Journal of Chromatography A 1757 (2025) 466098

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Chromatographic method to isolate valuable ingredients of saffron extracts

Mohsen Fotovati [a], Ju Weon Lee [a], Mohammadreza Taheri [b], Alireza Ghassempour [c], Hassan Rezadoost [c], Andreas Seidel-Morgenstern [a,*]

[a] Max Planck Institute for Dynamics of Complex Technical Systems, Sandtorstrasse 1, 39106 Magdeburg, Germany
[b] Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Planegg, Germany
[c] Shahid Beheshti University, Medicinal Plants and Drugs Research Institute, Tehran, , Iran

ARTICLE INFO

Keywords:
Saffron ingredients
Reversed-phase chromatography
Equilibrium theory
Henry constants
Step-gradient elution

ABSTRACT

The valuable spice saffron contains several valuable ingredients. This paper presents results of isolating picrocrocin, crocins I and crocin II from extracts generated of two grades of saffron using reversed phase chromatography exploiting step gradients. After performing a preliminary feasibility study using a low grade feed, isocratic pulse experiments were conducted to estimate and correlate the adsorption equilibrium constants for the target components for different constant mobile phase compositions. A graphical representation of the development of the chromatograms based on the equilibrium theory of chromatography was exploited for the design of a simple step gradient processes. The solvent gradient conditions identified were implemented and found to be applicable to efficiently solve the separation problem. Classical scale up rules were used to increase the productivity using a larger column volume. The tree target compounds could be isolated with purities of 97.3 %, 98.3 %, and 96.6 %, respectively. The component specific productivity of provision was for the feed processed between 0.6 and 1.7 g/h/L, which indicates potential for large-scale application of the separation method developed.

1. Introduction

1.1. Saffron: ingredients and analysis

The stigmas of *Crocus sativus Linnaeus* (Saffron) represent one of the most expensive spices in the world [1]. Picrocrocin (PC), crocin I (CI), crocin II (CII), and safranal are the major valuable ingredients in saffron extracts, which are responsible for the bitter taste, the red color, and odor of saffron, respectively [1]. Picrocrocin and crocins have considerable potential for treating human diseases and disorders, such as inflammatory diseases [2,3], multiple sclerosis [4], depressive disorder [5, 6], ophthalmic disease [7], and cancer [8]. In addition, crocins are red-color apocarotenoids, which are often used as a colorant and spice in the food industries [9,10]. Since there is no efficient production method for pure picrocrocin and crocins, their costs are very high. Despite of various efforts to synthesize picrocrocin and crocins, extraction from plant sources is attractive for provision in a commercial scale [11]. General aspects of extracting natural products are outlined comprehensively in [12]. An example exploiting the selective extraction of crocin from saffron stigmas using molecularly imprinted polymers (MIP) is described in [13]. The optimization of solid-phase extraction (SPE) conditions to efficiently separate picrocrocin and crocins from aqueous saffron extracts is reported in [14].

For analysis most frequently chromatographic techniques were applied. In recent years, many efforts have been devoted to develop high resolution HPLC methods capable to analyze ingredients of saffron extracts with a focus of quantifying the content of valuable components. The method reported in [15] was applied successfully for the quantification of crocins and picrocrocin in saffron. Application to analyze various saffron samples, facilitated quality control and differentiation from other Crocus species. Centrifugal partition chromatography was used for the purification of saffron ingredients, in particular crocin I [16]. In [17,18] semi-preparative reverse phase HPLC (RP-HPLC) is described as a tool for the purification of picrocrocin and crocins from certain saffron extracts. Crocin I was obtained by fast centrifugal partition chromatography (FCPC) with purity >90 % and yield of about 48 % in a single run without fractionating the solvent [19]. High-speed counter-current chromatography was reported to be applicable for purifying crocins I and II from *Gardenia Jasminoides* connected with relatively low productivity [2]. Low-pressure liquid chromatography

---

* Corresponding author.
E-mail address: seidel-morgenstern@mpi-magdeburg.mpg.de (A. Seidel-Morgenstern).

https://doi.org/10.1016/j.chroma.2025.466098
Received 10 March 2025; Received in revised form 26 May 2025; Accepted 27 May 2025
Available online 1 June 2025
0021-9673/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

was another chromatographic technique tested for purifying crocins [20]. Recently, an attractive method was reported suitable to isolate crocin-I from saffron extracts using a twin-column Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) system and pure ethanol as the solvent. The process delivered a crocin-I purity of 99.7 % at much higher recovery and productivity compared to traditional batch chromatography [21].

Besides chromatographic separations, crystallization based techniques were also considered as an alternative for isolating crocins [22], which are not further discussed in this paper.

1.2. *Chromatography separation using gradients*

Due to the wide retention time spectrum expected for the different ingredients of saffron extract, gradient chromatography was the method of choice. In contrast to simpler isocratic operation the elution strength a suitable modulated during gradient elution. Gradients can significantly reduce the run time required for the separation and improve the resolution around the target components present in complex mixtures [23–27].

There are numerous types of gradient regimes applicable. Mostly applied under HPLC conditions are solvent gradients and flow-rate gradients. Alternatives are offered by pH and temperature gradients. Rarely applied are mixed gradient altering simultaneously more than one parameter [26,27].

Since their introduction in the early 1950s in particular, solvent gradient elution are most widely used in liquid chromatography [26]. Among the infinite number of options for designing gradient shapes, a particular easy to implement mode is to impose a certain number of step changes of the solvent composition at the inlet of the column at distinct switch times. Thus, if there are M characteristic solvent changes intended, M switch times need to be specified [28].

1.3. *Goals of this study*

The aim of this study is to contribute to improved simultaneous isolation of the above-mentioned three major ingredients of saffron extracts, namely picrocrocin, crocin I and crocin II. Rational method development, scale up and productivity are reported. Due to available preliminary evidence, reverse-phase high performance liquid chromatography (RP-HPLC) was considered. Thermodynamic information acquired in a first stage of investigations combined with the subsequent application of the equilibrium theory of chromatography was used for a rational short-cut design of suitable step-gradient elution conditions. Finally, a theoretically predicted promising gradient regime was evaluated experimentally in a preparative scale for model validation and to estimate the process productivity.

2. Extraction, analytics, characterization of feed

Ground saffron stigmas (saffron powder) used for this study were acquired in Iran by the Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran). For this study there were two grades of saffron samples available: a Low Grade Feed (LGF), and a High Grade Feed (HGF). These two feed materials differed in their content. In a pre-extraction step with a Soxhlet, 12 g of saffron powder from both LGF and HGF were extracted with 100 ml dichloromethane at 40 °*C* for 7 h. In this pre-extraction step, numerous unwanted compounds in saffron powder such as fatty acids that can contaminate reverse phase chromatography columns were eliminated to support the subsequent extraction of the target molecules in the next steps.

Since DCM is a volatile and potentially hazardous solvent, its complete removal was ensured through air-drying of the pretreated saffron samples under a fume hood prior to the main extraction step. This approach guarantees that no residual DCM remained in the final extract, maintaining both the safety and integrity of the sample preparation process.

In the main extraction step, 10 *g* of the pretreated saffron powder (from both LGF and HGF) was added to the 1 L of 80 % ethanol aqueous solution. Ethanol was added to increase the solubility and also the stability of the components. Extraction was performed under stirring (350 *rpm*) for 15 min (equilibrium time) at room temperature. After the saffron powder has settled down, a solid-liquid separation was performed by decantation. The still wet saffron powder was exposed again to fresh 80 % ethanol aqueous solution. The procedure was repeated two more times to achieve a high degree of extraction (“exhaustive extraction”). Finally, all three obtained extract phases were concentrated with rotary evaporator (BUCHI, Switzerland) and dried by a freeze dryer (Zirbus technology GmbH, VaCo2, Germany).

A preliminary analysis of saffron crude extracts and a study of the retention behavior of essential constituents were performed under both isocratic and gradient conditions using an octadecyl silica (C18) reversed phase column (YMC-Triart Prep C18-S, 150 × 4.6 mm, particles of 15 μm diameter and 12 nm pore size). For this a HPLC system (Agilent 1260 series, Germany) was used, equipped with a degasser (1260 Degasser), a four-channel gradient pump (1260 Binary Pumps), an autosampler (1260 ALS), a column oven (1260 TCC), and a UV–Vis photodiode array detector (1260 MWD VL). An example of a separation of the components of a saffron crude extract performed using consecutively several linear ethanol-water gradients is shown in Fig. 1.

It should be mentioned that there are two groups of impurities present, namely early eluting impurities (designated as “Imp. I”) and late eluting impurities (designated as “Imp. II”). The components generating the three larger peaks were identified by LC-MS (Q-TOF-LC-MS 6530, Agilent Technology, CA, US) using a reduced flowrate of 0.4 ml/min. The detected sodium adduct masses $[M+Na]^+$ belong to picrocrocin, crocin II and crocin I (353.16, 837.36 and 999.36 m/*z*, respectively).

Table 1 shows the determined concentrations of picrocrocin (PC), crocin I (CI), and crocin II (CII) in the two saffron crude extracts based on calibration curves generated using samples pre-purified in our lab collected from numerous repetitive injections on the analytical column.

The error ranges for the mass fractions provided in Table 1 ($\Delta x_{S,i}^{Feed}$ *between* 0.015 to 0.025) are based on the variabilities observed in three replicate measurements.

LGF, which contained less target component, was employed for preliminary studies of purifying picrocrocin and a total crocin fraction which lumps CI, CII and further crocins. Subsequently in a more detailed study for HGF a refined gradient was developed which allows the separation and purification of picrocrocin, crocin I, and crocin II.

3. Theory

3.1. *Equilibrium dispersive model*

An important model capable to describe main features of preparative chromatography is the equilibrium dispersive model [23]:

$$\frac{\partial c_i}{\partial t} + F\frac{\partial q_i(c)}{\partial t} + u_L\frac{\partial c_i}{\partial z} = D_{app,i}\frac{\partial^2 c_i}{\partial z^2} \qquad i = 1, NC \tag{1}$$

Here c_i is the mobile phase concentration of component i in equilibrium with the stationary phase concentration of that component, q_i. The parameters, u_L, $D_{app,i}$, and NC are the phase ratio in the column, the linear velocity of the mobile phase, the apparent axial dispersion coefficient of component i, and the number of components, respectively [23, 25]. The phase ratio F is connected with the total column porosity ε_t as follows:

$$F = \frac{1-\varepsilon_t}{\varepsilon_t} \tag{2}$$

The total porosity is connected as follows with the column dead time,

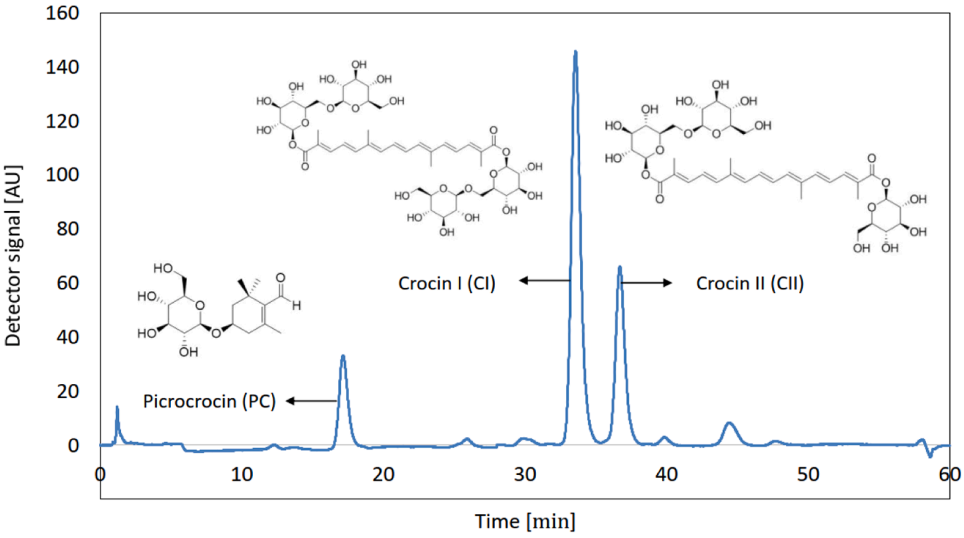

Fig. 1. Chromatogram separated three major components of saffron crude extract. Reverse phase mode with ethanol (A) – water (B) gradient elution. The linear gradient method used for analysis was: 90 % B (0–5 min), 90 % to 49 % B (5–50 min), 49 % to 50 % B (50–51 min) and 50 % B (51–55 min), 50 % to 90 % of B (55–56 min) and 90 % B (56–60 min). The mobile phase flow rate was $\dot{V}_L$=1 ml/min and the injection volume was V_{inj}=5 µl (sample: m_{inj}=1 mg dried extract dissolved in 1 ml of 80 % Ethanol solvent). UV detection at 254 nm (until 21 min) and 440 nm (after 21 min).

Table 1

Measured total solid phase mass fractions, $x_{S,i}^{Feed}$, of the three main ingredients for two grades of Iranian saffron (LGF and HGF). The values were determined using the exhaustive extraction technique. Solid phase sample sizes used for analysis: $m_S^0 = 0.1\ g_S$.

Components	Mass fraction of component i, $x_{S,i}^{Feed}$ $[\frac{g_{S,i}^{Feed}}{g_S}]$	
	LGF	HGF
Picrocrocin, PC	0.100 ± 0.015*	0.150 ± 0.020
Crocin I, CI	0.180 ± 0.020	0.250 ± 0.025
Crocin II, CII	0.065 ± 0.019	0.094 ± 0.023

* Errors estimated based on analyzing three extraction experiments performed under identical conditions.

t_0, the volume of the empty column, V_c, and the volume flow rate of the mobile phase, $\dot{V}_L$:

$$\varepsilon_t = \frac{t_0 \dot{V}_L}{V_c} \tag{3}$$

with

$$t_0 = \frac{L}{u_L} \tag{4}$$

The apparent axial dispersion coefficients ($D_{app,i}$) quantify as lumped parameters band broadening in the column. They are related as follows to the column length L and the widely used column efficiency expressed as the average value of the component specific numbers of theoretical plates (equilibrium steps), N_i, [19]:

$$D_{app,i} = \frac{u_L L}{2N_i} \tag{5}$$

It is well known that for analytical peaks characterized by Gaussian peak shapes the component plate numbers can be estimated analyzing the retention times, $t_{R,i}$, and corresponding measures of the peak width. For the latter can be used the standard deviation σ_i, the peak width at half height $w_{1/2,i}$, or the base peak width $w_{base,i}$ [23]:

$$N_i = \left(\frac{t_{R,i}}{\sigma_i}\right)^2 \cong 5.54\left(\frac{t_{R,i}}{w_{1/2,i}}\right)^2 \cong 16\left(\frac{t_{R,i}}{w_{base,i}}\right)^2 \qquad i = 1,\ NC \tag{6}$$

3.2. Equilibrium theory

Often N_i exceeds 100, and accordingly $D_{app,i}$ is very small. This leads to the fundamental equation of equilibrium theory [29–31]. This theory assumes that, Eq. (1) can be reduced as follows:

$$\frac{\partial c_i}{\partial t}\left(1 + F\frac{dq_i(c_i)}{dc_i}\right) + u_L\frac{\partial c_i}{\partial z} = 0 \qquad i = 1, NC \tag{7}$$

For dilute feeds, the equilibrium loadings of a component i depend usually in a linear manner on the fluid phase concentrations. Furthermore, they do not depend on the presents of other components. Thus holds:

$$q_i(c_i) = K_i c_i \qquad i = 1, NC \tag{8}$$

The equilibrium constants K_i are often called Henry constants. They capture the isotherm slopes.

$$K_i = \frac{dq_i}{dc_i} \tag{9}$$

Introducing this equilibrium information into Eq. (7) provides the propagation velocity of the component in the column as follows.

$$u_i = \frac{u_L}{1 + FK_i} \qquad i = 1, NC \tag{10}$$

This velocity quantifies how a certain component specific concentration propagates over time in the column:

$$u_i = \frac{\Delta z}{\Delta t} = \frac{z - z_{init}}{t - t_{init}} \qquad i = 1, NC \tag{11}$$

or

$$z = z_{init} + u_i(t - t_{init}) \qquad i = 1, NC \tag{12}$$

For $t_{init} = 0$ and $z_{init} = 0$, the retention times required to reach the column outlet are:

$$t_{R,i}(z = L) = \frac{z}{u_i} = t_0(1 + FK_i) \qquad i = 1, NC \tag{13}$$

In the above equation the column dead time t_0 corresponds to Eq. (4). Thus, knowing K_i and the phase ration, F, provides for a given linear velocity, u_L, and column length, L, the specific retention time of a component *i*.

3.3. Variation of Henry constants and design of gradient operation

To determine the Henry constant for given constant solvent compositions, K_i, using Eq. (13), the column dead time t_0, the phase ratio F and the retention time of the solute, $t_{R,i}$, need to be determined experimentally [23]. Since the values of these Henry constants are affected by the composition of the solvent, these measurements must be performed multiple times, for example by preparing different fractions of the stronger solvent applied to alter the solvent strength (below called modifier). It is convenient to characterize the solvent composition using the volume fraction of the modifier present in the solvent stream. We designate this modifier fraction by ε_m:

$$\varepsilon_m = \frac{\dot{V}_m}{\dot{V}_t} \tag{14}$$

$\dot{V}_m$ and $\dot{V}_t$ are the modifier and total volumetric mobile phase flow rates. It is assumed that the two solvent constituents are well-mixed and move un-retained with velocity u_L through the column.

The solvent dependence of the Henry constants on the solvent composition can be expressed using the well-established linear solvent strength model (LSS) [27,32], which provides the following exponential function which contains two component specific empirical parameters A_i and B_i:

$$K_i(\varepsilon_m) = A_i e^{B_i \varepsilon_m} \tag{15}$$

To capture unavoidable limitations of Eq. (15), a more flexible empirical extension applying four free parameters, A_i^*, B_i^*, C_i, D_i, is given by the following equation which is used in this work:

$$K_i(\varepsilon_m) = A_i^* e^{B_i^* \varepsilon_m} + C_i e^{D_i \varepsilon_m} \tag{16}$$

This extended equation contains Eq. (15) as a special case.

Quantitative knowledge of the solvent composition dependence of the Henry coefficients, $K_i(\varepsilon_m)$, and thus possible changes in the local and temporal migration velocities, $u_i\,(t,z)$ (eq. (10)), allows to design in a rational way solvent gradient processes. The design of simple step gradients (as applied below) requires specifying the solvent compositions in each step j, $\varepsilon_{m,j}$, the switch times between two steps, $t_{s,j}$, and the number of steps M. The corresponding gradient protocol describes the resulting local and temporal solvent compositions as follows:

$$\varepsilon_m(t,z) = \varepsilon_{m,j} \ \mathit{for} \ \left[t_{s,j-1} < t \le \left(t_{s,j} + \frac{z}{u_L}\right)\right] \qquad j = 1, M \tag{17}$$

Together with Eq. (12) the positions of the component specific bands can be predicted analytically using Eq. (7). Instructive and helpful are graphical solutions of the development of the chromatograms which presented in a space-time (z-t)-plane [29–31]. For illustration and to prepare the later presented application to the "pseudo-5-component-system" of interest in this work, we show first the development of a chromatographic separation of a two-component feed mixture exploiting first isocratic conditions (Fig. 2) and then a 2-step gradient (Fig. 3). In both cases a finite injection time, Δt_{inj}, is considered. In order to maximize the productivity, it is of interest to reduce the cycle time, i.e. the time difference between two consecutive injections. This time is defined as:

$$\Delta t_{cyc} = \sum_{n=1}^{N} t_{start\ \ injection\ \ n+1} - t_{start\ \ injection\ \ n} \tag{18}$$

Fig. 2 indicates the constant propagation velocities of the two components over the whole operation period by the constant component specific slopes. Fig. 3 shows the effect of a solvent step gradient introduced at $t_{s,1}$. At this time ε_m is changed from $\varepsilon_{m,1}$ to $\varepsilon_{m,2}$. If this new solvent composition has the potential to increase the propagation velocities (due to reduced Henry-constants) the slopes of the bands increase. There needs to be respected that the change in composition also travels through the column with a constant velocity, namely u_L. The figure shows that the accordingly designed gradient allows reducing the cycle time without creating overlaps of bands corresponding to different consecutive injections.

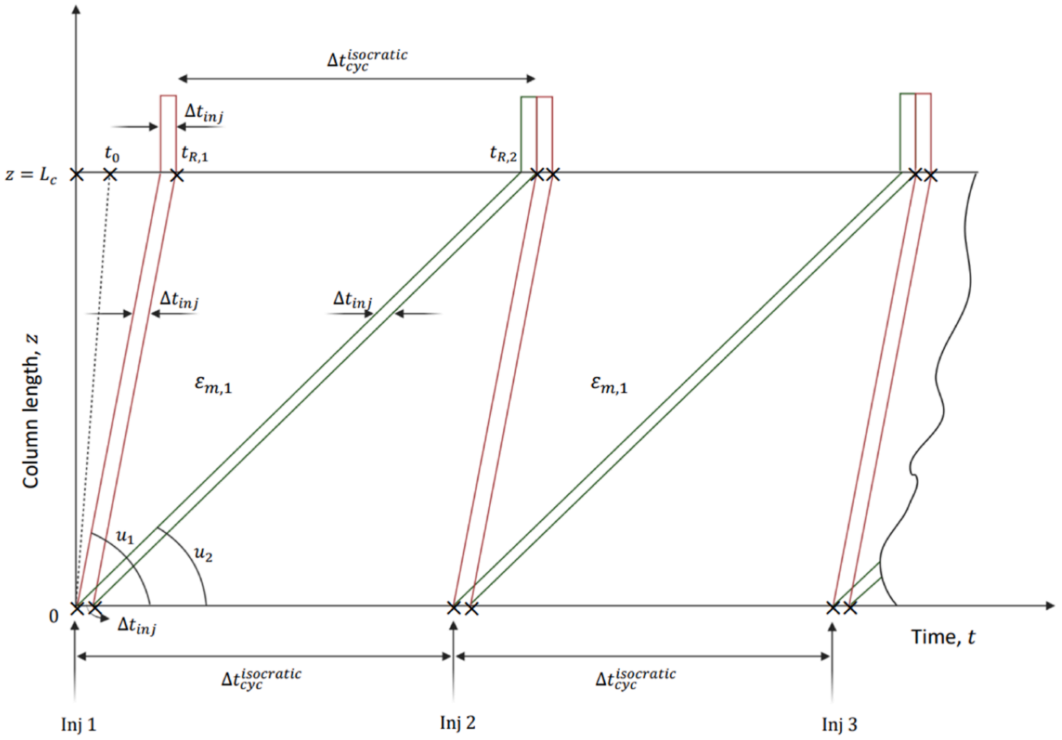

Fig. 2. Schematic illustration of three consecutive injections of a binary feed mixture (red and green) under isocratic conditions at a constant solvent composition characterized by $\varepsilon_{m,1}$. Δt_{inj} is the injection time and $\Delta t_{cyc}^{isocratic}$ is the cycle time as a measure of process productivity.

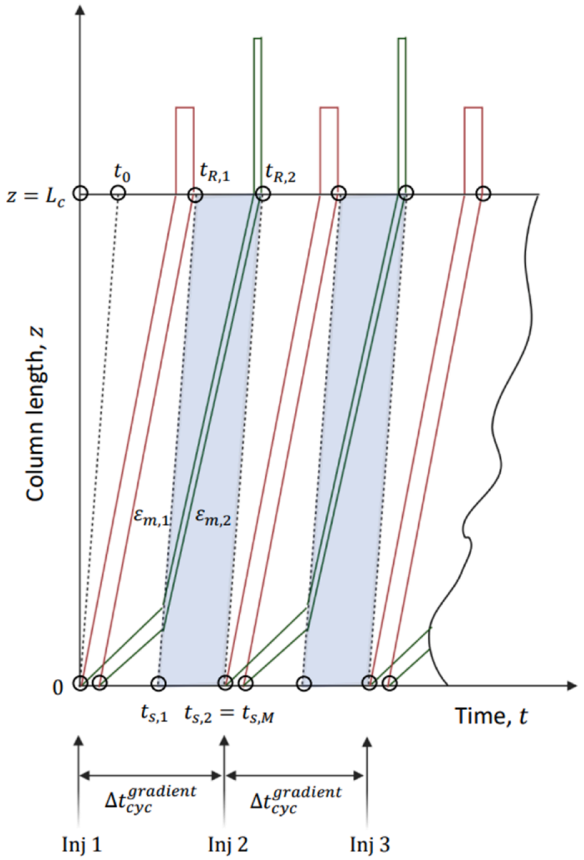

Fig. 3. 2-step solvent gradient elution ($M = 2$). The switch in solvent composition occurs at $t_{s,1}$. The white color section indicates Step1 ($\varepsilon_{m,1}$), the blue section indicates Step2 ($\varepsilon_{m,2}$, which causes an acceleration). The cycle time of the gradient operation is $\Delta t_{cyc}^{gradient}$. It is smaller than $\Delta t_{cyc}^{isocratic}$ (see Fig. 2).

3.4. Performance criteria and scale up

The performance of the separation processes can be evaluated for example exploiting purity, productivity, and yield as defined below. With these performance parameters it is possible to compare different rivaling process variants.

3.4.1. Purity

In crude extract processing (solid-liquid chromatography), purity is ratio of the mean concentration of the purified target component i in the fraction collected ($\bar{c}_i$) to the sum of all mean concentrations ($\sum_{i=1}^{N} \bar{c}_i$):

$$Pu_i = \frac{\int_{t_{i,\ coll.}^{start}}^{t_{i,\ coll.}^{end}} c_i(t)dt}{\sum_{i=1}^{N} \int_{t^{start}}^{t^{end}} c_i(t)dt} \times 100 = \frac{\bar{c}_i}{\sum_{i=1}^{N} \bar{c}_i} \times 100 \ [\%] \tag{19}$$

3.4.2. Productivity

A productivity of the separation can be defined as the rate of producing a target component, i, for the given scale (expressed for example by the empty column volume) by the following equation:

$$Pr_i = \frac{m_{i,coll}}{\Delta t_{cyc} \times V_c}; \ [g/h/l] \tag{20}$$

Where m_i is the collected mass of the target component i, V_c is the column volume, and Δt_{cyc} is the cycle time (Eq. (18)).

3.4.3. Recovery yield

The recovery yield is also of interest. The yield Y_i is for a component i the ratio of the mass collected, $m_{i,coll.}$, to the corresponding mass injected, $m_{i,inj.}$:

$$Y_i = \frac{m_{i,coll}}{m_{i,inj}} \times \ 100 \ [\%] \tag{21}$$

In case of complete resolution and recoveries of Y_i=100 %, the component specific productivities Pr_i vary according to the fractions of the components in the feed, $x_{S,i}^{Feed}$ (Table 1).

3.4.4. Scale-up rules

In the analytical scale the injected mass of a component i, $m_{i,inj}$, is small. It depends both on injection concentration and on injection volume, ($c_{i,inj}$, and V_{inj}). The latter is specified with the volumetric flowrate and the injection time, t_{inj}.

A well-known and widely used reliable scale-up method is based on the following relations which evaluates a scale up by increasing exclusively the column diameter and keeping the column lengths constant [23,25]. The following scale-up rule allows specifying the increase in injection mass required in case of larger columns as follows:

$$\frac{m_{inj}^{large}}{m_{inj}^{small}} = \frac{c_{inj}^{large}}{c_{inj}^{small}} * \frac{V_{inj}^{large}}{V_{inj}^{small}} = \frac{c_{inj}^{large}}{c_{inj}^{small}} * \left(\frac{d_c^{large}}{d_c^{small}}\right)^2 \tag{22}$$

This also allows to specify the flowrate adaptation required to match in both scales the residence times as follows:

$$\frac{\dot{V}_L^{large}}{\dot{V}_L^{small}} = \left(\frac{d_c^{large}}{d_c^{small}}\right)^2 \tag{23}$$

The symbols d_c^{large} and d_c^{small} refer to the inner diameters of the larger (preparative) column and the smaller (analytical) column, respectively.

This approach provides the following scale up factor, *SF,* caused by a dimension (diameter) increase:

$$SF = \left(\frac{d_c^{large}}{d_c^{small}}\right)^2 \tag{24}$$

It should be mentioned here that this simple scale-up approach ignores numerous unavoidable differences between operation in small and large columns, for example differences in the inlet distribution profiles and the development of radial concentration profiles [23].

4. Separating saffron ingredients

4.1. Preliminary empirical study with low grade feed (LGF)

At first a preliminary study was made with the cheaper LGF. The goal of this study was to evaluate the impact of altering the solvent composition and also the flowrate on the course of chromatograms. The runs also served to test a multi-port valve (CSEP C9812, KNAUER, Germany), which allowed to fix four columns on the lower movable part of the valve and to operate them in parallel. Thus, it was possible to implement in an easy way four steps gradients in which the time step sizes were identical for all steps. Four YMC-Triart Prep C18-S columns filled with the same particles were used, which differed in the length and diameter from the column used to generate Fig. 1. The dimensions were: ($L = 200$ mm and =50 mm). Goals of this preliminary study were: a) to eliminate the fast eluting impurities, b) to collect picrocrocin with high purity as a target T1 and c) to collect a fraction containing all crocins (total crocins) as target T2. To achieve these goals in a simple way it was tried to use only two solvent strength levels. As the strong solvent (modifier) pure ethanol was used (ε_m=1). To achieve residence times which allowed applying a pre-specified step time of 10 min an elution strength of the weaker solvent of ε_m=0.19 was empirically found to be suitable. Exploiting the potential multiport valve setups and following [28] the additional possibility of imposing flowrate variation was also tested. Fig. 4 illustrates a promising separation carried out, which successfully

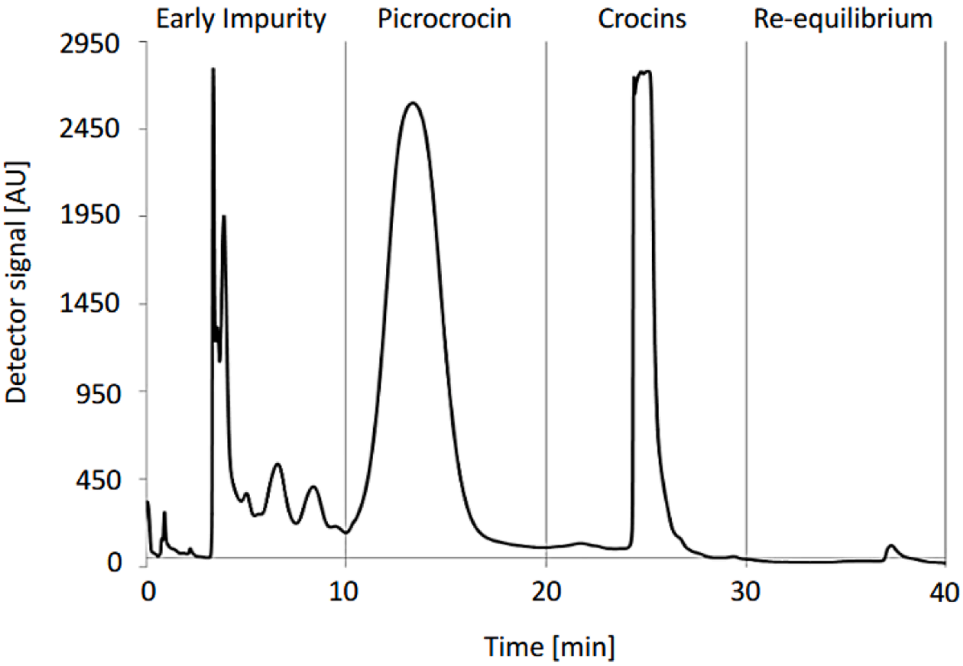

Fig. 4. Separation profile of the LGF extract on the preparative column using the gradient method described: $\varepsilon_{m,1} = \varepsilon_{m,2} = 0.19$, $\varepsilon_{m,3} = 1.00$, $\varepsilon_{m,4} = 0.19$. $\dot{V}_{L,1,2,3,4} = 107, 55, 35, 32\ ml/min$. Late impurities were eluted with total crocins. Hence, there are no last impurities, and there are only early impurities at the first step of elution. Last step is only for the re-equilibrium. m_{inj}^{Feed}=3 g, $t_s = 10\ min$, Δt_{cyc}=40 min.

reached the goals mentioned.

Table 2 provides the purity (Eq. (19)) of the collected fractions of picrocrocin and the total of all crocins corresponding to the chromatogram shown in Fig. 4 together with estimates for the productivity (Eq. (20)), and yield (Eq. (21)). These productivities serve below as a benchmark.

In the preliminary study with the LGF described an empirical design approach was followed characterized by the following shortcomings: a) there was no quantitative understanding acquired and exploited how the solvent strength influences the retention times, b) no resolution of the individual crocins was considered.

Therefore, a more rational and systematic investigation was carried out based on acquiring thermodynamic parameters and exploiting the equilibrium theory to predict suitable gradient regimes. This prediction then served as the basis for an experimental study with the valuable HGF.

4.2. *High grade feed (HGF): design and application of a 3-step gradient*

In this main study, a systematic solvent step-gradient separation method was developed for a fixed constant flow rate using equilibrium theory and the adsorption isotherms. Below we will designate this step-gradient separation method for the five characteristic “components” as follows:

1: lumped fraction of early eluting unidentified impurities,
2: picrocrocin,
3: crocin I,
4: crocin II,
5: lumped fraction of late eluting unidentified impurities.

Table 2
Results of the preliminary study with the LGF based on the chromatogram shown in Fig. 4. Purities, productivities and yields based on to Eqs. (19)–21.

Component (i)	Pu_i [%]	Pr_i $[g_i/h/l]$	Y_i [%]
Picrocrocin	96	0.77	97
Total Crocins (CI, CII and other crocins)	95	1.93	91

As described in Section 2, the processing of the HGF was performed using again the YMC-Triart C18-S column with $L = 150$ mm und $D = 4.6$ mm. with mobile phases consisting of ethanol as the organic modifier and water. To simplify the process, the flow rate was kept constant and adjusted to the column size. A volumetric flow rate of 1.2 mL/min was selected based on lowest HETP, efficiency and pressure drop evaluations.

The column dead time, t_0 (Eq. (4)), was experimentally determined based on the retention of uracil to be 1.12 min. This leads to a phase ratio F of 0.86, which corresponds to ε_t=0.538. Peak widths investigations were carried out to estimate band widths in order to improve ideal gradient switch times predicted by equilibrium theory (Eq. (6)).

4.2.1. *Estimation and correlation of solvent composition dependent henry constants*

In isocratic elution, the composition of the mobile phase is kept constant. The retention time of component i and column efficiency can vary with different mobile phase compositions. For systematic method development, the retention behavior of picrocrocin, crocin I, and crocin II and their peak widths in several different volumetric fractions of modifier (ethanol), ε_m =[0.05, 0.5], were studied isocratically (Table S.1). Fig. 5 shows the changes in Henry constants (using Eq. (7)) of these main three components and selectivity between them, defined as $\alpha_{ij}(\varepsilon_m) = \frac{k_j(\varepsilon_m)}{k_i(\varepsilon_m)}$.

The Henry constants determined as a function of the volume fraction of the modifier were correlated using Eq. (16). The empirical coefficients A_i^*, B_i^*, C_i, D_i obtained by nonlinear egression are provided in Table S.2. For picrocrocin, the ethanol volume fraction ε_m explored during the experiments ranged from 0.05 to 0.5, whereas for crocin I and II, it spanned 0.22 to 0.5 (Table S.1). At ε_m values below 0.22, crocins exhibit high affinity for the stationary phase, resulting in negligible elution and/or significant band broadening. The Henry constant of late impurities ($K_{5,3}$, purple solid dot for ε_m=0.5 in Fig. 5A) were only

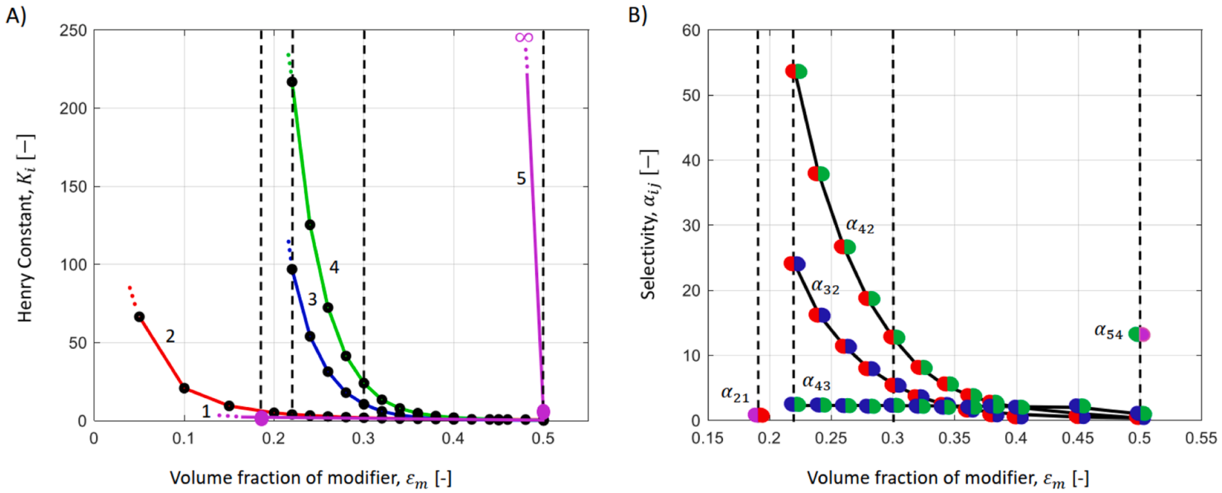

Fig. 5. Henry constant and selectivity vs. volume fraction of the organic modifier (ethanol) in reverse phase isocratic elutions. Figure A: Early impurities (1, purple), picrocrocin (2, red), crocin I (3, blue), crocin II (4, green), and late impurities (5, purple). Figure B: Selectivity between the mentioned components. t_0=1.12 min, V_c=2.5 ml, ε_t=0.538. Eq. (16) was used to correlate the data shown in figure A.

determined at this single solvent composition.

Eq. (16) can be now employed to estimate all relevant Henry constants, allowing for the subsequent prediction of component retention times at specified volume fractions of the modifier (utilizing Eq. (10)). This approach was utilized to develop and design a 3-step solvent gradient elution procedure with the goal in mind to reduce the cycle time and to increase the productivity.

4.2.2. *Design of a 3-step solvent gradient*

In this section we apply the concepts outlined in sub-Section 3.3 based on the equilibrium theory to separate the 5 characteristic components of this system indicated by different colors: impurities (1, purple), picrocrocin (2, red), crocin I (3, blue), crocin II (4, green), and late impurities (5, purple).

With the framework provided by equilibrium theory and illustrated above for the case of separating a simple binary mixture using a step-gradient (Fig. 3) it is possible to rationally identify and design a step-gradient regime capable to process the “pseudo-5-component” mixture of interest, which offers a minimum for the cycle time connected with a maximum for the productivity. The concept is based on knowing for characteristic key components the dependence of the Henry constants on the solvent compositions. Inspecting the retention order and the relative selectivities observed in the preliminary study, it is obvious that the early eluting impurities and the first target component (PC), and also the two crocins (CI and CII) can be relatively easy separated under specific isocratic conditions. It can be concluded, that the five mixture components can be obtained using just three different solvent compositions altered at three characteristic shift times. By fixing first three concrete solvent compositions, namely $\varepsilon_{m,1}$, $\varepsilon_{m,2}$ and $\varepsilon_{m,3}$, the development of the chromatogram can be predicted for three specific corresponding switch times using the theory and graphical solution described above. The graphical illustration allows to specify feasible switch times and to achieve short cycle times connected with high process productivities.

For solving the current separation problem, as illustrated in Fig. 6, the following three functions for picrocroin, crocin II and the late impurities are relevant: $K_2(\varepsilon_m)$, $K_4(\varepsilon_m)$, and $K_5(\varepsilon_m)$. The slopes shown in the *z*-*t*-diagrams result from the dependences of the Henry constants on the solvent composition shown in Fig. 5 and quantified by Eq. (16) (parameters in Table S.2).

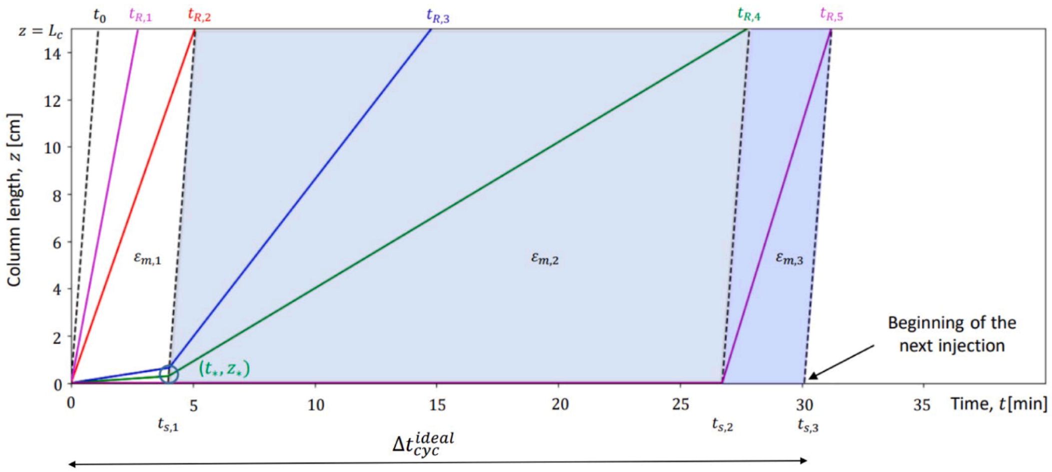

Fig. 6. Trajectories of the 5 components considered applying a 3-step solvent gradient elution. Early impurities (1, purple line), Picrocrocin (2, red line), crocin I (3, blue line), crocin II (4, green line) and late impurities (5, purple line). The specific values illustrated correspond to the case studied described in the text later. Gradient parameters: $\varepsilon_{m,1,2,3}$=0.22, 0.3, 0.5, $t_{s,1,2,3}^{ideal}$ =3.98, 26.73, 30.10 min, Δt_{cyc}^{ideal}=30.10 min (Table 3). In contrast to Figs. 2 and 3, a small (not indicated) Δt_{inj} is considered in this plot.

The trajectories and the characteristic times and positions indicated in Fig. 6 can be calculated using the three Henry-constant functions mentioned with the following equations:

$$t_{s,1} = \frac{L}{u_L}\left(1 + FK_{2,1}\left(\varepsilon_{m,1}(t,z)\right)\right) - t_0 \tag{25}$$

$$t_{s,2} = t_* + \frac{L - z_*}{u_L}\left(1 + FK_{4,2}\left(\varepsilon_{m,2}(t,z)\right)\right) - t_0 \tag{26}$$

The coordinates in Eq. (26) mark the position and time at which the new solvent compositions reaches component 4. These coordinates are:

$$t_* = \frac{1 + FK_{4,1}\left(\varepsilon_{m,1}(t,z)\right)}{FK_{4,1}\left(\varepsilon_{m,1}(t,z)\right)} t_{s,1} \tag{27}$$

$$z_* = u_L\left(t_* - t_{s,1}\right) \tag{28}$$

$$t_{s,3} = t_{s,2} + \frac{L}{u_L}\left(1 + FK_{5,3}\left(\varepsilon_{m,3}(t,z)\right)\right) - t_0 \tag{29}$$

As a main result of this analysis, an ideal cycle time can be estimated for this 3-step gradient exploiting the three pre-specified solvent composition levels:

$$\Delta t_{cyc}^{ideal} = t_{s,3} \tag{30}$$

The three solvent strength levels applied, namely $\varepsilon_{m,1}$=0.22, $\varepsilon_{m,2}$=0.3, and $\varepsilon_{m,3}$=0.5, were chosen because at $\varepsilon_{m,1}$=0.22 picrocrocin is eluting rapidly, but is still sufficiently separated from the weakly retained undefined impurities. Meanwhile the other three components remain in the column. To achieve sufficient separation between the well separated crocin I and II with acceptable elution time, the volume fraction of organic modifier for the second step, $\varepsilon_{m,2}$, was chosen to be 0.3. In the final step, the volume fraction of the organic modifier, $\varepsilon_{m,3}$, was chosen to be 0.5, which was sufficient to wash out all remaining late impurities, as several overlapped peaks. Therefore, the Henry constant for the late impurities was estimated from the estimated mean retention time of the overlapped peaks, which was $K_{5,3}$=3.70 (Table 3).

The pattern of all components shown in Fig. 6 corresponding to this gradient exploiting the three solvent compositions and step times. It leads to a relative short attractive cycle time compared to isocratic elution. Possible rigorous optimization would require further refining the solvent compositions and to recalculate corresponding step times. This option was considered to be outside the scope of this paper.

Table 3 provides a summary of the conditions resulting from the ideal analysis performed using equilibrium theory.

In addition to the results obtained from equilibrium theory based on assuming an infinite column efficiency, losses due to finites plate numbers can be taken into account. Component specific plate numbers of the key components were estimated for several isocratic conditions, $N_i(\varepsilon_m)$. For ε_m=[0.05–0.5], the plate numbers were between 66 and 784. To guarantee that a high purity is achieved, the broadening of the peaks can be incorporated into the gradient design by adding the times connected to 50 % of observed peak bases, $w_{base,i}$. Based on Eq. (6) these base widths can be estimated as function of the solvent composition as follows:

$$w_{base,i}\left(\varepsilon_{m,j}\right) = \sqrt{\frac{16 t_{R,i}^2\left(\varepsilon_{m,j}\right)}{N_i\left(\varepsilon_{m,j}\right)}} \tag{31}$$

Table 3

Design and developed 3-step solvent gradient elution, based on enough separation factor, shorter cycle time and enhanced productivity, along with ideal switch times. $\dot{V}_L$=constant=1.2 ml/min.

Step j	$\varepsilon_{m,j}$	$K_{i,j}$ (Eq. (16))	$t_{s,j}^{ideal}$ [min] (Fig. 6)
$j = 1$	0.22	$K_{2,1}$=4.13 $K_{4,1}$=216.96	3.98 (Eq. (25))
$j = 2$	0.30	$K_{4,2}$=24.07	26.51 (Eq. (26))
$j = 3 = M$	0.50	$K_{5,3}$=3.70	30.10=Δt_{cyc}^{ideal} (Eqs. (29) and 30)

Therefore, improved real switch times can be estimated from the idel values as:

$$t_{s,j}^{real} = t_{s,j}^{ideal} + \frac{w_{base,i}\left(\varepsilon_{m,j}\right)}{2} \tag{32}$$

For the specific system investigated holds for picrocrocin and crocin II N_{PC}=289 and N_{CII}=606 (Table S.3). Together with the retention times (Table S.3) Eq. (32) provides $t_{s,1}^{real}$ = 4.58 min, $t_{s,2}^{real}$ = 28.8 min.

As mentioned above, the late impurities can be completely eluted with $\varepsilon_{m,3}$=0.5. Several unspecified and partly overlapping peaks are observed. Because of the lack of a clear determination protocol for the late eluting components and the desire to achieve reliably a complete column re-equilibration an additional time was empirically selected as 10 min and added to the ideal value, which finally provides $t_{s,3}^{real}$ = Δt_{cyc}^{real}=40.10 min.

4.3. Implementation of solvent step gradient elution with HGF

4.3.1. First validation of gradient regime

At first, the applicability of the designed 3-step gradient was tested using a small column injecting a small sample size. The column length and diameter of the YMC-Triart Prep C18-S were L = 150 mm and D = 4.6 mm. Fig. 7 illustrates the recorded separation profile of HGF using the designed 3-step solvent gradient elution method (Table 3). A relatively small sample mass was injected using small injection volume of 5 µl in order to stay under linear conditions as assumed in the derivation of the model. The three switch times estimated using the column efficiency determined were: $t_{s,j}^{real}$=4.58, 28.8 and 40.10 min.

Fig. 7 shows that the desired simultaneous isolation of rather pure picrocrocin, crocin I and crocin II has been achieved successfully for a dilute feed using the parameters of the designed 3-step gradient. This fact is further confirmed by the relatively good agreement between predicted and observed retention times (Table 4).

After having seen the general applicability of the gradient concept, in order to increase the product, there is a first option to increase for the same column the product amount be increasing the injection volume and/or injection concentration (or both) maintaining cycle times. Here are constraints in solubility limits and in the not considered unavoidable non-linearity and competitions required in the equilibrium functions. We investigated the potential of increasing the column loading (m_{inj}=0.005 mg) while still maintaining sufficient separation striving to keep touching band situations and to avoid peak overlapping and yield reductions [19]. In experiments with this small column an upper limit for the injection mass of m_{inj}=12.5 mg was identified. Respecting solubility restriction the injection volume could be increased from V_{inj}=0.005 ml to V_{inj}=0.167 ml.

A second option to generate more product is to increase the column diameter keeping the column length, the relative column loading and the gradient regime unchanged. This requires respecting the scale up rules given by Eqs. (22)–24.

We combined these both options mentioned.

4.3.2. Scale-up to larger column diameter and productivity estimation

For scale-up an increase column diameter was increased from 4.6 to 25 mm. This corresponds to a scale up factor of approximately SF=30 (Eq. (24)). The volumetric flow rate, $\dot{V}_L$, and the injection volume, V_{inj}, required for the larger column are given in Table 5 together with the values for the small column.

The results of implementing the 3-step solvent gradient elution using the conditions given in Table 3 are illustrated in Fig. 8. After having performed preliminary test runs with the larger column, the second and

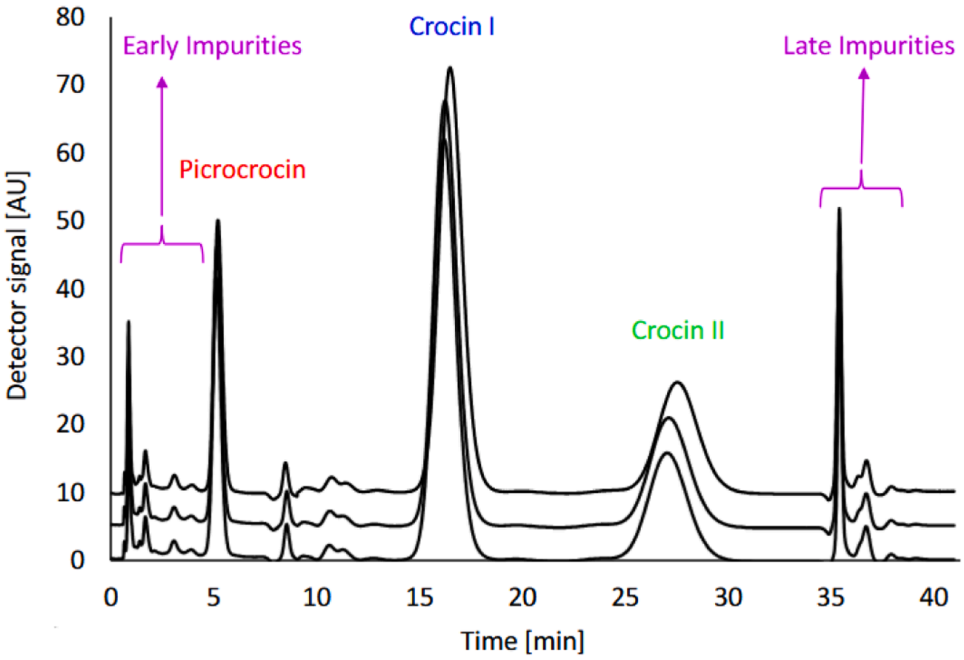

Fig. 7. Real separation profile of HGF by the designed 3-step solvent gradient elution (Table 3) on small scale for a small injection amount. Three subsequent injections are superimposed to indicate reproducibility. Injection: m_{inj}=0.005 mg, V_{inj}=5 μl $L = 150$, $D = 4.6\ mm$ $\dot{V}_L$=1.2 ml/min, $\Delta t_{inj}\ = 0.00416\ min$, Δt^{real}_{cyc}=40.10 min Detection: UV at 254 nm (until 8 min) and at 440 nm (later). Further parameters in Tables S.3 and S.4.

Table 4
The predicted and experimental retention times (see Fig. 7) of picrocrocin, crocin I, and crocin II using the designed 3-step solvent gradient elution method for linear conditions of the elution profile.

Component (i)	$t^{pred.}_{R,i}$ $[min]$	$t^{exp.}_{R,i}$ $[min]$
Picrocrocin	5.09	5.10
Crocin I	15.31	16.20
Crocin II	27.64	27.70

Table 5
Parameters of the analytical to preparative columns (in both cases: $c^{tot}_{inj}\ = 75\ mg/ml$). Reverse phase chromatography (YMC-Triart Prep C18-S, column lengths: 150 mm).

Scale	d_c $[mm]$	m_{inj} $[mg]$ (Eq. (22))	V_{inj} $[ml]$ (Eq. (22))	$\dot{V}_L$ $[ml/min]$ (Eq. (23))
Analytical	4.6	12.5	0.167	1.2
Preparative	25	375	5	36

third switch time used during the small scale experiments (i.e., $t^{real}_{s,2}$=28.8 min, $t^{real}_{s,3}$=Δt_{cyc}=40.10 min) were empirically slightly extended. These corrections were required due to the increased band broadening caused by the significant column overloading and retention time variations of the late-eluting impurities observed. The deviations compared with smaller column behavior are likely also due to the lower efficiency of the self-packed column and the application of a non-standard feed distributor. Consequently, $t^{real}_{s,2}$was extended to 35 min and the cycle time in a conservative manner to Δt_{cyc}= 45 min. The resulting chromatogram is shown in Fig. 8.

The shift to earlier retention times, an increase in band broadening, and band deformation compared to the analytical situation is visible. The retention times for picrocrocin, crocin I, and crocin II are approximately 0.4, 1.4, and 2.2 min shorter, respectively, compared to the not-overloaded case shown in Fig. 7. Three fractions of picrocrocin, crocin I, and crocin II were manually collected in the time intervals indicated in Table 6. The fractions collected (Fig. 9A) were subsequently concentrated to reach approximately 100 ml using an industrial rotary evaporator (BUCHI, Switzerland). Pure fine powders (Fig. 9B) were obtained using freeze dryer (Zirbus technology GmbH, VaCo2, Germany). The picrocrocin powder had a purity of 97.3 % (as obtained by analytical HPLC, see Fig. 1) and was of light yellow color. In contrast, crocin I powder (98.3 % purity) displayed a red color, and crocin II powder (96.64 % purity) was of deep red color (Fig. 9B). A summary of purities (Eq. (19)), productivities (Eq. (20)), and yields (Eq. (21)), of the three fractions is also given in Table 6. Due to the relative high yields achieved the ratio of component specific productivities correspond well to the feed composition (Table 1).

It is worth noting that, due to the shorter cycle time of the gradient methods applied, the productivity of picrocrocin from HGF is approximately 24 % higher than from LGF (Table 2). Compared to the recently reported productivity of isolating exclusively crocin I using a more complex multi-column solvent gradient configuration (5.7 $g/h/l$, [21]), the results achieved in providing three valuable target simultaneously in high purity is seen is very satisfactory.

For possible further industrial application the size of a preparative column required to deliver a desired production rate can be estimated from the productivity results provided in Table 6.

Due to limited availability of feed samples no further experiments could be carried out to perform a desirable statistical analysis of the reproducibility of the results. The qualitative agreement between experimental results and predictions by the simple short-cut model applied is seen as rather satisfactory. A possible development of a more detailed model incorporating unavoidable isotherm nonlinearities and/or a more profound analysis of scale-up issues is pending and subject of future work.

5. Conclusion

This study presents results regarding the provision and purification of picrocrocin, crocin I, crocin II, and total crocins from saffron extracts using reversed-phase gradient (C18) elution chromatography. Both a low-grade feed (LGF) and a high-grade feed (HGF) of Iranian saffron

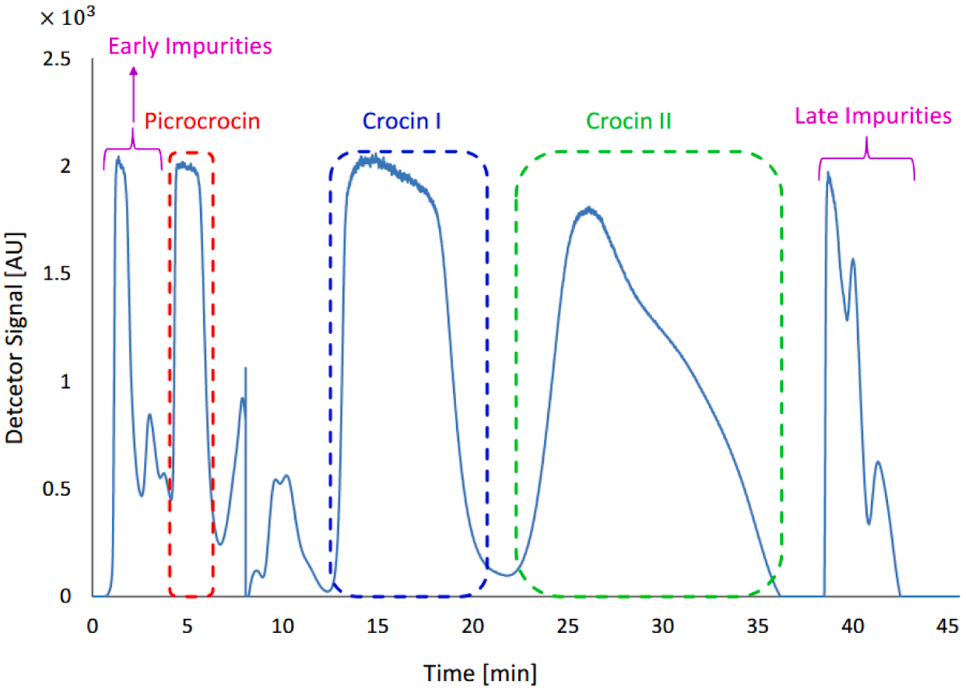

Fig. 8. Preparative separation profile of HGF by the designed 3-step solvent gradient elution (Table 3). YMC-Triart Prep C18-S (L_c=150 mm, d_c=25 mm). t_0=1.12 min, c_{inj}=75 g/l, V_{inj}=5 ml, m_{inj}=375 mg, $\dot{V}_L$=36 ml/min, Δt_{cyc}= 45 min.

Table 6
Purity, productivity and yield evaluation of the picrocrocin, crocin I, and crocin II fractions collected (coll.) from the HGF at the outlet of the preparative column. c^{Feed}_{inj}=75 mg/ml, V_{inj}=5 ml, $\dot{V}_L$=36 ml/min, Δt_{inj}=0.14 min, m^{Feed}_{inj}=375 mg, $\Delta t^{exp.}_{cyc}$=45 min, V_c=73 ml.

Component (i)	$m_{i,inj}$ [g]	$t^{start}_{i,\ coll.}$ [min]	$t^{end}_{i,\ coll.}$ [min]	$m_{i,coll.}$ [g]	Pu_i [%]	Pr_i [g_i /h /l]	Y_i [%]
Picrocrocin	0.05625	4.2	6.5	0.05371	97.34	0.96	95.50
Crocin I	0.09375	12.7	21	0.09187	98.30	1.68	98
Crocin II	0.03525	22.5	35	0.03313	96.64	0.60	94

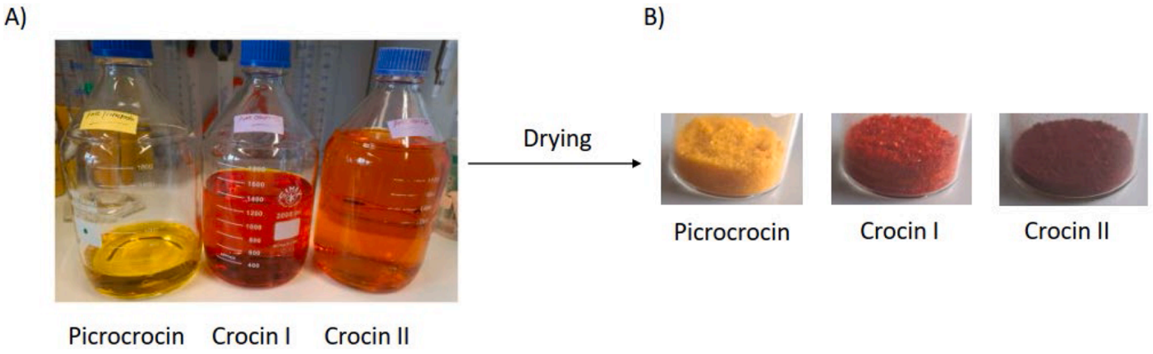

Fig. 9. Fractions with picrocrocin, crocin I, and crocin II collected form the run with the preparative column.

were processed. In an initial empirical study with the LGF, a simple step-gradient method enabled the successful isolation of picrocrocin and a combined crocin fraction, with purities of 96 % and 95 %, and yields of 97 % and 91 %, respectively.

A more systematic study was then carried out using the HGF to simultaneously purify picrocrocin, crocin I, and crocin II. The method development involved preliminary isocratic retention studies to determine Henry constants across varying ethanol concentrations. The retention factors were empirically correlated with the solvent composition. Exploiting equilibrium theory, this information enabled rationally designing a 3-step gradient method, targeting high purity and, yield, and minimal cycle time to achieve a high productivity.

The method was first validated under linear conditions using an analytical column, and subsequently scaled up to a preparative column of 30 times larger volume operated under overloaded conditions. Despite increase of loading, the separation maintained high performance: purities of 97.3 % (picrocrocin), 98.3 % (crocin I), and 96.6 % (crocin II) were achieved, with recovery yields of 95.5 %, 98 %, and 94 %, respectively. The component-specific productivities were 0.96 g/h/L (picrocrocin), 1.68 g/h/L (crocin I), and 0.60 g/h/L (crocin II), and thus attractive for industrial production.

In summary, the developed 3-step gradient method enables robust and efficient purification of saffron's key bioactive ingredients with high reproducibility and productivity. The short-cut model used was found to be very useful. It can be applied for estimations of the separation costs. Improved predictions of the model may be achievable through consideration of unavoidable nonlinearities in the adsorption equilibrium functions. Application of the straightforward simple conceptual approach to other complex multi-component separation problems can be recommended.

CRediT authorship contribution statement

Mohsen Fotovati: Writing – review & editing, Writing – original draft, Validation, Software, Investigation, Formal analysis, Data curation. **Ju Weon Lee:** Writing – review & editing, Supervision, Methodology. **Mohammadreza Taheri:** Writing – review & editing, Validation, Methodology, Data curation. **Alireza Ghassempour:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Hassan Rezadoost:** Writing – review & editing, Resources, Funding acquisition. **Andreas Seidel-Morgenstern:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This research was supported by the Max Planck Society. We sincerely thank Jacqueline Kaufmann and Stefanie Leuchtenberg for their valuable collaboration and support.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.466098.

Data availability

Data will be made available on request.

References

[1] F. Mosca, G.I. Hidalgo, J. Villasante, M.P. Almajano, Continuous or batch solid-liquid extraction of antioxidant compounds from seeds of Sterculia Apetala plant and kinetic Release study, Molecules 23 (7) (2018) 1759.

[2] Sh. Guan, Q. Pu, Y. Liu, H. Wu, W. Yu, Z. Pi, Sh. Liu, F. Song, J. Li, D.-A. Guo, Scale-up preparation of crocins I and II from Gardenia jasminoides by a two-step chromatographic approach and their inhibitory activity against ATP citrate lyase, Molecules 26 (11) (2021) 3137.

[3] M. Pashirzad, M. Shafiee, A. Avan, M. Ryzhikov, H. Fiuji, A. Bahreyni, M. Khazaei, S. Soleimanpour, S.M. Hassanian, Therapeutic potency of crocin in the treatment of inflammatory diseases: current status and perspective, J. Cell Physiol. 234 (9) (2019) 14601–14611.

[4] M. Ghiasian, F. Khamisabadi, N. Kheiripour, M. Karami, R. Haddadi, A. Ghaleiha, B. Taghvaei, S.S. Oliaie, M. Salehi, P. Samadi, A. Ranjbar, Effects of crocin in reducing DNA damage, inflammation, and oxidative stress in multiple sclerosis patients: a double-blind, randomized, and placebo-controlled trial, J. Biochem. Mol. Toxicol. 33 (12) (2019) e22410.

[5] Y. Xie, Q. He, H. Chen, Z. Lin, Y. Xu, C. Yang, Crocin ameliorates chronic obstructive pulmonary disease-induced depression via PI3K/Akt mediated suppression of inflammation, Eur. J. Pharmacol. 862 (2019) 172640.

[6] A. Talaei, M. Hassanpour Moghadam, S.A. Sajadi Tabassi, S.A. Mohajeri, Crocin, the main active saffron constituent, as an adjunctive treatment in major depressive disorder: a randomized, double-blind, placebo-controlled, pilot clinical trial, J. Affect. Disord. 174 (2015) 51–56.

[7] S. Sepahi, S.A. Mohajeri, S.M. Hosseini, E. Khodaverdi, N. Shoeibi, M. Namdari, S. A. Sajadi Tabassi, Effects of crocin on diabetic maculopathy: a placebo-controlled randomized clinical trial, Am. J. Ophthalmol. 190 (2018) 89–98.

[8] A. Colapietro, A. Mancini, A. Maria D'Alessandro, C. Festuccia, Crocetin and crocin from Saffron in cancer chemotherapy and chemoprevention, AntiCancer Agents Med. Chem. 19 (1) (2019) 38–47.

[9] X. Pu, Ch. He, y. yang, W. Wang, K. Hu, Zh. Xu, J. Song, In vivo production of five crocins in the engineered *Escherichia coli*, ACS. Synth. Biol. 9 (5) (2020) 1160–1168.

[10] S.Z. Bathaie, A. Farajzade, R. Hoshyar, A review of the chemistry and uses of crocins and crocetin, the carotenoid natural dyes in saffron, with particular emphasis on applications as colorants including their use as biological stains, Biotech. Histochem. 89 (6) (2014) 401–411.

[11] F. Liu, F. Ding, W. Shao, B. He, G. Wang, Regulated preparation of crocin-1 or Crocin-2′ triggered by the Cosolvent DMSO using Bs-GT/At-SuSy one-pot reaction, J. Agric. Food Chem. 67 (45) (2019) 12496–12501.

[12] J.M. Prado, M.A. Rostagno, Natural product extraction: principles and applications, RSC Green Chem. Ser. (2022).

[13] S. Mohajeri, H.R. Beheshti, Extraction of crocin from saffron (Crocus sativus) using molecularly imprinted polymer solid-phase extraction, J. Sep. Sci. 33 (14) (2010) 2103–2109.

[14] P.-K. Revelou, E. Kougianou, M. Xagoraris, Haralambos Evangelaras, Kanakis C. D. Papadopoulos, I.F. Strati, C.S. Pappa, P.A. Tarantilis, Optimization of a solid-phase extraction procedure for the separation of picrocrocin and crocins from saffron extract, Compounds 3 (1) (2023) 233–243.

[15] P.A. Tarantilis, A.B. Tsoupras, M.G. Polissiou, Quantitation of crocins and picrocrocin in saffron by HPLC: application to quality control and phytochemical differentiation from other crocus taxa, J. Agric. Food Chem. 53 (20) (2015) 7872–7878.

[16] E. Karkoula, A. Angelis, N.Stavros Koulakiotis, E. Gikas, M. Halabalaki, A. Tsarbopoulos, A.L. Skaltsounis, Rapid isolation and characterization of crocins, picrocrocin and crocetin from saffron using centrifugal partition chromatography and LC-MS, J. Sep. Sci. 41 (22) (2018) 4105–4114.

[17] M. Kabiri, H. Rezadoost, A. Ghassempour, A comparative quality study of saffron constituents through HPLC and HPTLC methods followed by isolation of crocins and picrocrocin, LWT 84 (2017) 1–9.

[18] J. Feng, Xi. He, Sh. Zhou, F. Peng, J. Liu, L. Hao, H. Li, G. Ao, Sh. Yang, Preparative separation of crocins and geniposide simultaneously from gardenia fruits using macroporous resin and reversed-phase chromatography, J. Sep. Sci. 37 (3) (2014) 314–322.

[19] M. Lautenschläger, M. Lechtenberg, J. Sendker, A. Hensel, Effective isolation protocol for secondary metabolites from Saffron: semi-preparative scale preparation of crocin-1 and trans-crocetin, Fitoterapia 92 (2014) 290–295.

[20] H. Zhang, Y. Zeng, F. Yan, F. Chen, X. Zhang, M. Liu, W. Liu, Semi-preparative isolation of Crocins from Saffron (Crocus sativus L.), Chromatographia 59 (11) (2004) 691–696.

[21] M. Hooshyari Ardakani, C. Nosengo, S. Felletti, M. Catani, A. Cavazzini, C. De Luca, H. Rezdoost, Enhancing the purification of crocin-I from saffron through the combination of multicolumn countercurrent chromatography and green solvents, Anal. Bioanal. Chem. 416 (2024) 2553–2564.

[22] F. Hadizadeh, S.A. Mohajeri, M. Seifi, Extraction and purification of crocin from saffron stigmas employing a simple and efficient crystallization method, Pak. J. Biol. Sci. 13 (14) (2010) 691–698.

[23] G. Guiochon, A. Felinger, D.G. Shirazi, Fundamentals of Preparative and Nonlinear Chromatography, Academic Press, 2006.

[24] C. Fanali, P.R. Haddad, C. Poole, M.-L. Riekkola, Liquid chromatography: Fundamentals and Instrumentation, 2nd Edition, 1, Elsevier, 2017.

[25] H. Schmidt-Traub, M. Schulte, A. Seidel-Morgenstern, Preparative Chromatography, 3rd Edition, Wiley-VCH, 2020.

[26] P. Jandera, J. Churacek, Gradient elution in column liquid chromatography: theory and practice, J. Chromatogr. Libr. 31 (1986).

[27] L.R. Snyder, J.W. Dolan, High Performance Gradient Elution: The Practical Application of the Linear Solvent Strength Model, Wiley, 2007.

[28] D. Antos, A. Seidel-Morgenstern, Continuous step gradient elution for preparative separations, Sep. Sci. Technol. 37 (7) (2002) 1469–1487.

[29] H.-K. Rhee, R. Aris, N.R. Amundson, On the theory of multicomponent chromatography, Philos. Trans. A 267 (1182) (1970) 419–455.

[30] H.-K. Rhee, R. Aris, N.R. Amundson, First-order Partial Differential Equations, I, Prentice-Hall, Englewood Cliffs, 1989. Volumeand Volume II.

[31] M. Mazzotti, A. Rajendran, Equilibrium theory-based analysis of nonlinear waves in separation processes, Annu Rev. Chem. Biomol. Eng. 4 (2013) 119–141.

[32] L.R. Snyder, J.W. Dolan, The linear-solvent-strength model of gradient elution, Adv, Chromatogr. 38 (1998) 115–187.