Separation of Mixtures of Rutin and Quercetin: Evaluating the Productivity of Preparative Chromatography

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Dedicated to Prof. Dr. rer. nat. Jörg Kärger on the occasion of his 80th birthday

The flavonoid rutin is present in significant amounts in the flower buds of *Sophora japonica* L. It offers numerous desired pharmacological effects. Under certain extraction conditions quercetin is found as a hydrolysis product which needs to be separated from rutin. This paper describes the application of liquid chromatography to solve this task. Based on the determination of adsorption equilibrium constants and column efficiencies, the productivity of the separation process is estimated, and scale-up considerations are presented. A comparison with alternatively directly crystallizing rutin from raw extracts is also reported.

Keywords: Crystallization, Equilibrium dispersion model, Preparative chromatography, Quercetin, Rutin, Scale-up

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1 Introduction

Flavonoids are the predominant class of polyphenolic secondary metabolites distributed widely in plants. Their structure is characterized by two aromatic rings with phenolic hydroxyl groups. The number of flavonoids identified so far exceeds 9000 [1, 2].

The flavonoid rutin (quercetin-3-O-rutinoside), also designated as rutoside or sophorin, is a flavonol glycoside, which consists of aglyconequercetin and rutinose linked to the aglycone hydroxyl group (Fig. 1). Rutin is widely distributed in the flower buds of *Sophora japonica* L, apples, berries, citrus, onions and tomatoes [3]. Rutin is of interest due to potential pharmacological benefits such as observed anti-oxidant, anti-diabetic, anti-inflammatory, kidney protection, anti-asthma, cardioprotective, vasoprotective, neuroprotective, anti-cancer and antithrombotic activities effects [3].

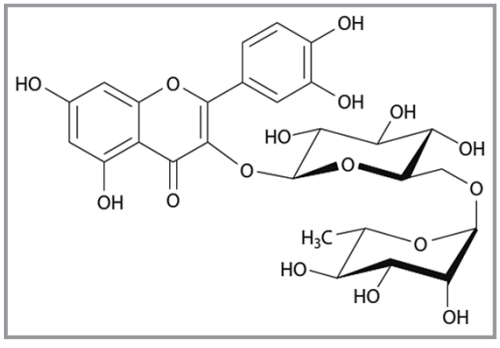

Figure 1. Molecular structure of rutin.

Quercetin (Figure 2) belongs to the class of aglycone forms of rutin. It exists naturally along with rutin or appears as a hydrolysis product of rutin exploiting specific extraction conditions. Pharmacological actions of quercetin are angioprotective, anti-carcinogenic, antioxidant, anti-obesity, anti-inflammatory, anti-aggregatory, gastroprotective and vasodilating effects [4].

In comparison with the other potential natural sources of rutin mentioned above, the flower buds of *Sophora japonica*

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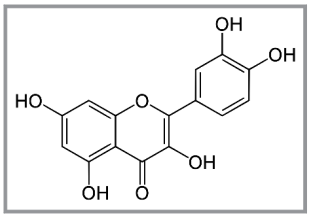

Figure 2. Molecular structure of quercetin.

L. have an exceptional high content reaching up to 22 wt % [5, 6]. These buds are in Vietnam and China the main raw material for producing rutin. Extraction is typically carried out exploiting diluted alkaline solutions [3]. This provides also quercetin as a major impurity, which hampers the pharmacological actions of rutin. Therefore, to achieve pharmaceutical grade purity the raw rutin obtained using extraction techniques must be further processed.

2 Analytical Chromatography to Determine Contents of Rutin and Quercetin

Zu et al. [7] described an efficient liquid chromatography method using conventional reversed phase chromatography with octadecylsilica (RP18). This stationary phase is capable to separate rutin and quercetin and to determine their amounts in extracts. To achieve a high selectivity and to resolve also other components present in the extracts a mobile phase consisting of acetonitrile, methanol and acetic acid was applied. Samples of dried raw extracts are typically redissolved in methanol, which is used as the injection solvent. Further analytical techniques to characterize rutin und rutin-containing mixtures are summarized in [8].

In our preliminary work [9] and also for the investigations described here we adapted the method developed in [7]. A Luna 18 reversed phase analytical column was applied (length 25 cm, inner diameter 0.46 cm, particle size 10 µm, Phenomenex GmbH, Germany) The solvents acetonitrile, methanol and acetic acid (VWR Chemicals, Germany) were of HPLC grade. Deionized water was prepared with a Millipore Milli-Q water-purification system (Merck, Germany). The chromatographic separations were carried out using an HPLC system (Agilent Technologies 1200 Series) consisting of a quaternary pump, a vacuum degasser, an autosampler, a thermostated column compartment, and a diode-array detector (DAD). UV-signals were recorded for a wavelength of 254 nm. The column temperature was set to 20 °C and a constant mobile phase flowrate of 1.0 mL min^{-1} applied. Primary data analysis was performed using the Agilent HPLC Chem-Station software.

Dried extract was prepared at Hanoi University of Science and Technology (Vietnam). Fig. 3 shows a chromatogram revealing its multi-component character typical for natural product extracts. Only the target product rutin and the decomposition product quercetin could be clearly identified and after calibration with available pure standard components quantified. The rutin content in the crude extract was determined to be 81 %. The mass ratio between the amounts of rutin and quercetin was 96:4.

3 Preparative Chromatography

Main goal of this paper is to evaluate the potential of applying the chromatographic method described above for preparative purposes. The focus was set on efficiently providing highly pure rutin.

Scale-up chromatography to isolate larger amounts of a specific target component is typically performed following two approaches. In a first step analytical scale columns are overloaded by injecting larger sample sizes. This can be done by increasing the injection concentrations, the injection volumes or both [10, 11]. Corresponding experimental results provide insight regarding the degree and effects of nonlinearity of the underlying phase equilibria. This knowledge allows estimating upper limits regarding the injection amounts that can be processed. The experimental results also offer the opportunity to parametrize adsorption isotherm models, which are subsequently valuable input parameters for predicting the second scale-up approach, namely the application of larger chromatographic columns.

The described procedures were applied considering binary model-mixtures of rutin and quercetin. The experimental investigations were focused on estimating the equilibrium data from breakthrough studies [12] to parametrize a mass balance based column model. This model could then be used for productivity estimations and scale-up considerations.

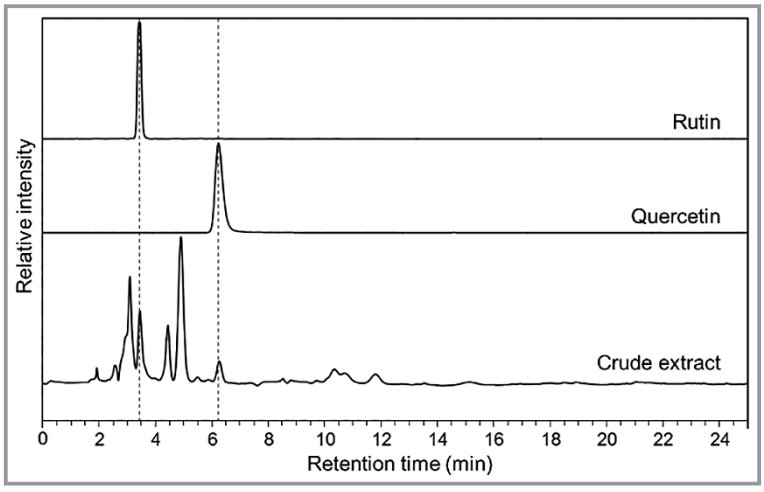

Figure 3. HPLC chromatogram of the crude rutin extract compared to pure rutin and quercetin [9].

3.1 Materials, Column Characterization and Preliminary Overloading Studies

Rutin and quercetin were purchased from TCI Germany GmbH (Eschborn, Germany) and used without further purification.

In a first step the established chromatography method [7] was re-evaluated testing different ratios of the solvent constituents. The percentages were varied for methanol between 25 and 85, for acetonitrile between 5 and 30 and for water between 10 and 60. The best results were obtained for a solvent that contained methanol, acetonitrile and water in the following ratio: 40/15/45 v/v/v. Further improvement of resolution was achieved by adding 1 % of acetic acid.

In order to allow for estimating retention factors the bed porosity was estimated recording elution profile of injections of small amounts of solvent which differed slightly in its composition compared to the mobile phase. For the applied flowrate of 1.0 mL/min a column dead time of t_0 = 2.75 minutes was determined from these pulse experiments. This dead time corresponds to a total column porosity of ε_T = 0.66 or a phase ratio $F = (1 - \varepsilon_T)/\varepsilon_T$ of 0.5.

Another important feature of the separation process is the column efficiency. It is typically expressed by the number of theoretical plates N. This value can be estimated recording and analyzing the responses to small injections of the components of interest. Under diluted conditions and in case of well-packed columns the peaks are symmetrical and resemble Gaussian distributions. This allows estimating N using the following formula [10, 11]:

$$N \cong 5.54\left(\frac{t_R}{w_{0.5}}\right)^2 \quad (1)$$

In this equation t_R stands for the mean retention time and $w_{0.5}$ for the peak width at its half height. Evaluating in this way recorded elution profiles of both rutin and quercetin an averaged plate number of N = 302 was determined for the specific flowrate used.

In a first series of overloading experiments, we examined 50/50 % feed mixtures of rutin and quercetin with a concentration of 0.02 g L^{-1} for each component. The resulting profiles are presented in Fig. 4. The band profiles of rutin and quercetin are completely separated for the smaller injection volumes of 1 mL and 3 mL. For increased injection volumes (4 mL, 5 mL and 6 mL) the band profiles of the solutes overlapped. Subsequently, higher feed concentrations of each solute (0.03 g L^{-1} and 0.04 g L^{-1}) were studied using a fixed injection volume of 5 mL. The recorded absorbance values are given in Fig. 5.

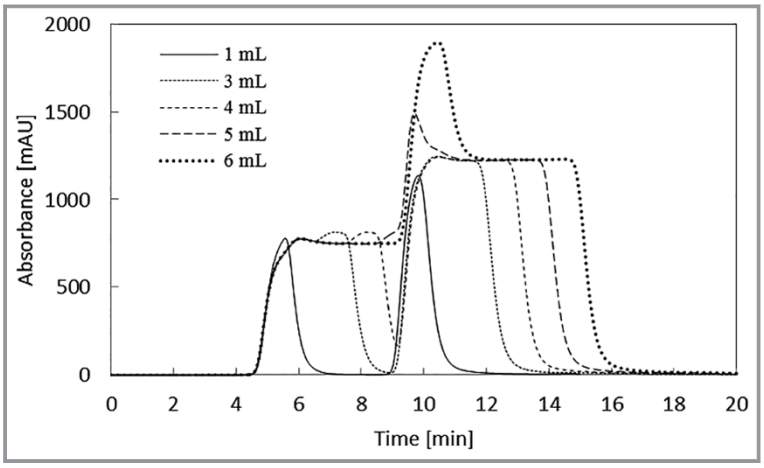

Figure 4. Experimental elution profiles for different injection volumes (1, 3, 4, 5 and 6 mL). Sample: 50/50 % rutin/quercetin mixture with a concentration of 0.02 g L^{-1} (for each solute).

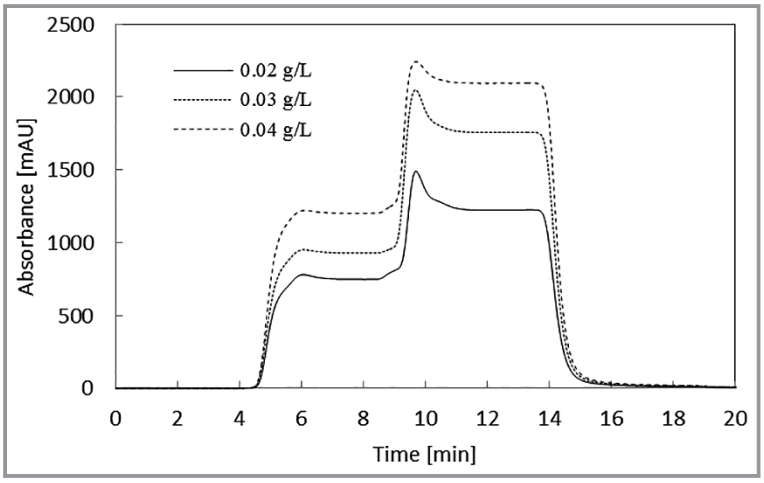

Figure 5. Experimental elution profiles of 50/50 % rutin/quercetin mixture with different concentrations of each solute (0.02 g L^{-1}, 0.03 g L^{-1} and 0.04 g L^{-1}) for an injection volume of 5 mL.

3.2 Estimation of the Adsorption Equilibria

Among the different methods available for determination of single-component isotherms using liquid chromatographic techniques frontal analysis is known to be precise and reliable [12]. The method is based on performing and recording a series of elution profiles characterized by different injection amounts large enough to fully saturate the columns.

Using this method, we measured at ambient temperature single component isotherm data of rutin and quercetin injecting 5 mL for three different concentrations. The experiments were carried out through switching between pure solvent and the sample solutions using two pumps offered by the HPLC system. The breakthrough curves for each injection were recorded using the UV-detector at a wavelength of 254 nm.

Each breakthrough curve measured was used to calculate the loading of the solute adsorbed on the stationary phase (q) that is in equilibrium with the inlet concentration of the solute (c). For this the following mass balance was used [10, 11]:

$$q_i = \frac{t_{R,i} - t_0}{F t_0} c_i, \quad i = \text{Rutin, Quercetin} \tag{2}$$

where t_R denotes the determined mean retention time of a breakthrough curve and t_0 and F are the mentioned column dead time and the phase ratio, respectively. Hereby, the values of t_R were estimated from the times corresponding to the half-heights of the plateaus ("middle point method").

The chromatograms recorded for rutin and quercetin are shown in Fig. 6. The curves reveal for both components the influence of unidentified impurities, which were present in the samples used. In the concentration range covered for the two components the specific breakthrough times did not change significantly. Thus, both isotherms are linear in this range. The mean values of the retention times are $t_{R,Rutin}$ = 4.9 min and $t_{R,Quercetin}$ = 9.4 min. This allows to estimate the adsorption equilibrium constants H_i of the two components with the balance equation Eq. (2) [10, 11]:

$$H_i = \frac{q_i}{c_i} = \frac{t_{R,i} - t_0}{F t_0}, \quad i = \text{Rutin, Quercetin} \tag{3}$$

The adsorption equilibrium constants estimated are H_{Runtin} = 1.55 and $H_{Quercetin}$ = 4.71. This corresponds to a rather satisfying separation factor of approximately 3.

3.3 Column Model and Validation

To further analyze the separation process we performed simulations using the equilibrium dispersive model of chromatography [10, 11]. The mass balance equation of this model is for a component i:

$$\frac{\partial c_i}{\partial t} + F\frac{\partial q_i(c_i)}{\partial t} = -u\frac{\partial c_i}{\partial z} + D_{app}\frac{\partial^2 c_i}{\partial z^2} \tag{4}$$

In addition to the above introduced variables in Eq. (4) t and z stand for time and axial coordinate, u is the interstitial velocity (related to the volumetric mobile phase flowrate) and D_{app} represents a mean apparent dispersion coefficient. The equilibrium loadings of the two components were calculated using the two Henry constants H_i given above. The dispersion coefficient was estimated from the determined average plate number N and the column length L as follows [10, 11]:

$$D_{app} = \frac{Lu}{2N} \tag{5}$$

Eq. (4) was solved using a finite difference method based on the Rouchon algorithm [10].

To verify the model one of the breakthrough experiments was repeated. For the injection of 5 mL with 0.04 g L^{-1}, inlet concentrations of the two solutes in the relevant region of the chromatogram fractions of the outlet stream were collected. Subsequently small amounts of each sample (1 µL) were reinjected on the same column. The fractions were collected in the mixed band zone (between 8 and 12 min) in one-minute time interval. Between 10 and 11 min, additional fractions were collected every 6 s in order to acquire a more detailed view. In Fig. 7 are given the results compared together with the model predictions. The concentration profiles of rutin and quercetin are presented together with the total (sum) concentration of both substances. The continuously recorded (overall) UV-signals generated by the mixture could be compared with corresponding theoretical signals calculated using the two available component specific detector calibration factors (Fig. 7).

The agreement between the simulation results and the experimental observations can be considered as rather satisfying. Therefore, the validated model is seen as a good basis for further process simulation and evaluation.

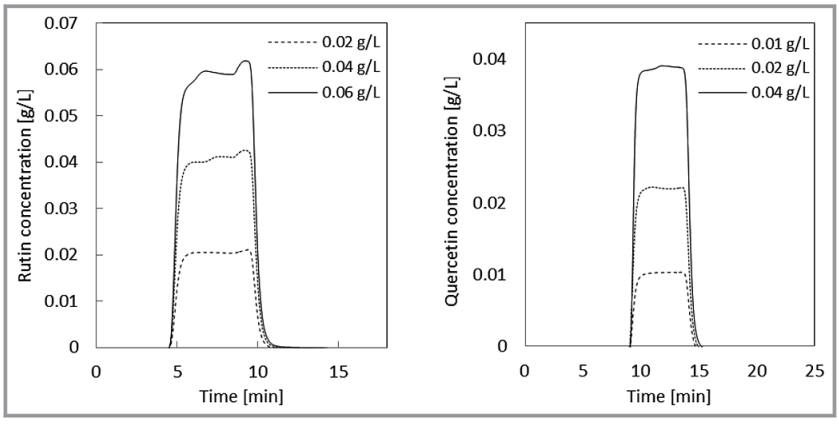

Figure 6. Experimental band profiles of rutin (left) and quercetin (right) recorded for different concentrations, used for frontal analysis. Injected volumes: 5 mL.

3.4 Application of the Validated Model: Productivity Estimation and Scale-up Considerations

One option to apply the described chromatographic system for isolating highly pure rutin from rutin/quercetin-mixtures is to apply repetitive injections and to collect always the sufficiently pure target product fraction. To achieve this goal several characteristic retention times need to be respected. This is illustrated schematically in Fig. 8 assuming a 1/1 feed mixture.

In a periodic regime the averaged mean product flow, $\dot{m}_i$, is a measure of a compo-

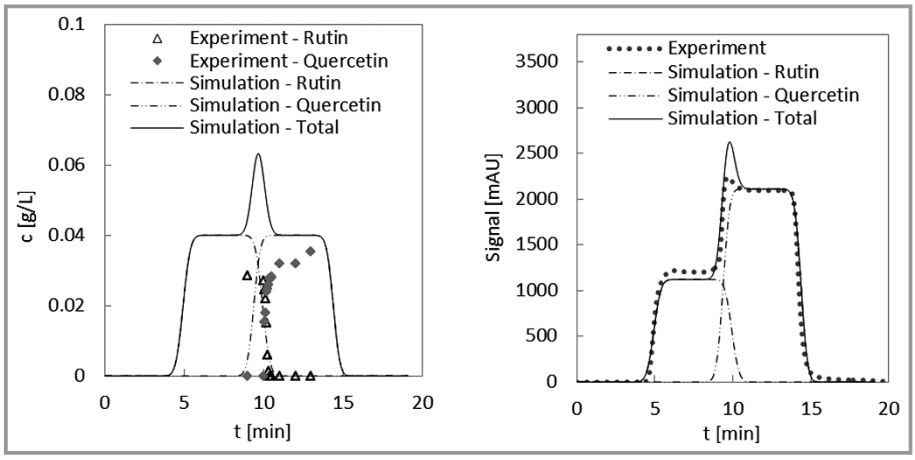

Figure 7. Simulation of the outlet concentration profiles of the 50/50 % mixture of rutin and quercetin compared to experimental results (left) and simulated and experimentally observed detector responses (right). Injected volume: 5 mL; concentrations of each component: 0.04 g L^{-1}.

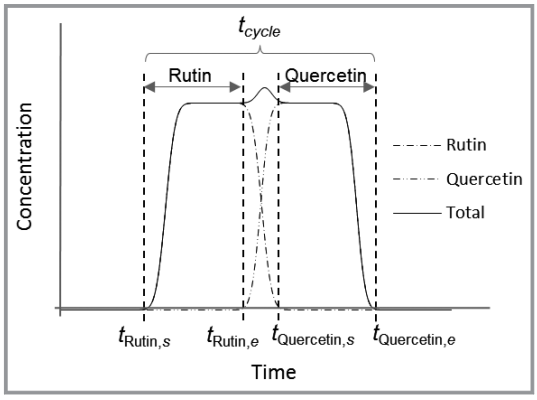

Figure 8. Illustration of the cut times relevant for a periodic repetitive injection regime. (s: start of collecting, e: end of collecting).

nent and scale dependent productivity, Pr_i. This quantity is inversely proportional to the cycle time t_{cycle}, i.e., the shortest time difference between two consecutive injections:

$$\dot{m}_i = Pr_i = \frac{m_{collected,\,i}\left[t_{i,s}, t_{i,e}\right]}{t_{cycle}}, \quad \text{with} \quad t_{cycle} = t_{Quercetin,e} - t_{Rutin,s} \tag{6}$$

In this equation $m_{collected,i}[t_{i,s},t_{i,e}]$ stands for the mass of the component i (rutin or quercetin) collected between two characteristic times, $t_{i,s}$ (start of the component collection) and $t_{i,e}$ (end of the collection). The characteristic times are indicated in Fig. 8. They can be specified providing a threshold concentration, which is related to detection limits and purity requirements. The threshold was set to 10^{-5} g L^{-1}. This assured that the purity of the collected fractions was higher than 99 %. To have access to a scale independent productivity the Pr_i values can be divided by the column volume or the volume of the adsorbent (V_{Ads}). Taking the second option a normalized productivity, $Pr_{N,i}$, is calculated as:

$$Pr_{N,i} = \frac{Pr_i}{V_{Ads}} = \frac{m_{collected,\,i}\left[t_{i,s}, t_{i,e}\right]}{t_{cycle} V_{Ads}} \tag{7}$$

Knowledge regarding this normalized productivity supports scale-up considerations and comparisons with other separation techniques.

One goal was to identify the chromatographic process parameters that would provide the highest productivity for the provision of highly pure rutin. In agreement with typical extract compositions mixtures of 96 % rutin and 4 % quercetin were considered. To investigate the course of the productivity elution profiles were predicted for six gradually increasing amounts injected. Fig. 9 shows the six elution profiles. For the profiles a) and b) still complete peak resolution (100 % recovery) is achieved. However due to the small amount injected into the system, productivity is low. When larger amounts are injected (profiles c–f), increasingly mixed fractions form in the intermediate part of the chromatogram, which results in recovery losses.

To estimate the process productivity for the analysis of the simulation results the cut times were identified to keep the purity over 99 %. By increasing the injected amount, the rutin productivity increased until a maximum was reached beyond which the components could not be separated anymore well enough. The highest not normalized productivity was 2.10 mg h^{-1} for rutin when 0.46 mg of the mixture was injected. The corresponding productivity for isolating quercetin was 0.056 mg h^{-1}. These results are summarized in Fig. 10. The maximal normalized productivity values (Eq. (7)) for the 96:4 mixture are $Pr_{N,Rutin}$ = 1.50 g $h^{-1}L_{ADS}^{-1}$ and $Pr_{N,Quercetin}$ = 0.0397 g $h^{-1}L_{ADS}^{-1}$. The course of the normalized rutin productivity over the feed composition is shown in Fig. 11. Obviously, the achievable productivity of isolating rutin increases with its amount in the feed mixture.

The determined normalized productivities are useful for scale-up considerations. These $Pr_{N,i}$-values remain constant with the column volume increases. In preparative chromatography a successful simple scale-up strategy consists in keeping the column length identical and increasing the column diameter, d [10, 11]. To keep over all scales the same retention and, thus, the same cycle times, the following adaptions of the injected amounts and the flowrates in the small and large columns need to be respected [13]:

$$\frac{m_{inj}^{large}}{m_{inj}^{small}} = \frac{Flowrate^{large}}{Flowrate^{small}} = \left(\frac{d^{large}}{d^{small}}\right)^2 \tag{8}$$

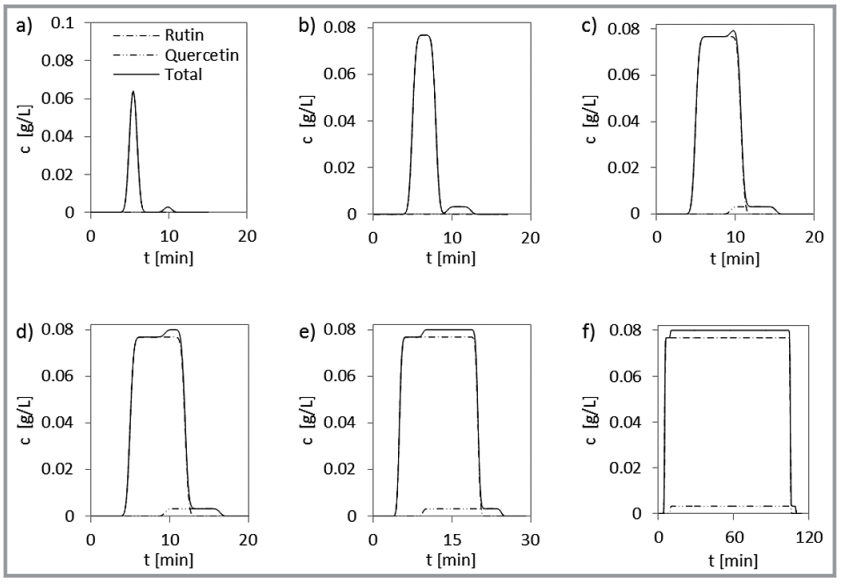

Figure 9. Elution profiles for the 96/4 % rutin/quercetin mixture for increasing amounts injected: Total feed concentration: 0.08 g L^{-1} (= 0.077 + 0.003). Injection volumes: 1, 3, 5.8, 7, 15 and 100 mL (corresponds to total injection masses of 0.08, 0.24, 0.46, 0.56, 1.2 and 8 g).

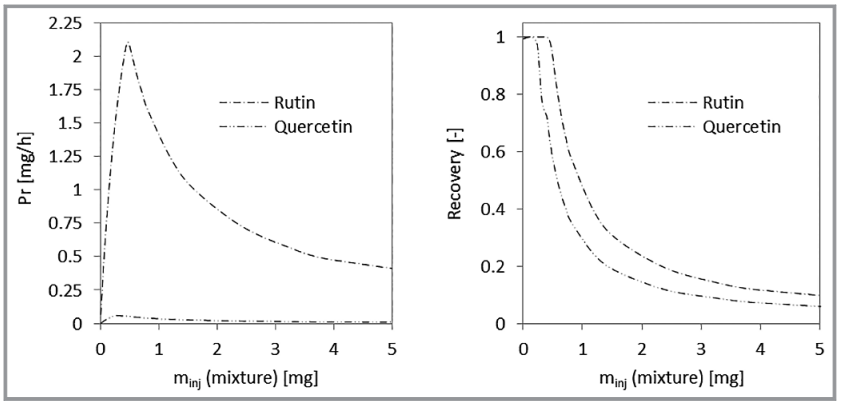

Figure 10. Change of the not normalized productivity of collecting pure rutin and pure quercetin over the total injected mass of a 96/4 % rutin/quercetin mixture. Feed concentrations: rutin 0.077 g L^{-1}, quercetin 0.003 g L^{-1}.

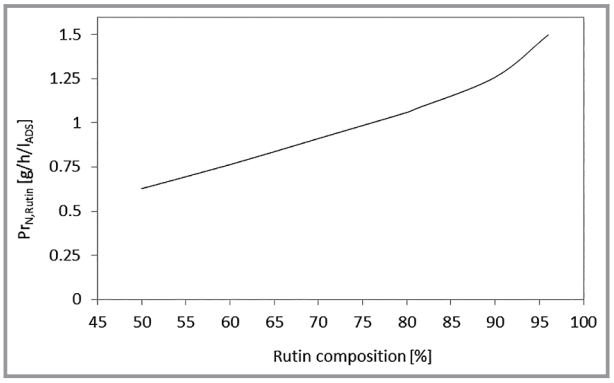

Figure 11. Influence of the feed composition on the achievable normalized rutin productivity. Total feed concentration: 0.08 g L^{-1}. Injected volumes: correspond to the conditions that provide highest productivities.

Predicted scale-specific not-normalized productivities can then be estimated as follows:

$$Pr_i^{large} = Pr_i^{small}\left(\frac{d^{large}}{d^{small}}\right)^2 \quad (9)$$

Keeping the column length at 25 cm the column diameter was varied from the analytical column size value of 0.46 cm to 1 m. The latter value is seen as the currently implementable upper limit of high performance preparative HPLC columns [11]. Fig. 12 shows the development of the productivity using this scale-up concept. The values are given for both substances in kilograms per year. For the largest scale a rutin production rate of almost 900 kg a^{-1} is predicted to be achievable. In this case approximately 16 g are injected periodically using 47 L min^{-1} solvent.

4 Crystallization

Recently, in parallel work an alternative crystallization-based approach was followed to isolate rutin from the raw extract [9, 14, 15]. Since the results are instructive in comparison with the potential of the preparative chromatography method described above, they will be briefly summarized in this section.

XRPD studies confirmed that rutin trihydrate ($Ru \cdot 3H_20$) is the stable solid-state form at ambient conditions and is also present in the extract. Detailed studies of solubility and metastable zone width of rutin in alcoholic solvents methanol, ethanol and isopropanol lead to selection of ethanol as low-toxic and comparatively cheap solvent for further crystallization work. The key step exploits the crystallization of a rutin-ethanolate phase as an intermediate. Starting with an unsaturated solution of about 8 wt % extract in ethanol at 50 °C, cooling to 25 °C and seeding with a suspension of rutin ethanolate phase led to quick crystallization and almost complete depletion of the target molecule in the solution as result of the high supersaturation applied and the low solubility of the ethanolate phase in the solvent used.

The primarily formed rutin-ethanolate phase undergoes in presence of ethanol quickly a phase transition to the desired trihydrate phase when exposed to ambient air conditions. The recently patented crystallization process [16] is capable to isolate after extraction 99 % pure rutin with overall process

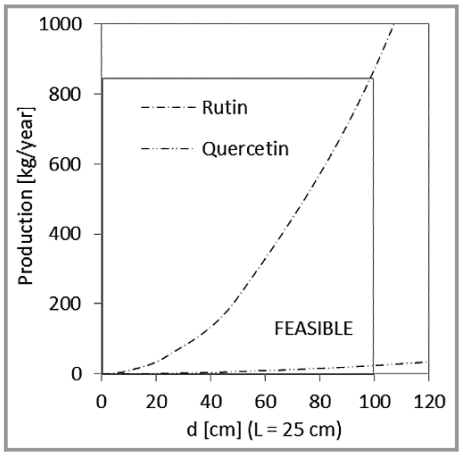

Figure 12. Predicted increase of the production rate of rutin and quercetin for increased column diameters (96/4 feed mixtures).

yields of 95 % and 87 % in batch and semi-continuous operation, respectively. Evaluating the performance of the robust process which can be performed on-site an exploitable rutin productivity of 3.1 $g\,h^{-1}L_{solv}^{-1}$ was estimated [15].

5 Conclusions

This paper demonstrated the feasibility of using repetitive batch chromatography to isolate rutin from mixtures with quercetin. Such mixtures originate from extracting rutin from *Sophora japonica* L. Estimating basic chromatographic parameters a model was parametrized and could be applied to estimate the productivity of isolating rutin form binary rutin/quercetin mixtures as 1.5 $g\,h^{-1}L_{ADS}^{-1}$. Production rates for different column dimensions were also estimated. Comparing with the performance and simplicity of the mentioned alternative crystallization technique capable to provide rutin directly from the extract, the chromatography method analyzed in this paper is not competitive. Other pairs of stationary and mobile phases as well as more sophisticated chromatographic configurations definitely possess potential for further improvement. For such developments the conceptional work presented here might prove helpful.

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