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# Purification of Artemisinin from the Product Solution of a Semisynthetic Reaction within a Single Crystallization Step

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**ABSTRACT:** Artemisinin, a natural compound isolated from the *Artemisia annua* L. plant, represents an indispensable ingredient in artemsisinin-based combination therapies (ACT) against malaria. Starting from dihydroartemisinic acid, a biosynthetic precursor of the target, a semisynthetic pathway described previously provides in the reactor effluent approximately 69% artemisinin together with structurally similar byproducts. Successful purification of artemisinin was achieved by a combination of continuous chromatography and crystallization. This work is directed to isolate crystalline artemisinin of high purity from the effluent of the catalytic reaction by crystallization only. On the basis of knowledge of artemisinin solubility and nucleation behavior in the reaction medium, a seeded cooling crystallization was designed and implemented subsequent to organic synthesis. It provided crystalline artemisinin with a final purity of 99.9% in a single straightforward crystallization step, simplifying the overall purification process significantly. The new purification method was further demonstrated to successfully isolate pure crystalline artemisinin from a synthesis solution produced directly using crude *A. annua* extract as initial feed.

**KEYWORDS:** *artemisinin, purification, complex mixtures, crystallization*

## 1. INTRODUCTION

Artemisinin (ARTE), a natural ingredient of sweet wormwood, still constitutes the most active component of novel antimalarial drugs.[1−4] Because the molecular structure of ARTE is very complex and it is difficult to achieve commercial value of the drug via implementation of the total synthesis,[5−8] plant extraction represents a primary pathway for ARTE isolation.[9] The ARTE content in the *Artemisia annua* leaves is very low and varies considerably between 0.01 and 1.4% due to seasonal and geographical differences.[10] To become more independent of the low yield plant-derived supply of ARTE and to cover the drug demand, an alternative pathway for ARTE production has been considered within the last few years.[11] Artemisinic acid (AA) or dihydroartemisinic acid (DHAA), both biosynthetic precursors of ARTE in the plant and molecules of a significantly less complex structure which remain after plant extraction in the raffinate stream as unspent residue, were utilized as the starting compounds for the production of semisynthetic ARTE. The breakthrough was achieved by a successful conversion of DHAA to ARTE by means of a photocatalytic reaction.[12] In particular, using the highly selective photocatalyst 9,10-dicyanoanthracene (DCA) and toluene as the reaction medium, ARTE was gained with 69% of the total solids content, with DCA, unreacted educt DHAA, and other structurally very similar and mostly not yet identified byproducts remaining as impurities in the synthesis solution.

According to the World Health Organization (WHO), a minimum acceptable purity of ARTE for antimalarial drug formulation was defined as >97%.[13] In the same report, the maximum content of a single impurity in crystalline ARTE was limited to 0.5%.[13] Because the contribution of unknown byproducts to the overall therapeutic effect has not been sufficiently investigated, ARTE has to be isolated from the plant-based mixtures or related synthesis solutions to meet the quality requirements. A novel hybrid process for ARTE separation from the plant extract was published by Qu et al.[14] By coupling column chromatography and a two-step antisolvent crystallization, crystalline ARTE with a purity of 95% and a yield of 30% was isolated. It has also been shown that in particular, the crystallization is an indispensable process step for purification of ARTE from the chromatographic fractions.[15]

In our previous work,[16] we presented a process for isolation of artemisinin from the above-mentioned synthesis solution, the product of the photocatalytic reaction.[12] In this earlier separation process development, we demonstrated that through use of a binary solvent mixture containing toluene and ethanol at a ratio of 20/80 wt % in a seeded cooling crystallization, ARTE with a purity of 98.8% can be provided within a single crystallization step. However, traces of the photocatalyst DCA in the final crystalline product prevented further processing of the drug. As observed during the crystallization process and confirmed via solubility studies, the excess amount of ethanol in the solvent mixture noticeably reduced the solubility of DCA in the synthesis solution. Thus, DCA became supersaturated shortly after cooling started in the system and spontaneously nucleated in the solution. As a result, the final crystalline product was contaminated with 1.2% DCA. Accordingly, to enhance the purity of ARTE, spontaneous nucleation of DCA has to be avoided.

For this reason, in the present study, pure toluene is considered as a potential solvent for the development of the seeded cooling crystallization process of ARTE from the synthesis solution. This is also motivated by the fact that the photocatalytic reaction as a prior step is conducted in

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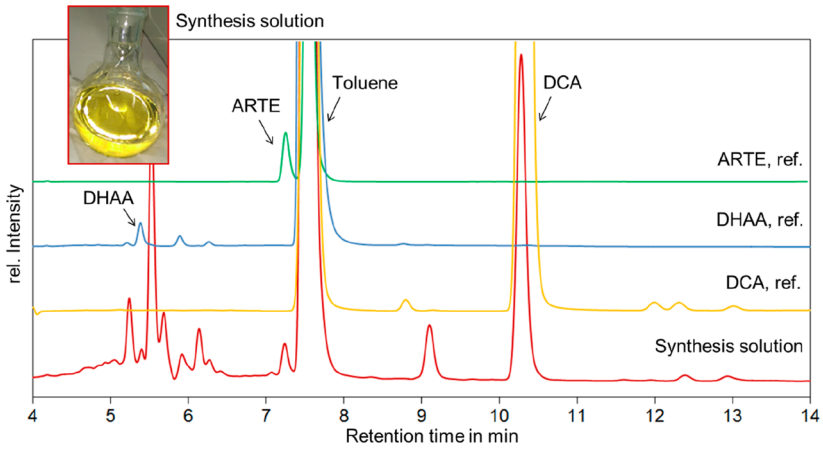


**Figure 1.** Analytical HPLC chromatograms of the reactor effluent (synthesis solution) (red), the target ARTE (green), and two impurities: DHAA (blue) and DCA (yellow).

toluene.[12] Thus, the isolation of crystalline ARTE is facilitated in a straightforward manner from the reactor effluent without any solvent change.

Also, just recently, new results have shown that DCA can be avoided when exploiting the chlorophyll, a naturally occurring component in the plant extract, as catalyst for production of semisynthetic ARTE.[17] In the synthesis, a mixture extracted from *Artemisia annua* leaves and some added DHAA (0.5 M) was fed directly into the flow reactor to convert DHAA to ARTE in an acidic medium. In the present study, this crude plant extract-based solution was further used to demonstrate the wider applicability of the developed crystallization process for purification of ARTE.

## 2. MATERIALS AND METHODS

**2.1. Materials.** Solid standards of artemisinin (>97%, TCI Chemicals), 9,10-dicyanoanthracene (>98%, TCI Chemicals), and dihydroartemisinic acid (>98%, Cfm Oskar Tropitzsch GmbH, Marktredwitz, Germany) were used as standards for HPLC analysis, for solubility and nucleation studies. Mobile phase components were acetonitrile (>99.8%, HiPerSolv CHROMANORM, VWR Chemicals, Germany) and water purified via Milli-Q Advantage devices (Merck Millipore). Toluene (>99.8%, HiPerSolv CHROMANORM, VWR Chemicals, Germany) was used as the crystallization solvent and for related solubility and nucleation studies. The filter cake obtained after solid−liquid separation at the end of the crystallization process was washed with ethanol (>99.8%, HiPerSolv CHROMANORM, VWR Chemicals, Germany).

Crystallization experiments were conducted directly from the effluent of the photo flow reactor[12] representing a bright yellow solution with a total solid content of 12.2 wt %. As seen from the HPLC chromatograms in Figure 1, the synthesis solution is a complex mixture containing, besides the solvent toluene, mainly the target component ARTE, the unconverted precursor DHAA, and the photocatalyst DCA with respective solution contents of 8.5, 0.08, and 0.05 wt %. The results of the isolation of ARTE from this synthesis solution are presented in Section 3.1.

In Section 3.2, the wider applicability of the developed purification strategy to the crude plant extract-based synthesis solution[17] with an initial ARTE content of 9.0 wt % in toluene as solvent is evaluated. Therein, plant-inherent chlorophyll was exploited as photosensitizer instead of DCA and is responsible for the dark green color of the solution. The extract contains besides ARTE, unreacted DHAA, and chlorophyll also a grand variety of other plant-derived compounds. These have not been quantified in this study but were elucidated in previous publications, e.g. ref 18. Thus, it will be shown if the newly introduced method is capable to separate ARTE from theses coextracted impurities as well as the byproducts of the semisynthesis.

**2.2. Analytical Methods and Crystallization Studies.** An analytical HPLC unit (Agilent 1200 Series, Agilent Technologies Germany GmbH) was used to characterize the solid standards to quantify the ARTE, DHAA, and DCA content in the synthesis solution[12] as well as for purity analysis of the crystalline product. The mobile phase composition was fixed to 50/50 vol % acetonitrile/water, and the eluent flow rate was fixed to 0.5 mL/min. Solid samples preliminarily dissolved in acetonitrile were injected (injection volume 1 $\mu$L) in the column (Kinetex C18, 250 × 4.6 mm; 5 $\mu$m, Phenomenex GmbH, Germany, column temperature 20 °C) and analyzed at a wavelength of 254 nm. Purity analysis of the final product from the extract-based synthesis solution in Section 3.2 was conducted by means of HPLC connected to an evaporative light scattering detector, as used in previous synthesis studies.[17]

Solubility investigations of ARTE and DCA in toluene were carried out via the classical isothermal method.[19] Suspensions containing solid material in excess and 5 mL of solvent were introduced in glass vials. To guarantee efficient mixing of the prepared suspensions, vials were equipped with a magnetic stirrer and sealed. Samples were placed in a thermostatic bath and allowed to equilibrate at constant temperatures between 5 and 40 °C for at least 48 h under stirring. Afterward, samples of equilibrated slurries were withdrawn with a syringe and filtered through a 0.45 $\mu$m PTFE filter. Obtained liquid phases were analyzed for solute content by HPLC. To ensure the corresponding solution composition in saturated liquid phases equilibrated at 5 °C, syringes and filters were precooled before usage.

Metastable zone width data of ARTE in toluene were ascertained by means of the multiple reactor system Crystal16 (Avantium Technologies BV, Amsterdam). Suspensions containing known excessive amounts of solid in solvent were prepared in standard HPLC glass vials, equipped with magnetic stirrers, and subjected to a heating step from 5 to



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60 °C and a subsequent cooling step from 60 to −15 °C, both at a moderate rate of 0.1 °C/min. Temperatures of a "clear" and "cloud" point represent the respective saturation and nucleation temperatures and were obtained via turbidity measurement.

Batch crystallization experiments were conducted in a jacketed 100 mL glass vessel equipped with a Pt-100 resistance thermometer (resolution 0.01 °C) and connected to a thermostat (RP845, Lauda Proline, Germany) to control the system temperature. A magnetic stirrer was used for agitation. Solution composition data (Table 1) and the applied crystallization procedures are given and discussed in connection with the crystallization process design in the following Section 3.1.

## 3. RESULTS AND DISCUSSION

**3.1. Crystallization Process Design and Successful Isolation of ARTE from Toluene Solution.** To select appropriate conditions for the selective cooling crystallization process, the solubility and nucleation behavior of pure ARTE in toluene as well as in the presence of the solution impurities were studied. The temperature range was selected in accordance with the cooling procedure previously applied for the 20/80 wt % toluene/ethanol solvent mixture.[16] Figure 2 shows solubility and nucleation curves of ARTE in toluene constructed from the experimental data. To highlight the differences in crystallization conditions selected for isolation of ARTE from toluene in comparison to the 20/80 wt % toluene/ethanol solution,[16] both derived crystallization processes are presented in the diagram. As observed for the 20/80 wt % toluene/ethanol solution, the presence of impurities slightly decreased the artemisinin solubility in toluene. In contrast, Malwade et al.[15] reported an increase of ARTE solubility in different *n*-hexane/ethyl acetate binary solvents in a combined chromatography fraction of an *A. annua* extract. At 5 °C, the saturation concentration of ARTE in toluene in the presence of impurities is 10.8 wt %, which is about twice as high as that measured in the 20/80 wt % toluene/ethanol solvent mixture. Further, the solubility of ARTE in toluene reaches a value of 24.5 wt % at 40 °C. Accordingly, in toluene, ARTE shows significantly higher solubilities and a stronger temperature dependency of solubility as in the binary solvent mixture. The observed differences result from the fact that ethanol acts as an antisolvent for ARTE in toluene.[16]

With respect to the determined solubility and nucleation curves and in consideration of the selected start temperature of the cooling profile, the initial ARTE content in the feed solution for crystallization was set to 22 wt %. This concentration value ensures an undersaturated ARTE solution at 40 °C at the beginning of the process ($c_{start}$, Figure 2a).

**Table 1. Overview of the Amount of Solute and Solvent in the Original Synthesis Solution and Feed Solution for Crystallization**

| | original synthesis solution[12] | feed solution for crystallization |
|---|---|---|
| solution | 191.5 g | 73.5 g |
| toluene | 168 g | 50 g |
| total solids | 12.2 wt % | 31.9 wt % |
| ARTE | 8.5 wt % | 22.0 wt % |
| DCA | 0.05 wt % | 0.12 wt % |

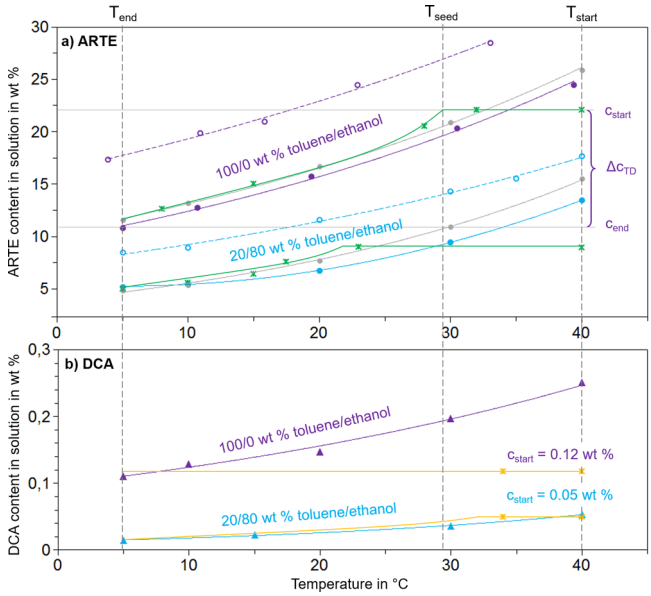


**Figure 2.** Solubility curves (solid blue and purple curves), nucleation border (dashed blue and purple curves), and crystallization trajectories of the solute (a) ARTE (green symbols and curves) and (b) DCA (yellow symbols and curves) in the solution during the cooling crystallization of ARTE. Solubility data of ARTE in pure solvents are presented as gray solid circles and curves for comparison. Symbols represent experimental data, and curves fitted trajectories as guides to the eye. The related data for ARTE and DCA in a 20/80 wt % toluene/ethanol solution (blue symbols and curves) are adapted from ref 16. $T_{start}$, $T_{end}$, and $T_{seed}$ represent the start and end temperature of the cooling step and the temperature of seed addition, respectively; $c_{start}$ and $c_{end}$ refer to the initial and final solute content in the solution, and $\Delta c_{TD}$ characterizes the maximal depletion of ARTE from solution.

Because the synthesis solution from the effluent of the photoreactor contains about 8.5 wt % ARTE,[12] in a first process step, the initial concentration of ARTE was adjusted to the starting value via evaporation of the excess amount of toluene. Table 1 gives an overview of the amount of the solute and solvent in the original synthesis solution and in the prepared feed solution for crystallization.

The seeded cooling crystallization of ARTE was conducted in a second step following the process trajectory shown in Figure 2a (upper green line). Starting at 40 °C, the clear undersaturated solution with 22 wt % ARTE was cooled to 5 °C at a linear rate of 0.1 °C/min. After exceeding the saturation temperature (at around 34 °C), 50 mg of pure ARTE seeds was added to the clear supersaturated solution at 29 °C. When reaching the set end temperature of the cooling profile, the suspension was stirred for a further 0.5 h. Subsequently, solid−liquid phase separation was carried out using filter paper of 0.6 μm pore size. To remove residual toluene and adhering mother liquor from the filter cake, product crystals were washed with 5 °C cold ethanol, dried at 40 °C for about 1 h, and finally were weighed and analyzed by HPLC to check for purity and yield. Therefore, to also quantify the residual amount of toluene in the crystallization product, a sample of the product crystals was dissolved in acetonitrile, which represents the major solvent component of the HPLC eluent. A picture of the filter cake (white product crystals) and the results of HPLC purity analyses are shown in Figure 3.



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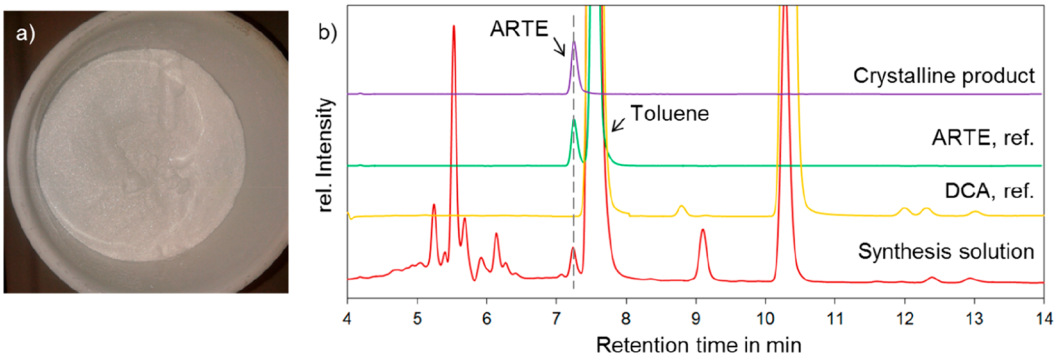


**Figure 3.** (a) Picture of filtered product crystals and (b) HPLC chromatograms of the crystalline product, pure standards of ARTE and DCA (indicated as ref.), and the synthesis solution.

The detected chromatogram contains only a single peak at 7.2 min, which can be clearly assigned to the target ARTE. As already assumed from the color of the filter cake, the gained crystalline product was free of DCA but also of toluene and other impurities initially present in the synthesis solution and was specified to 99.9% ARTE.

The removal of toluene (118 g, Table 1) from the original synthesis solution in the first process step led to the increase in DCA concentration from 0.05 to 0.12 wt % in the feed solution (Table 1). As seen from the DCA solubility behavior in Figure 2b (upper yellow line) and similar to ARTE, DCA has higher solubility in pure toluene than in the 20/80 wt % toluene/ethanol solvent mixture. Accordingly, in contrast to the previous process,[16] DCA remains undersaturated in the toluene solution along the cooling trajectory up to 8 °C and does not spontaneously crystallize, leaving the crystalline product DCA-free. In Table 2, the quantitative results of the ARTE purification from toluene as solvent are summarized and compared to those previously obtained from the toluene/ethanol solvent mixture.

**Table 2. Results of the ARTE Separation Process from Toluene Compared to the 20/80 wt % Toluene/Ethanol Solvent Mixture**[a]

| | toluene | 20/80 wt % toluene/ethanol |
|---|---|---|
| product mass (g) | 8.15 | 3.1 |
| target purity (%) | 99.9 | 98.8 |
| target mass (g) | 8.14 | 3.06 |
| yield (%) | 50.2[b]/98.5[c] | 43.8[b]/96[c] |
| productivity (g $g^{-1}$ $h^{-1}$) | 0.028[d] | 0.0076[d] |

[a]Data given in the right column were taken from ref 16. Mass of solvent: $m_{toluene}$ = 50 g; $m_{20/80\ wt\ \%\ toluene/ethanol}$ = 70 g. [b]Yield = $m$(ARTE in crystalline product)/$m$(ARTE in feed solution) × 100%. [c]Fraction of the theoretical crystallization yield at equilibrium calculated based on the solubility data. [d](ARTE mass in product)/(mass of solvent)/(time of batch = 5.83 h).

The data show that by using toluene as solvent, i.e. the synthesis effluent directly, 8.14 g of crystalline ARTE of 99.9% purity were produced from 16.2 g of ARTE initially present in the synthesis solution within a single crystallization step. This amount corresponds to 50.2% yield and is equal to 98.5% of the theoretical crystallization yield calculated based on the solubility data.

The implemented separation process in toluene led to the desired ARTE purity and a 6.4% higher yield than via crystallization in the binary solvent mixture (Table 2). From the illustrated ARTE crystallization trajectories in Figure 2a, it can be derived that the higher ARTE content in the toluene feed solution as well as the lower solvent consumption resulted in a nearly threefold product mass and a productivity improvement by a factor of almost four when applying the same cooling procedure. The newly presented and previously published[16] results demonstrate that the selection of the solvent for crystallization can be decisive to reach the desired purity of the final crystalline product.

**3.2. Application of the Designed Crystallization Process to Isolate ARTE from a Crude *A. annua* Extract-Based Synthesis Solution.** A solution provided from a recently published study[17] was applied to investigate a potential wider applicability of the designed crystallization process. This solution contained a similar amount of semisynthetic ARTE of 9.0 wt %, no DCA (because the plant's own chlorophyll acted as conversion photocatalyst), but a great variety of other plant-derived compounds coextracted from the plant and being, besides the byproducts formed in the semisynthesis, impurities to be separated from ARTE. So, it was examined if crystalline ARTE of comparably high quality can be recovered from that reactor effluent within a single crystallization step by applying the separation process conditions used above. Due to the fact that only a limited amount of the crude solution was provided (approximately 190 g), the crystallization experiment was carried out without performing any additional solubility or metastable zone width studies.

Thus, the excess amount of toluene was evaporated from the green colored crude synthesis solution in the first step. The obtained concentrated solution (approximately 88 g) containing around 23 wt % ARTE in toluene was transferred to the double-jacketed vessel, and the seeded cooling crystallization was conducted in the second step. At the end of the cooling procedure, the suspension was stirred for further 1 h. The filter cake, obtained after solid−liquid phase separation, was rinsed with cold ethanol to remove residual toluene and adhering mother liquor and was finally analyzed by HPLC to specify purity and yield.

The performed crystallization process from the crude extract-based synthesis solution provided 6.42 g of solid product. The picture of the filter cake in Figure 4a reveals the desired white crystals, and HPLC-ELSD analysis in Figure 4b confirmed that ARTE of 99.9% purity was isolated within a single crystallization step from the crude extract mixture. The yield achieved (37.5%) was lower than in the process presented in Section 3.1, which might be a result of the slow


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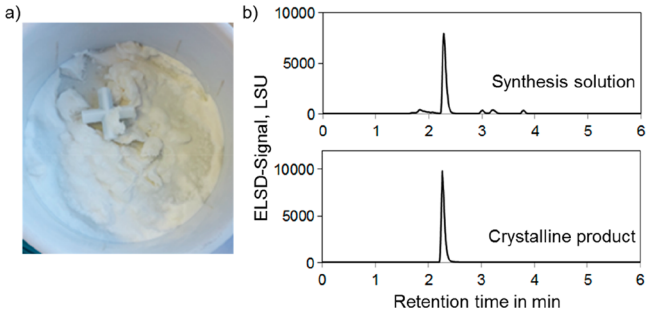


**Figure 4.** (a) Picture of filtered product crystals; (b) HPLC-ELSD chromatograms of the synthesis solution and crystalline product.

crystallization kinetics of ARTE in the presence of the increased variety of impurities in the crude extract-based solution. Also, Malwade et al.[15] observed a direct dependency of the ARTE solubility on increasing amount of impurities in the plant extract, which was more pronounced at lower temperatures. Accordingly, the presence of plant-derived impurities could also increase the solubility of ARTE in the solution and therewith directly affect the product yield.

## 4. CONCLUSIONS AND PERSPECTIVES

The purification of semisynthetically produced ARTE from two different crude multicomponent mixtures composed of structurally similar and to a great extent unidentified constituents was studied. On the basis of the knowledge of fundamental solution equilibria and metastable zone widths, a highly selective crystallization process was designed and successfully implemented. As a result and independent of the origin of the crude but similar solution with regard to the target content, the process provided 99.9% pure ARTE in the final solid product without any solvent change through application of only a single crystallization step. This is, to the best knowledge of the authors, demonstrated for the first time. 50.2 and 37.5% of the ARTE could be recovered from the respective crude solution. Principally, the method used should be applicable to similar semisynthetic ARTE mixtures and plant extracts as a first try. Of course, as such crude mixtures may contain a number of unpredictable constituents (that may also be different from the ones in the mixtures used in this study), an adaptation of the presented process may be necessary.

It should be noted that the crystallization procedures were not yet fully optimized in terms of yield; also, more experiments are required to support the studies done. The maximum yield of a cooling crystallization depends on the content of the component to be crystallized in the feed solution and the implemented cooling procedure and thus is limited by the saturation value of the target component in the solution at the final temperature. Accordingly, to enhance the target yield, the designed processes can be performed either at higher ARTE contents in the feed solution or by selecting lower final temperatures of the cooling profile in the crystallization step. In both cases, the impact of impurities in the crude mixture on the crystallization behavior of ARTE has to be considered. Further, due to its high solubility in toluene, a substantial amount of ARTE remained in the mother liquor after solid–liquid phase separation. Therefore, recycling of the mother liquor is a strong tool to additionally improve the yield.

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### Notes

The authors declare no competing financial interest.

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