphs, an orthorhombic form (QNGHSU02)[19] and a triclinic form (QNGHSU01).[19] The received artemisinin crystals were analyzed with X-ray powder diffraction (XRPD) and identified as the orthorhombic form, which is the thermodynamically stable form at room temperature. The artemisinin crystals were used as received.

The solubilities of artemisinin in different organic solvents are shown in Figure 2 against the Hildebrand solubility parameters of the solvents, which briefly represent the polarities of the solvent. Artemisinin has been reported to be insoluble in water and oil but soluble in most aprotic organic solvents. It can be seen from Figure 2 that the polarity alone cannot account for the solubility of artemisinin. The two structurally similar solvents, DCM and chloroform, provided remarkably high solubility of artemisinin, which was about 5 times higher than the solubility of artemisinin in EtOAc and acetone, although the Hildebrand solubility parameters of these four solvents are close to each other. It has been reported that acetone and chloroform can form a strong complex in their mixtures.[20,21] Since artemisinin contains a carbonyl group, it is possible that similar complexes are also formed between the molecules of chloroform or DCM and artemisinin, which may explain the remarkably high solubility of artemisinin in these two solvents. The solubilities of artemisinin in acetone, EtOAc, and acetonitrile are close to the ideal solubility of artemisinin, which implies that the intermolecular interaction between the solute and the solvent is not significantly different from the intermolecular interaction of the solvent. The solubilities of artemisinin in the alcohols (EtOH, MeOH, 1-butanol, 1-propanol) and hexane are low, probably due to the solvents' polarities being different from that of artemisinin.

The solubility of artemisinin in binary solvent mixtures of hexane−EtOH, EtOH−water, EtOAc−acetonitrile, and aceto-

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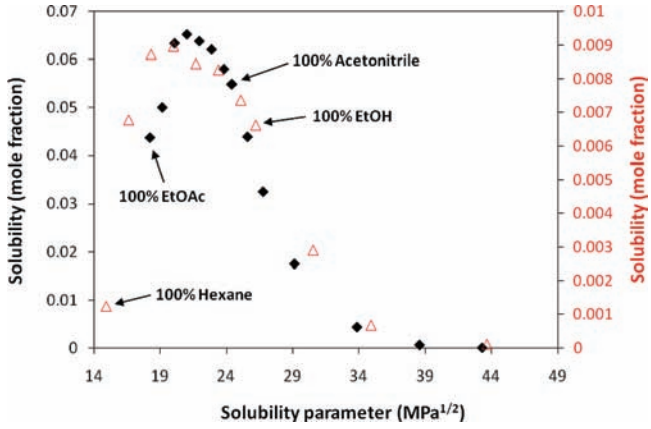

Figure 3. **Solubility of artemisinin against the solubility parameters of the solvent mixtures: (◆) EtOAc−acetonitrile and acetonitrile−water (primary *Y*-axis); (Δ) hexane−EtOH and EtOH−water (secondary *Y*-axis).**

nitrile−water was investigated. The solubilities are shown in Figure 3 against the Hildebrand solubility parameter of each of the mixed solvents. The Hildebrand solubility parameter of the mixed solvents is computed as follows:

$$\bar{\delta} = \frac{\sum_i \phi_i \delta_i}{\sum_i \phi_i} \qquad (1)$$

where ϕ_i is volume fraction and δ_i is the Hildebrand solubility parameter of the component solvent.

It has been observed that the solubility of a substance in a solvent mixture can be much higher than its solubility in either of the pure solvents.[22,23] The underlying principle of this phenomenon is that a certain mixture of two solvents, one more polar and another less polar than the solute substance, will best match the polarity of the solute. This solvent mixture will provide the highest solubility of the solute. The binary solvent mixtures studied in this work covered a wide range of polarity. It can be observed that the solubility profiles shown in Figure 3 exhibit one maximum in the solvent mixtures with relatively low polarity, and the solvent solubility parameter corresponding to this maximum solubility is between 19−21 $MPa^{1/2}$ regardless of the nature of the component solvents. This observation suggests that the shape of the solubility profile in a solvent mixture is governed by the overall polarity of the solvent. However, the absolute value of solubility is affected by the nature of the component solvents. As it can be seen from Figure 3 the solubility of artemisinin in the mixtures of hexane−EtOH is much lower than that in EtOAc−acetonitrile. For the mixtures of EtOH−water and acetonitrile−water, the solubility of artemisinin remarkably decreased with increasing water concentration, which suggested the feasibility of performing an antisolvent crystallization of artemisinin by using water as the antisolvent.

Artemisinin is capable of forming two different polymorphs; the orthorhombic form with higher density and lower solubility in water is considered as the thermodynamically stable form at room temperature, and the triclinic form is the metastable one.[19] The antisolvent crystallization of artemisinin was performed from acetonitrile solution by using water as the antisolvent. It was observed that the orthorhombic form always crystallized out, regardless of the feeding rate of the antisolvent.

Hybrid Chromatography−Crystallization Separation Process for the Isolation and Purification of Artemisinin from *A. annua.* DCM was selected as the solvent for extraction of artemisinin from the leaves of *A. annua* due to its high solubilisation potential and high volatility, which can minimize the solvent consumption and also the energy costs for the solvent recycling. A series of TLC was performed on the extracts of *A. annua* to test the separation effect of the different solvents and solvent mixtures. The separation efficiencies of the pure organic solvents used in the solubility experiments and binary mixtures of EtOAc−MeOH were tested. Finally it was decided to use pure DCM as the eluent for CC due to its good separation efficiency and also to facilitate the recycling of the eluent. In the first separation by CC with the ratio of adsorbent to solute 30:1, the TLC showed that 2500 mL of the eluate contained artemisinin, whereas in the second separation, with the ratio of adsorbent to solute 15:1, the total volume of the eluate containing artemsinin was 1700 mL. On the basis of the TLC analysis, the eluate fractions containing the same components were combined, and the composition of the combined fractions was analyzed by HPLC. The volume and the concentration of artemisinin in the combined fractions from the first and second separation on CC are shown in Table 1. The HPLC chromatograms of the four combined fractions from the first separation on CC are shown in Figure 4. It can be seen that all the combined fractions are complex and contain several other compounds besides artemisinin. The second separation on CC with a lower ratio of adsorbent to solute (15:1) yielded a much more complex mixture of compounds both in number and concentration. In principle, the separation efficiency of chromatography can be improved by selecting an appropriate stationary phase and/or by modifying and optimizing the mobile phase parameters, such as composition, pH, and gradient profile. However, both stationary phase selection and mobile phase optimization require the composition of the feed, which is an unknown variable in the present work. Therefore, for the crude extract separation, it is advantageous to utilize a hybrid separation process of chromatography−crystallization, which can combine the advantages of both techniques.

Cooling crystallization has been used to separate artemisinin from the EtOH extracts of *A. annua*.[15] In the present work, antisolvent crystallization was selected to separate the artemisinin crystals from the mixtures resulting from the separation by CC. One of the most important advantages of antisolvent crystallization compared with those of cooling and evaporative crystallization processes is that antisolvent crystallization has much lower energy consumption. Since supersaturation is generated by mixing the antisolvent into the solution, no energy is required to change the temperature or to create a vacuum, which is needed in cooling or evaporative crystallizations. As shown in Figure 5, a two-step crystallization process was utilized to isolate artemisinin. The combined fractions resulting from

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Table 1. **Volume and Concentration of Artemisinin in the Combined Fractions (mixtures) from the CC Separations**

	mixtures from CC (first separation)					mixtures from CC (second separation)			
combined mixtures	S1CF1	S1CF2	S1CF3	S1CF4	S1CF5	S2CF1	S2CF2	S2CF3	S2CF4
artimisinin concn (mg/mL)	0.99	0.90	0.61	0.43	0.03	1.08	1.63	0.51	0.2
volume (mL)	490	550	185	356	750	185	355	355	700

separation by CC were treated individually, following the procedures shown in Figure 5, due to their different compositions. The aim of the first-step antisolvent crystallization process was to replace the water-immiscible solvent, DCM, with a water-miscible solvent, acetonitrile. After evaporating 80% of the DCM from the solution, crystallization was performed by feeding acetonitrile to the solution, which caused the precipitation of a white powder that was identified as 1-nonadecanol by ^{1}H and ^{13}C NMR spectroscopy. After filtration, the clear filtrate was evaporated under vacuum to completely remove DCM, which also led to the precipitation of more 1-nonadecanol. A TLC analysis showed that 1-nonadecanol and artemisinin have similar chromatography characteristics, and therefore the concentration of the former in the combined fractions was quite high. However, the solubilities of 1-nonadecanol and artemisinin in acetonitrile were very different, which led to a good separation of 1-nonadecanol through this first-step antisolvent crystallization process. This observation demonstrated the potential of combining the advantages of chromatography and crystallization, since the compounds possessing similar chromatography characteristics might have different solubility behaviour in certain solvents, and vice versa.

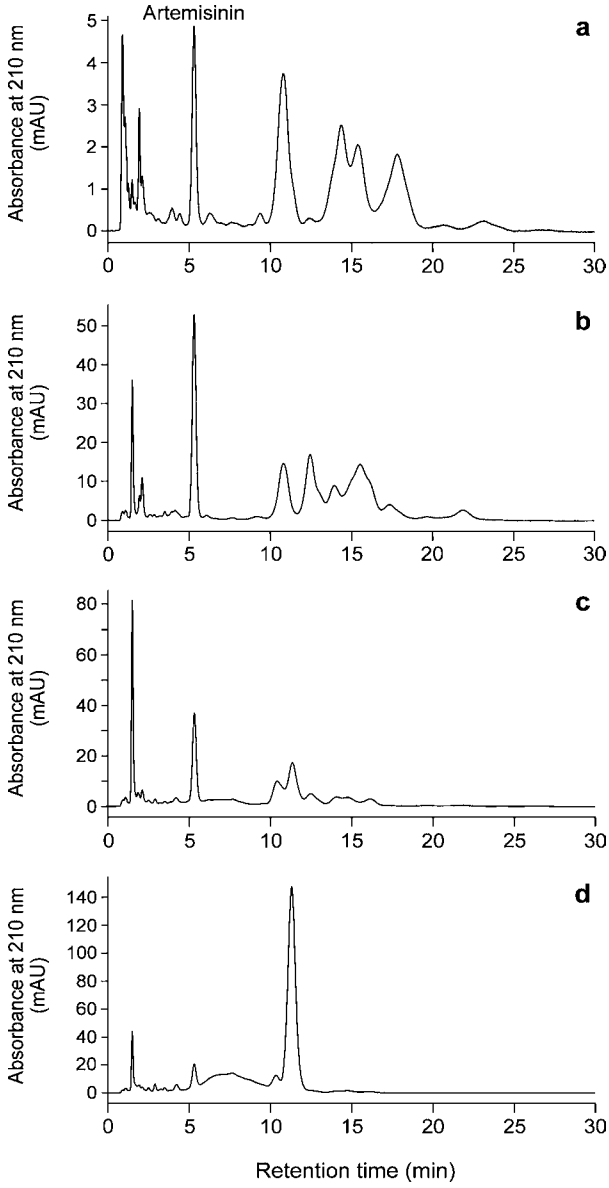

Figure 4. **HPLC chromatograms of the combined fractions (Table 1) from the first CC separation with a ratio of adsorbent to solute of 30:1. (a) S1CF1 (b) S1CF2, (c) S1CF3, and (d) S1CF4.**

The second step in the antisolvent crystallization was induced by feeding water to the acetonitrile solution. Artemisinin crystals were produced from this step, and the purity of these products was determined by HPLC and TLC. The yield of artemisinin was calculated as follows:

$$\text{Yield} = \frac{M_{\text{art}}X}{V_{\text{i}}C_{\text{i}}} \qquad (2)$$

where M_{art} and X are the mass and purity of the raw artemisinin crystals produced from the crystallization, respectively, and V_{i} and C_{i} are the volume and artemisinin concentration of the initial solutions which are shown in Table 1.

From the combined solutions S1CF1 and S1CF2, artemisinin crystals with 95% purity were harvested when the volume ratio of water and acetonitrile was 1:1. Further feeding of water resulted in the crystallization of more artemisinin; however, purity was lower at 73%. For the solutions from the second CC separation, the artemisinin crystals were collected when the volume ratio of water and acetonitrile in the solution was 4:1. As shown in Figure 5, the purity of the artemisinin crystals was 40−71%, depending on the initial composition of the mixture. The filtrate was kept in a refrigerator at 5 °C overnight, which yielded more artemisinin. The residual solutions from all experiments were analyzed by HPLC, and the concentration of artemisinin was lower than 0.2 mg/mL.

The total yield of artemisinin resulting from the first and the second separation by CC were similar, i.e. about 80%. However, the average purity of the artemisinin crystallized from the combined fractions resulting from the first separation by CC was much higher than that from the combined fractions resulting from the second CC separation. This result is consistent with the composition of the combined fractions as the first separation by CC with a higher ratio of adsorbent to solute clearly resulted in more pure fractions. The better separation effect of the first CC separation subsequently facilitated the crystallization of artemisinin.

In order to further understand the underlying mechanism of the second-step antisolvent crystallization process, the artemisinin concentration change during the crystallization was followed by taking samples with time. The artemisinin concentration profile during the antisolvent crystallization is shown in Figure 6 together with the solubility of artemisinin in acetonitrile−

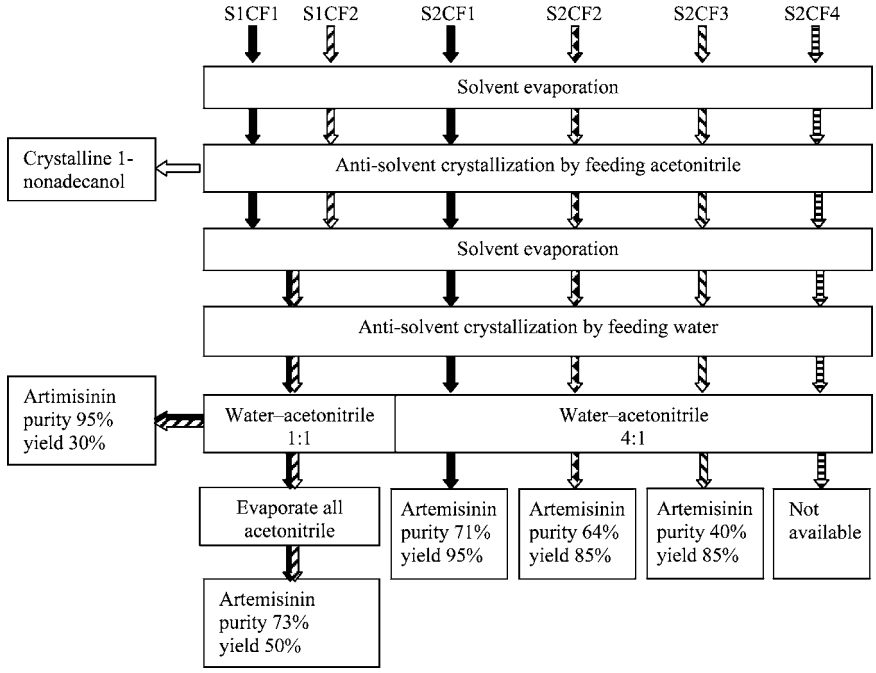

Figure 5. **Schematic process flow diagram of the two-step crystallization process for the isolation of artemisinin.**

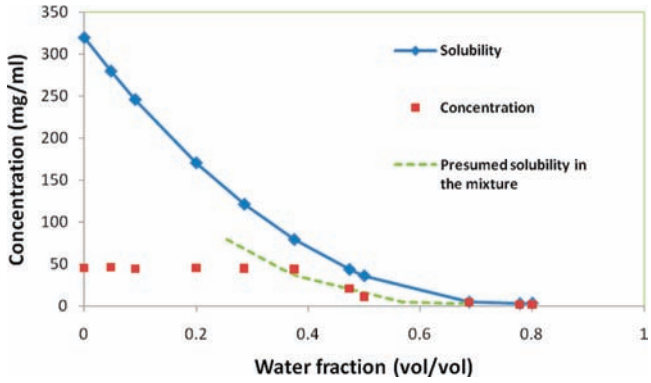

Figure 6. **Solubility of pure artemisinin in acetonitrile−water mixtures, and the concentration profile during the antisolvent crystallization of artemisinin in the mixture S2CF2 from the second CC separation.**

water mixtures. It can be observed that the concentration of artemisinin started to decrease when the water fraction reached 40 vol % at which point the concentration was still below the solubility curve. This is not a surprising result, considering the multicomponent nature of the mixture in which the crystallization was conducted. The existence of other compounds obviously decreased the solubility of artemisinin in the acetonitrile−water mixtures. A presumed solubility of artemisinin in the multicomponent environment has been drawn in Figure 6 on the basis of the de-supersaturation profile after the occurrence of the crystallization.

Since a large percentage of the produced artemisinin crystals was with a relatively low purity, further purification was done using recrystallization. The raw crystals were dissolved in acetonitrile at room temperature, and then water was fed to the solution until the volume ratio of water to acetonitrile was greater than 4:1. The crystallized artemisinin was harvested by filtration and was analyzed by HPLC. The HPLC chromatograms of the pure artemisinin from the supplier, the artemisinin crystallized out from the combined eluates from the first (purity 95%) and the second (purity 64%) separation by CC, respectively, and the crystals after two times purification with recrystallization are shown in Figure 7. It can be seen that the compounds that are more polar than artemisinin can be effectively removed by recrystallization. The compounds with longer retention times (less polar than artemisinin), for example the ones with retention times of 14.5 and 18 min, can also be removed by the recrystallization. However, the compound with a retention time of 10.5 min was difficult to remove, and it turned out to be the main impurity in the recrystallized crystals. As it can be seen from Figure 7, the compound with retention time of 10.5 min appeared in all the artemisinin crystals produced from the eluate mixtures from both the first and the second CC separation. This observation possibly indicated that this compound has solubility characteristics similar to those of artemisinin in acetonitrile−water mixtures, and thus it crystallized out with artemisinin during the antisolvent crystallizations. The similar solubility characteristics between this impurity and artemisinin also suggested that the complete separation of this main impurity needed to be done in the CC separation process, possibly by optimizing the operation conditions of CC, such as using an appropriate gradient elution.

Conclusions

In conclusion, in this paper we have described a novel hybrid chromatography−crystallization separation process for the isolation and purification of natural pharmaceutical target compounds from crude medicinal herb extracts consisting of a very complex multicomponent mixture. This separation process is challenging since many of the compounds in crude extracts are unknown. The hybrid chromatography−crystallization separation process was used to isolate and purify the antimalarial drug artemisinin from a crude extract of the Chinese medicinal herb *Artemisia annua*. The results showed a great potential of having the synergistic effect of chromatography and

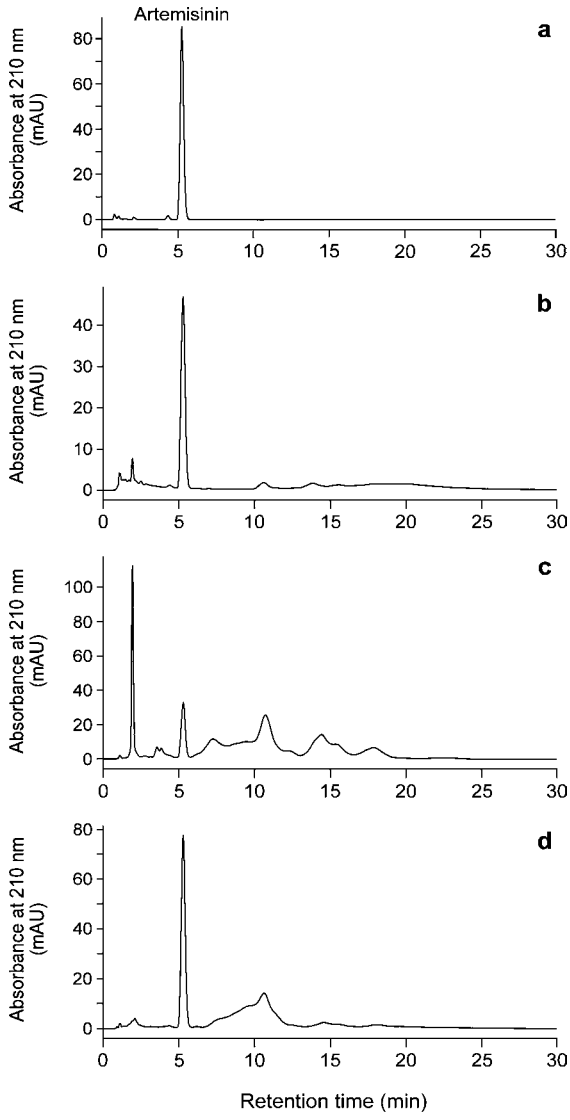

Figure 7. **HPLC chromatogram of (a) artemisinin from the supplier (purity 99%), (b) artemisinin crystallized from the first separation by CC (purity 95%), (c) artemisinin crystallized from the second separation by CC (purity 64%), and (d) artemisinin purified twice by recrystallization (purity 90%).**

crystallization processes. Compared with crystallizing the target compound artemisinin directly from the crude extract, the separation effect of crystallization was significantly enhanced when the crude extract was first fractionated by CC. The purity of the artemisinin crystals was improved by recrystallization. However, the purification effect of crystallization is determined by the solid−liquid equilibrium of both artemisinin and the impurity compounds with the solvents used in the crystallization. The impurities with solubility characteristics similar to those of artemisinin will crystallize out with artemisinin and thus cannot be eliminated by the crystallization process. This observation also highlighted the importance of having the synergistic effect of chromatography and crystallization. Furthermore, it was observed that the compounds possessing similar chromatography characteristics had different solubilities in certain solvents and vice versa. A properly designed CC aiming at the removal of the impurities that have solubility characteristics similar to those of artemisinin will significantly enhance the synergistic effect of chromatography and crystallization and thus improve the efficiency of the whole separation process. This further optimization of the hybrid separation process requires the identification and structural elucidation of the impurities that coexist with artemisinin in the recovered crystals.

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