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Simultaneous determination of loading capacity and selectivity in preparative off-line two-dimensional separation: An application for purification of corilagin from Pomegranate flower extracts

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ABSTRACT

This work describes the development of capacity orthogonal chromatography (COC), a new technique for simultaneously determining the loading capacity and orthogonality during the construction of two-dimensional (2D) separations. Three steps were required for the construction of a COC based on the correlation between the selectivity factor (α) and both orthogonality and loading capacity. (1) α values of the impurities-target compound were used to normalize the retention of the impurities around the target compound. (2) α values were input into four quadrants of a coordinate system to identify correlations between orthogonality and loading capacity. α values of the impurities must be greater in the first dimension than the second dimension, with iterated analyses performed until an α_{max} is obtained for the two purification methods. (3) Touch-peak separation using the first-dimensional α_{max} was performed and the target compound was collected. The co-eluted impurities are further separated in the second dimension. To test the efficiency of this technique, a COC using two methods on a standard C18 column was developed to purify corilagin from pomegranate flower extract. Despite its low abundance, 288 mg of corilagin was obtained by COC and further purified by LH-20 gel chromatography to obtain the compound with an 80.0% recovery and 98.4% purity. Compared to COC, the purity of corilagin independently obtained using the same purification methods and identical loading capacity was poor (60.1% and 61.6%). These results indicate that COC is a useful tool for extending loading capacity in the development of preparative 2D separations.

1. Introduction

Two-dimensional (2D) separation, consisting of columns with different separation mechanisms, is favored for the removal of co-eluted impurities during the purification of a target compound from a complex sample [1,2]. The degree of complementarity between the two methods used for 2D separation is characterized by the concept of "orthogonality". For most low-performance chromatography using silica gel or octadecylsilyl (ODS) columns, 2D separation is empirically constructed based on the adsorption characteristics of the stationary phase and the compound to be purified [3-5]. This is necessary because there are currently no established principles for studying the degree of orthogonality and mediating the correlation between orthogonality and loading capacity for these systems. Unfortunately, this often results in a need for repetitive purifications under overloading conditions. For 2D separation using high-performance chromatography, the evaluation of orthogonality has been studied in more detail. Gilar [6] introduced a widely accepted method for estimating orthogonality based on calculating the rate of occupancy of orthogonal space after normalizing the retention time of the peaks. This method effectively evaluates the orthogonality of the entire 2D system and has been successfully used for the separation and analysis of complex samples, such as traditional Chinese medicine [7-9]. Despite the reliability of this technique, evaluating the orthogonality of 2D purifications under overloading conditions remains difficult because the correlation between orthogonality and loading capacity is unclear. When the column is overloaded, the individual components of a complex mixture are often poorly resolved, which affects the purity of the collected fractions. The evaluation of orthogonality in prepara-

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tive 2D separation was largely independent of loading capacity. To address the resolution problems associated with overloading, it is necessary to develop an approach that considers loading capacity in the calculation of orthogonality.

Three key features affect the peak width in liquid chromatography (LC) under overloading conditions: selectivity factor (α), plate number (N), and retention factor (k) [10]. The k value is generally maintained at approximately 0.5-20.0 to optimize the time and efficiency of purification [11]. N can easily be increased in preparative LC using newly developed materials and applying advanced column packing techniques, such as dynamic axial compression [12,13]. As k and N are readily controlled, loading capacity is closely connected with α [10]. Although the α value is unique for each compound and purification method, α for the target compound is always maintained at 1.0. This specification provides the basis for normalizing the retention time for all peaks across the two purification methods using a uniform standard. As α plays in role in both orthogonality and loading capacity, it is possible to develop an α-based approach for the simultaneous evaluation of these features in preparative 2D separation.

To allow for the simultaneous evaluation of orthogonality and loading capacity, we developed a new technique, termed capacity orthogonal chromatography (COC). This method used α of the impurities-target to normalize the retention of the impurities around the target compound. The orthogonal space [6] was then simulated to allow for the α of different purification methods to be grouped into four quadrants of a coordinate system, which provided the analytical basis for identifying essential correlations. COC is the combination of two methods to ensure a maximum α, making it possible to get the highest loading capacity while the peak is broadening. To validate the effectiveness of our technique, LC-MS and computational simulations were used to develop a COC for the purification of corilagin from pomegranate flower extracts. Corilagin is a hydrolyzable tannin reported to display anti-hyperalgesic [14], anti-tumor [15], and anti-inflammatory [16] effects, making its efficient isolation medically pertinent.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). HPLC grade H_2O was purchased from Wahaha Co. Ltd (Hangzhou, China). Analytical grade formic acid was purchased from HongYan Chemical Reagent Co. (Tianjin, China).

Pomegranate flowers were extracted by refluxing in 60% ethanol two times and concentrated at 40°C. The concentrated solution was loaded onto a D101 macro-porous resin column and washed with H_2O to remove sugars and proteins. The sample was then eluted with 70% ethanol and the solution was concentrated and lyophilized to obtain the crude extract.

2.2. HPLC and MS Instrumentation

An HPLC-tri-Q-LIT-MS (AB Sciex, Concord, ON, Canada) equipped with an electrospray ionization (ESI) interface was used to identify the compounds in the pomegranate flow extract under the protection of N_2. The raw data were analyzed using Analyst 1.6.2 software. Analytical HPLC was performed on an ACCORM C18 (250 mm × 4.6 mm, 10 μm) column using a Dionex UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA). Analysis was performed at room temperature (22±2°C) with detection wavelengths of 254 nm and 366 nm. The pilot-scale preparation was performed on an ACCORM C18 (250 mm × 80 mm, 10 μm) column.

2.3. COC construction for the purification of corilagin from pomegranate flower extracts

2.3.1. Identification of impurities around corilagin by LC-MS

HPLC-MS and Drylab software were used to develop the methods required for the construction of COC **Section 1**, Supplementary Material). The retention time of corilagin and the impurities in the pomegranate flower extract was analyzed on an ACCORMC18 (250 mm × 4.6 mm, 10 μm) column. Four suitable mobile phases were identified: ((1) **A1**: CH_3OH-0.1% $HCOOH/H_2O$ (27.4:76.6, v/v); (2) **A2**: CH_3OH-0.1% $HCOOH/H_2O$ (21.9:78.1, v/v); (3) **B1**: ACN-0.1% $HCOOH/H_2O$ (10.8:89.2, v/v); (4) **B2**: ACN-0.1% $HCOOH/H_2O$ (13.7:86.3, v/v). The velocity for the HPLC-MS^2 was 0.6 mL/min. The retention time (t_R) and molecular and fragment peaks of the compounds were recorded and the capacity factor (k) was calculated using the equation $k=(t_R-t_0)/t_0$, where t_0 is the dead time indicated by the solvent peak. The sample solution was filtered through a nylon membrane (0.45 μm) prior to HPLC analysis.

2.3.2. Computational simulation and normalization of the retention factor

DryLab software was used to simulate the chromatograms using the retention times identified by two different purification methods [17-19]. For the analysis, k of corilagin was maintained between 3.0 and 7.0. α between the target and impurities was calculated using the equation $\alpha=k_1/k_2$ ($k_1>k_2$), where k_1 and k_2 are the retention factors for the two different purification methods. The α values of the quicker and slower eluting impurities are negative and positive, respectively. Origin 8.5 was used to obtain the distribution curves based on the calculated α values. Corilagin was set at X=0 for the analysis.

2.3.3. Purification of corilagin from pomegranate flower extracts by COC

Pomegranate flower extracts (48 mg) were separated on an ACCORM C18 (250 mm × 4.6 mm, 10 μm) column under **B1** conditions and the fraction eluting between 15.2 min and 18.8 min was collected. This fraction was then re-loaded and separated on the same column using **A1** conditions. The fraction eluting between 16.9 min and 21.0 min was collected, concentrated, and lyophilized. A pilot-scale preparation (16.8 g crude extracts) on an ACCORM C18 (250 mm × 80 mm, 10 μm) column was conducted using the same purification method. The velocity for the analytical and preparative methods was 0.6 mL/min and 180 mL/min, respectively.

2.4. Further purification of corilagin on LH-20 columns

LH-20 columns were packed as a slurry in methanol. The crude corilagin sample obtained from COC was dissolved in 200 μL methanol, loaded onto the columns, and successively eluted with methanol. Fractions were collected in terms of volume (V_1, V_2,...V_i,..., V_j) and analyzed by HPLC. The peak areas of **Impurity 8** (I8) and corilagin were recorded as A_i and B_i in the fraction of V_i. The occupancy ratios (η_A, η_B, %) of corilagin and **I8** were calculated using the equations: $\eta_A = A_i{*}V_i/\Sigma A_i{*}V_i{*}100\%$ and $\eta_B = B_i{*}V_i/\Sigma B_i{*}V_i{*}100\%$ (i=1-j), respectively. The effect of column length on separation was investigated by purifying the crude corilagin samples (50 mg) on 7.0 cm, 13.5 cm, and 20.0 cm LH-20 columns, with the diameter maintained at 1.0 cm across the experiments. To study the effect of loading quantity on the separation, 50 mg, 75 mg, and 100 mg crude extracts were individually loaded onto LH-20 column (13.5 cm×1.0 cm). A scale-up purification was performed on an LH-20 column using 288 mg crude corilagin (13.5 cm × 2.5 cm). The column was eluted with methanol and 11 fractions were collected. Fractions 6-9 were combined, concentrated,

and lyophilized to give 228 mg of a white powder, which was characterized by HPLC, HRMS, and NMR.

Recovery was calculated by preparing calibration curves. Aqueous solutions of pure corilagin were prepared with concentrations (C) of 0.01 mg/mL, 0.03 mg/mL, 0.05 mg/mL, 0.07 mg/mL, 0.30 mg/mL, 0.50 mg/mL, and 0.70 mg/mL. These standards were analyzed on an ACCORM C18 (250 mm × 4.6 mm, 10 μm) column to obtain the peak area (A), which was plotted against C to give the equation A=519.21*C-6.35 (R^2=0.999). The HPLC conditions used throughout these analyses were: mobile phase, ACN-0.1% HCOOH/H_2O (10:90, v/v); flow rate, 1.0 mL/min; temperature, 30°C.

3. Results and discussion

3.1. Theoretical, fundamental, and practical procedure for constructing COC

COC is a coordinating method to optimize orthogonality and loading capacity for the development of 2D separations. Coordinating these two properties helps to ensure a balance between purity and the production rate. Although α affects both orthogonality and loading capacity, the coordination of these properties is the key issue for COC. It was assumed that a complex system containing n number of impurities (**1, 2**,..., ***i+2, i+1, i***, target compound, ***j, j+1, j+2***,...***n***) were separately determined by a total x number of methods ($\mathbf{M_1}$,...$\mathbf{M_k}$,...$\mathbf{M_l}$,...$\mathbf{M_x}$) to produce x groups of α. Therefore, for a 2D separation, there are potentially $x^*(x\text{-}1)$ combinations of methods. In addition, this formula is currently only applicable for the combination of reverse-phase liquid chromatography and reverse-phase liquid chromatography. For this work, we selected $\mathbf{M_k}$ and $\mathbf{M_l}$ in our discussion of the procedure for constructing COC (**Scheme 1a**).

The first step is to normalize the retention of the compounds in the complex mixture. There are two methods generally reported for performing this evaluation. A universal and convenient approach is to calculate the ratio of the area occupied by normalized points of retention time or organic phase components in an orthogonal space [6,20]. This technique describes orthogonality that is highly correlated to the physical characteristics of both the sample and the chromatographic conditions. An alternative method, introduced by Dumarey et al. [23], normalizes retention using a hydrophobic subtraction model. The coefficients for this system were described by a single function, the column selectivity function F_S, which clearly revealed the contribution and interconnectivity of each parameter. We hypothesized that a combination of these two methods would be useful for the development of a new evaluation. For our evaluation, all points of α in $\mathbf{M_k}$-$\mathbf{M_l}$ were scattered within an X-Y coordinate system (**Scheme 1b**). The location of the target compound was localized at X=0 (α=1) and the remaining α points were distributed about the target compound. The space of all four quadrants was fully exploited for this analysis.

Several key points must be noted when using α as the standard for constructing a COC:

(1) Impurities around the target compound with α<3.0 were identified and their α were input into coordinate system for the establishment of COC. The impact of impurities that eluted far away from the target compound on purification was viewed as very limited, which were therefore not considered in COC. Too high sample loading may lead to injection problems like solubility limitation or solvent effect. Another critical factor that had to be considered in the construction of COC is the different speed of peak broadening between impurities (located at the right hand of the target compound) with high content and the target compound. If the peak broadening of impurities were faster, residual impurities would remain in the component of the first dimension of COC. In the construction of COC, their specific location at a given α should also be mastered through overloading assays. If the final loading capacity of COC were greatly reduced by these impurities, they would have to be dismissed during the construction of COC. To remove them, additional method may be inevitable except for COC.

(2) Compared to the target compound, the value of α is negative for faster-eluting impurities (left side of the coordinate system) and positive for slower-eluting impurities (right side of the coordinate system).

(3) It is assumed that all impurities can be removed by COC; however, impurities with α < 1.1 across the different purification methods are not included in this analysis [21]. In other words, it is not possible to remove these impurities using these methods, even though the methods were combined for COC.

(4) For impurities that could not be separated by the first dimension of the purification, the α of the first-dimensional separation should be greater than that for the second dimension. A better separation of these impurities could be achieved by the second dimension of the purification.

(5) Computer simulations maintain the k value of the target compound at 0.5 or 20.0. During preparative LC, k was maintained at 3.0 or 7.0, since neither shorter nor longer retention times are beneficial for pilot-scale preparation. However, k values outside of this range are not prohibited for this technique.

(6) The effect of N loading capacity should be ignored. In addition, we also did not consider the effect of k. Considering that k in the two methods of COC was always maintained at 3.0 or 7.0, but not very different values, the effect of k on the loading capacity was expected to be remained limited although it greatly affected the loading capacity.

There are several merits of an α-based normalization:

(1) α for all of the impurities is calculated in terms of a uniform standard (the target compound) regardless of the method selected for purification. As α for the target compound is 1.0, the comparison of selectivity across various methods can be performed based on a uniform standard. Notably, the interconnectivity between multiple impurities is clearly observed when using the uniform standard. This method is distinct from techniques focused on the separation of multiple targets and is more beneficial for the purification of a specific sample, which is rarely described in the literature [22].

(2) α is defined as the critical factor affecting the loading capacity in the development of touch-peak (T-P) separation on 1D-LC. Simultaneous consideration of the distribution of α in two methods could allow for the development of T-P separation in 2D chromatography.

(3) Following α-based normalization, areas of the chromatogram without peaks are removed.

The second step for constructing a COC is to visually display the orthogonality and identify the interconnectivity of the impurities on $\mathbf{M_k}$ and $\mathbf{M_l}$. Four data points belonging to $\mathbf{M_k}$ and $\mathbf{M_l}$ were connected to form a closed-cycle (**Scheme 1c**). The smallest cycle (**cycle 1**) occurs when we connect the innermost four points of **Impurity *i*** and ***j*** (the value of $\alpha_{\text{Impurity}\,i}$ is higher than $\alpha_{\text{Impurity j}}$) from $\mathbf{M_k}$-$\mathbf{M_l}$ Here, we selected the impurity with the largest α as the standard to describe the connection (**Impurity *i***), and adopted $\mathbf{M_k}$ as the first dimension.

Using $\mathbf{M_k}$ as the first dimension, the peak of the target compound broadens to overlap the tail of **Impurity *i***. A fraction con-

taining the target compound and **Impurity *j*** was collected and re-separated on $\mathbf{M}_I$. We next investigated whether the α of **Impurity *j*** increased upon transferring the fraction from $\mathbf{M}_k$ to $\mathbf{M}_I$. If the α increases, additional connections are made in the coordinate plane. In particular, the outer four points belonging to **Impurity *i+1*** and **Impurity *j+1*** were connected to produce a larger cycle (**cycle 2**) for use in additional investigations. If α does not increase, α for **Impurity *i*** is selected to represent the loading capacity for the first dimension (α_1). If α of the impurities co-eluted with the target compound in the first dimension increases in the second dimensional purification, the loading capacity will probably continue to increase. For the purpose of our work, α was assumed to increase and we further connected four points belonging to **Impurity *i+2*** and **Impurity *j+2*** (**cycle 3**) and obtained α_3. This method was iterated until the α value ceased to increase and the maximum α was obtained (α_{max}). Additionally, the dimension of the purification was investigated by using $\mathbf{M}_I$ as the first dimension. From this work, α_{max} was found to occur in **cycle 2**, belonging to **Impurity *j+1***.

The third step for constructing a COC is to conduct the overloading experiment in terms of α_{max} based on the principle of non-linear chromatography. For preparative LC, the peak width of the target compound (W) can be calculated using equations (1) and (2) [10]:

$$W^2 = W_0^2 + W_{th}^2 \quad (1)$$

where W_0 is the peak width obtained from analytical separation and W_{th} is the added contribution to W from overloading. This equation can be rewritten as:

$$W^2 = (16/N_0)t_0^2(1+k)^2 + 4t_0^2k^2(w_x/w_s) \quad (2)$$

where N_0 is the plate number of the analytical column, t_0 is the dead time, k is the retention factor, w_x is the injection weight, and w_s is the saturation capacity. Based on equation (2), the peak front of the target compound is able to reach the designated position on the chromatogram in the case of sample overloaded. Notably, the overloading of sample shall influence the retention times of compounds, including the target compounds. Minor adjustments of loading capacity is occasionally inevitable in order to reach the specified position, because displacement effect may form between the overlapping peaks.

α_{max} is the distance from **Impurity *j+1*** to the target compound (**Scheme 1d**). We assumed that the peak front of **Impurity *j+1*** broadens to the peak tail of the target compound (blue area) and the peak front of the target compound broadens to overlap **Impurity *i*** and **Impurity *i+1*** with the same distance (pink area). We also assumed that **Impurity *j*** broadens with the same distance (red area). By collecting the totality of the target compound in the pink area, it follows that **Impurity *i+1*, *i***, and ***j*** would co-elute into the fraction. All of these impurities obtained better separation on $\mathbf{M}_I$ than on $\mathbf{M}_k$, suggesting that the construction of the COC was successful.

3.2. COC for purifying corilagin from pomegranate flower extracts

3.2.1. Identification and normalization of impurities in four methods

We developed four separation methods, termed **A1, A2, B1**, and **B2** for a single C18 column. Using Drylab software, the k value of corilagin for these methods was maintained at 3.0 or 7.0, based on its reported retention using two known separation methods [19]. The simulated k value for corilagin was in excellent agreement with the experimental results (**Table 1**). LC-MS using **A1, A2, B1**, and **B2** identified seven impurities (**I1, I3, I4, I5, I6, I7**, and **I8**) around corilagin, which were simulated using the Drylab software (**Table S2**). Utilizing the method described in **Section 3.1**, we normalized the retention of these compounds as α (**Table 2**) and arranged them in order of greatest to least value. Notably, the order of α differed among **A1, A2, B1**, and **B2**, indicating the possibility for constructing an orthogonal separation. In particular, the distribution of α in **A1** (k=3.0) and **A2** (k=7.0) displays selectivity for methanol-H_2O, while α in **B1** (k=7.0) and **B2** (k=3.0) demonstrates selectivity with ACN-H_2O. The influence of solvent strength on the selectivity was directly obtained from the comparison of **A1** and **A2**, as well as **B1** and **B2**. The comparison of α between **A1** and **B2** or **A2** and **B1** reflects the effect of solvent type on the selectivity. This work clearly displays the convenience of this procedure. Unfortunately, the α of **I8** was less than 1.25 across all the methods, suggesting that it could not be removed using COC.

Table 1
Comparison between simulation and experimental k values.

Methods	Simulation value	Experimental value	SD (%)
A1	3.00	2.95	1.60
A2	7.00	7.02	0.20
B2	3.00	3.03	1.00
B1	7.00	6.90	1.40

3.2.2. Inner correlations of α in A1, A2, B1, and B2

After completing the normalization, **A1, A2, B1**, and **B2** were coupled as **A1-A2, A1-B1, A1-B2, B1-B2, A2-B2**, and **A2-B1** to allow for an investigation into their inner correlations. After inputting α into the coordinate system, points were connected to form closed-cycles following the principles described in **Section 3.1**, (**Figure 1a**~**Figure 1f**). For this work, it could be concluded that all of the impurities (**I1, I3, I4, I5, I6, I7**, and **I8**) around corilagin were present only in trace amounts based on the HPLC analysis and overloading assay. The broadening of the peak front from the overloading experiments only affected corilagin and the impurities described below. The **A1-B1** group was selected as the starting point for a detailed description of this procedure. Four points in the coordinate system, **I1** and **I8** from **A1** and **I8** and **I4** from **B1**, were connected to form **cycle 1** (**Figure 1b**). Setting **A1** as the first dimension, **I8** (α_{max}=-1.25) was used as the marker for the optimization. When the peak front touched the peak tail of **I8**, the peak front of **I1** overlapped with the peak of corilagin. **I1** and corilagin were collected as a single fraction and re-separated using **B1**. As **I1** displayed a greater α (-1.30) in the second dimension than the first one (α=+1.13), the cycle was enlarged to produce **cycle 2** by connecting the outer four points of **I6** and **I7** from **A1** and **I3** and **I5** from **A2. I6** (α_{max}=-2.00) was used as the marker for optimization. When the peak front reaches the peak tail of **I6**, the peak of corilagin overlaps with **I1, I7**, and **I8**. Although α of **I1** and **I7** increased from the first dimension to the second, the α value for **I6** decreased from 2.00 in **A1** to 1.47 in **B1**, indicating that co-elution of **I6** and corilagin cannot be prevented using **A1-B1**. At this point,

Table 2
α of corilagin* and the seven relevant impurities determined using **A1, A2, B1**, and **B2**.

Methods	α1	α2*	α3	α4	α5	α6	α7	α8
A1	+1.13	+1.00	<-5.00	<-5.00	-4.28	-2.00	+1.73	-1.25
A2	-1.15	+1.00	<-3.00	<-3.00	-2.80	+1.34	+1.69	+1.16
B2	-1.30	+1.00	+1.00	+1.20	+1.33	+1.43	+1.57	+1.00
B1	-1.59	+1.00	-1.13	+1.11	+1.33	+1.47	+1.63	-1.00

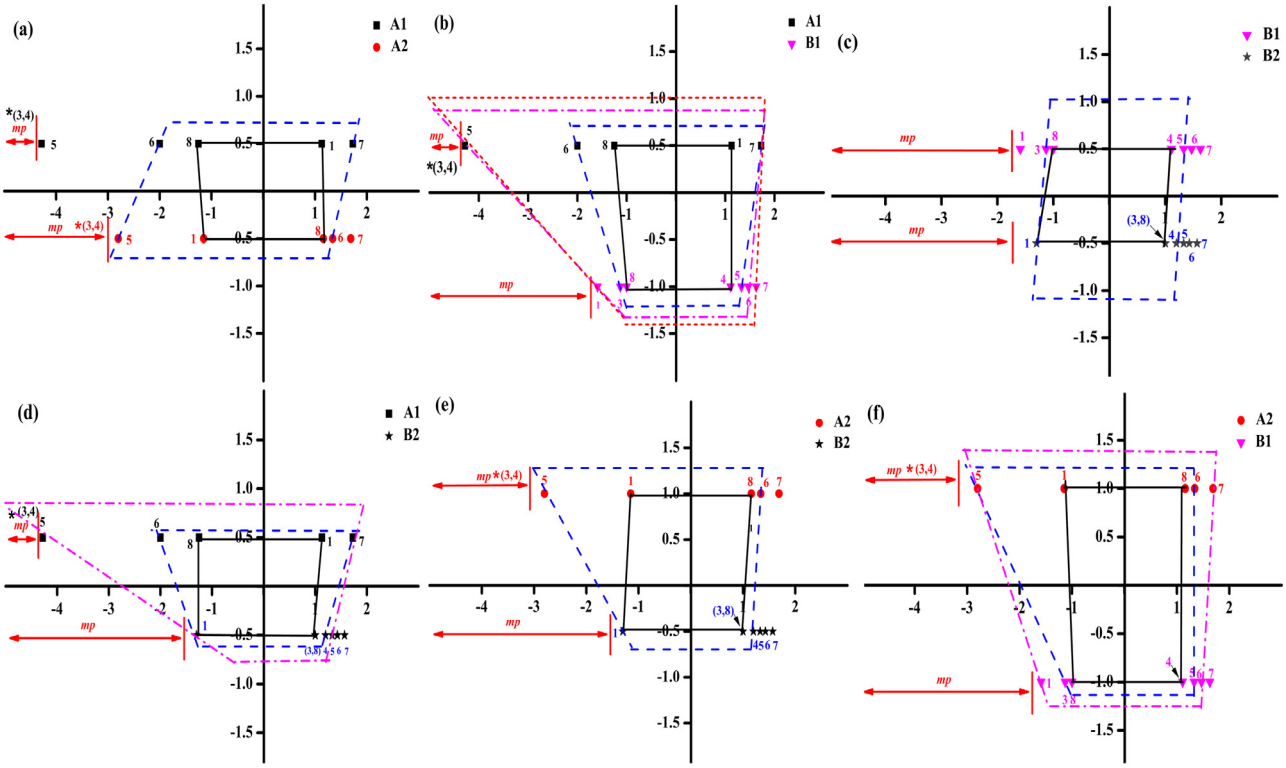


Figure 1. The closed-cycles of (a) **A1-A2** (**A2-A1**), (b) **B1-A1** (**A1-B1**), (c) **B1-B2** (**B2-B1**), (d) **A2-B2** (**B2-A2**), (e) **A2-B1** (**B1-A2**), and (f) **A1-B2** (**B2-A1**). (*mp*): multiple points for overlapping peaks. *(3,4): points of **I3** and **I4**.

the investigation into this system was stalled with the α_{max} of **A1-B1** designated as 1.25.

B1-A1 was next investigated. For the first cycle, **I4** of **B1** was selected as the marker. **I8** could gain better separation using **A1** because the value of α increased from 1.00 to 1.25. In the second cycle, **I5** was defined as the marker. Using this method, the α value increased for three of the co-elution peaks, **I4, I8**, and **I3**. A third cycle was prepared by connecting the outer four points of **I1** and **I6** on **B1** and **I7** and **I5** on **A1**, with **I1** (α_{max}=-1.59) chosen as the marker. **I3, I8, I4, I5**, and **I6** remained in the fraction containing corilagin and all of their α values increased from the first dimension to the second. The loading capacity from this cycle was α=-1.59 belonging to **I1**. A fourth cycle was prepared by connecting the four points of **I1** and **I7** on **B1** and **I7** and **I5** on **A1** using **I7** as the marker. Unfortunately, α of **I1** decreased from 1.59 on **B1** to 1.13 on **A1**. No further cycles were prepared and the maximum loading capacity of **B1-A1** was obtained from the third cycle (α_{max}=1.59). A comparison of the α_{max} values obtained from **A1-B1** and **B1-A1** clearly demonstrates the effect of the method order on the loading capacity of 2D separation.

α_{max} for all other method couplings was also obtained (**Table 3**). The α_{max} difference between two methods with different organic phases is expected to be greater than between methods with different organic phase content. As such, it is easily imagined that α_{max} differences will be even greater when two methods are developed with respect to different columns. Having obtained the maximum loading capacity for this work, we constructed COC based on **B1-A1**.

3.2.3. Overloading experiment on COC

The overloading experiment was performed using **B1** to develop a T-P separation between **I1** and corilagin using **A1**. The peak widths of corilagin are 2.0 min and 2.25 min using 1 mg and 8 mg crude samples, respectively. As expected, peak width proportionally increased with the loading quantity. Using equations (2) and (3), the loading quantity of crude extract was determined to be 48 mg for the analytical column. The peak of corilagin accurately expands to the predicted location and overlaps the peaks of **I3, I4, I5, I6, and I8** (**Figure 2b**). As expected, these impurities were better separated using **A1** (**Figure 2c**). Theoretically, all impurities could be directly removed using **B1-A1**. However, as described earlier, the peak of **I8** could not be isolated from the target compound (**Figure 2d**) owing to a minimal increase in the α_{max} value of **I8** across the two purification methods (1.00 on B1 to 1.25 on A1). The peak front of corilagin likely overlaps with **I8** using **A1** when samples were heavily overloaded. To address this issue, new guidelines are being developed and will be detailed in our future work. The results from our current study suggest that COC is a highly efficient technique for purifying compounds from complex samples.

Table 3
α_{max} obtained from closed-cycles of **A1-A2** (**A2-A1**), **A1-B1** (**B1-A1**), **A1-B2** (**B2-A1**), **A2-B2** (**B2-A2**), **B1-B2** (**B2-B1**), and **A2-B1** (**B1-A2**).

Groups	α_1 (Cycle 1)	α_2 (Cycle 2)	α_3 (Cycle 3)	α_4 (Cycle 4)
A1-A2	1.25	-	-	-
A2-A1	1.16	-	-	-
A1-B1	1.25	-	-	-
B1-A1	1.33	1.33	1.59	-
A1-B2	1.25	-	-	-
B2-A1	1.30	1.30	-	-
A2-B2	1.25	-	-	-
B2-A2	1.30	-	-	-
B1-B2	1.11	-	-	-
B2-B1	1.13	-	-	-
A2-B1	1.16	-	-	-
B1-A2	1.11	1.33	-	-

3.3. Purification of corilagin by COC from pomegranate flower extract

3.3.1. Separation of corilagin by COC and LH-20 chromatography

The constructed COC was successfully transferred from an analytical column to a pilot-scale column (**Figure S2(a)-2(c)**). Al-

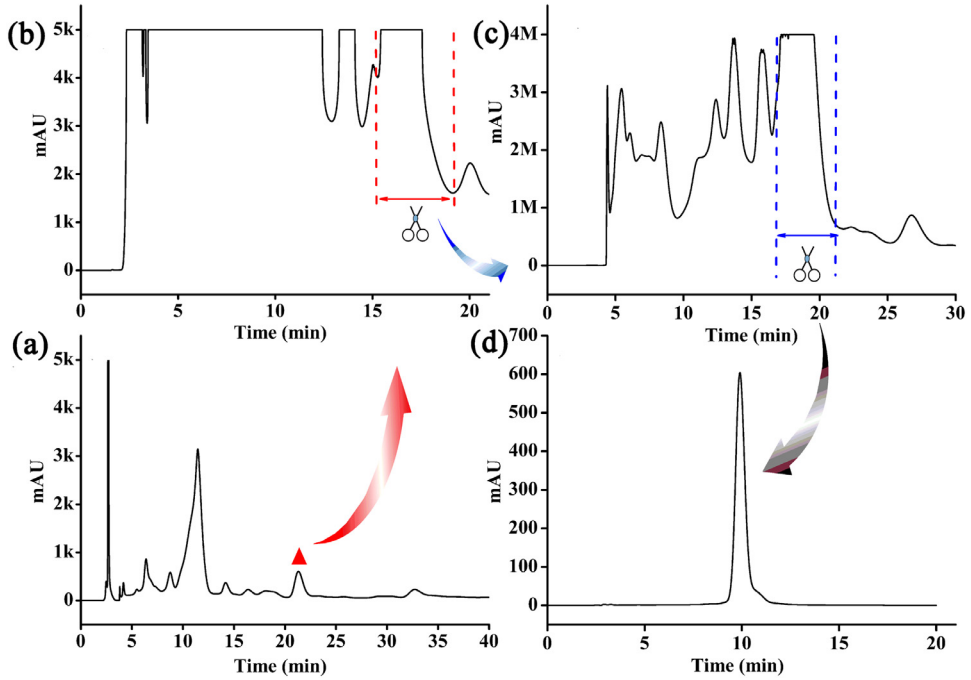


Figure 2. Chromatograms related to the purification of corilagin from pomegranate flower extracts: (a) pomegranate flower extract, (b) first-dimensional separation on **B1**, (c) second-dimensional separation on **A1**, and (d) purity analysis by HPLC using ACCORM C18 (250 mm×4.6 mm, 10 μm) column. CH_3OH-0.1% $HCOOH/H_2O$ (27.4:72.6, v/v) was used as the mobile phase. The temperature was kept at 22±2°C, and flow rate was 0.6 mL/min.

though the abundance of corilagin was low (1.6%), 288 mg of crude corilagin was purified from 16.8 g crude extracts by COC. As observed for the analytical method, **I8** remained after COC purification.

I8 was removed on an LH-20 column after investigating the effects of column length and loading quantity on purification. Figure 3 details the distribution of **I8** and corilagin on LH-20 columns of various lengths. The corilagin content of each fraction obtained from the various column lengths is described in Table 4. These results indicate that separation improves with increasing column length. However, a key focus of our work is to develop methods for purifying targets in shorter times. By collecting the 6^{th}-9^{th} fraction, we were able to obtain high-purity corilagin (>98%) with a satisfactory recovery (97.4%). From this work, the ideal column length was determined to be 13.5 cm. Separation is negatively affected by increasing the loading quantity (>50 mg) and the saturated capacity was determined to be 25 mg crude corilagin per 1 g gel (**Table S3**). The distribution of **I8** and corilagin on gel chromatography was very similar in both the preliminary experiments and the scale-up (**Figure S3, Table S4**). As a result of this work, 228 mg pure corilagin was obtained from the purification of a 288 mg crude sample on an LH-20 column (13.5 cm × 2.5 cm).

Using the area normalization method, the HPLC purity of corilagin was determined to be 98.4%. ESI-HRMS identified the major molecular ions at m/z = 633 $[M\text{-}H]^-$ and 301 (ellagic acid), which correspond to the literature values for this compound [24]. The ^{13}C and ^{1}H NMR (CH_3OD) spectra for the sample were listed in **Section 7,** Supplementary Material. These spectra agree with those reported in the literature [25], confirming that the isolated compound is corilagin.

3.3.2. Merits of COC for the purification of corilagin

The efficiency of the COC purification of corilagin was compared with previously reported methods (Table 5). **A1** and **B1** mobile phases were individually employed for the purification of corilagin. Using the same loading capacity described for the COC-based purification, the purity of corilagin was 60.1% and 61.6%, respectively. It is also worth noting that, apart from the commonly employed C18 column, our purification was performed without the need for special separation materials. Compared to the open-tube column or high-speed countercurrent chromatography (HSCCC), the high column efficiency and flow rate of HPLC allows COC to produce its production ratio at a high level. Apart from COC, separations using a normal C18 column are generally ineffective when a sample is overloaded. In addition, the orthogonality of a purification system based on the same purification mechanism (same column material) is notably low. However, in this work, orthogonality was obtained using the same column with distinct mobile phases to provide excellent purification. These results support the development of a new route for the construction of orthogonal separation; in other words, the construction of a 2D separation protocol for a given target should not just consider the orthogonality of the system, but should also account for the actual ability to remove impurities. Of course, the preliminary applicability of this theory in 2D separation composed of different stationary phases, such as now common combination of hydrophilic interaction liquid chromatography (HILIC) and RPLC, was also discussed in **Section 8** of Supplementary material.

Accounting for the actual ability to remove impurities is the key feature of COC. In this work, impurities around corilagin were effectively removed using two methods developed from a single column. Undoubtedly, COC can be applied to develop orthogonal separations using methods obtained from different columns; however, additional factors will need to be included for optimizing these protocols. We envision that computational programming (particularly C++) will play a vital role in this procedure and will be investigating this detail in the near future. The application of COC to the purification of multiple targets will be greatly enhanced upon optimizing the technique for the particular characteristics described above. In addition, even if column efficiency is poor, COC may be applicable to open-tube column or HSCCC if the distribution of the

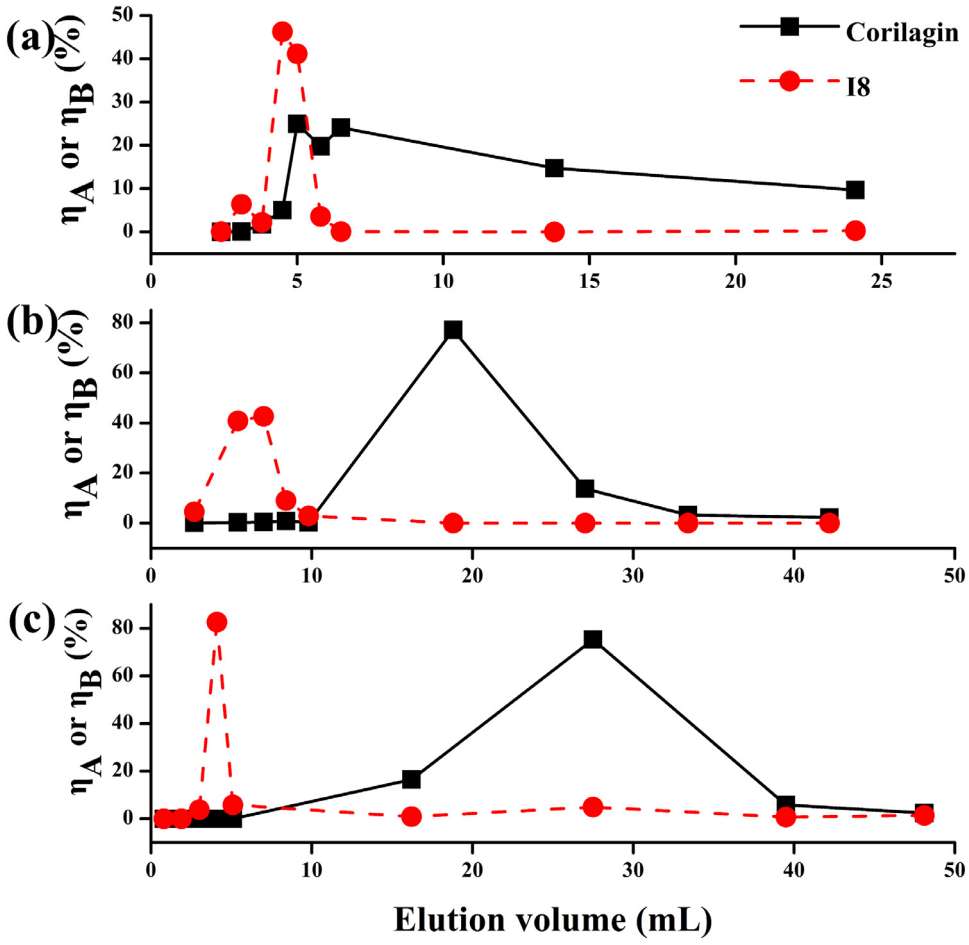


Figure 3. The distribution of **I8** and corilagin in the fractions collected from purifications on LH-20 columns with lengths of (a) 7.0 cm, (b) 13.5 cm, and (c) 20.0 cm.

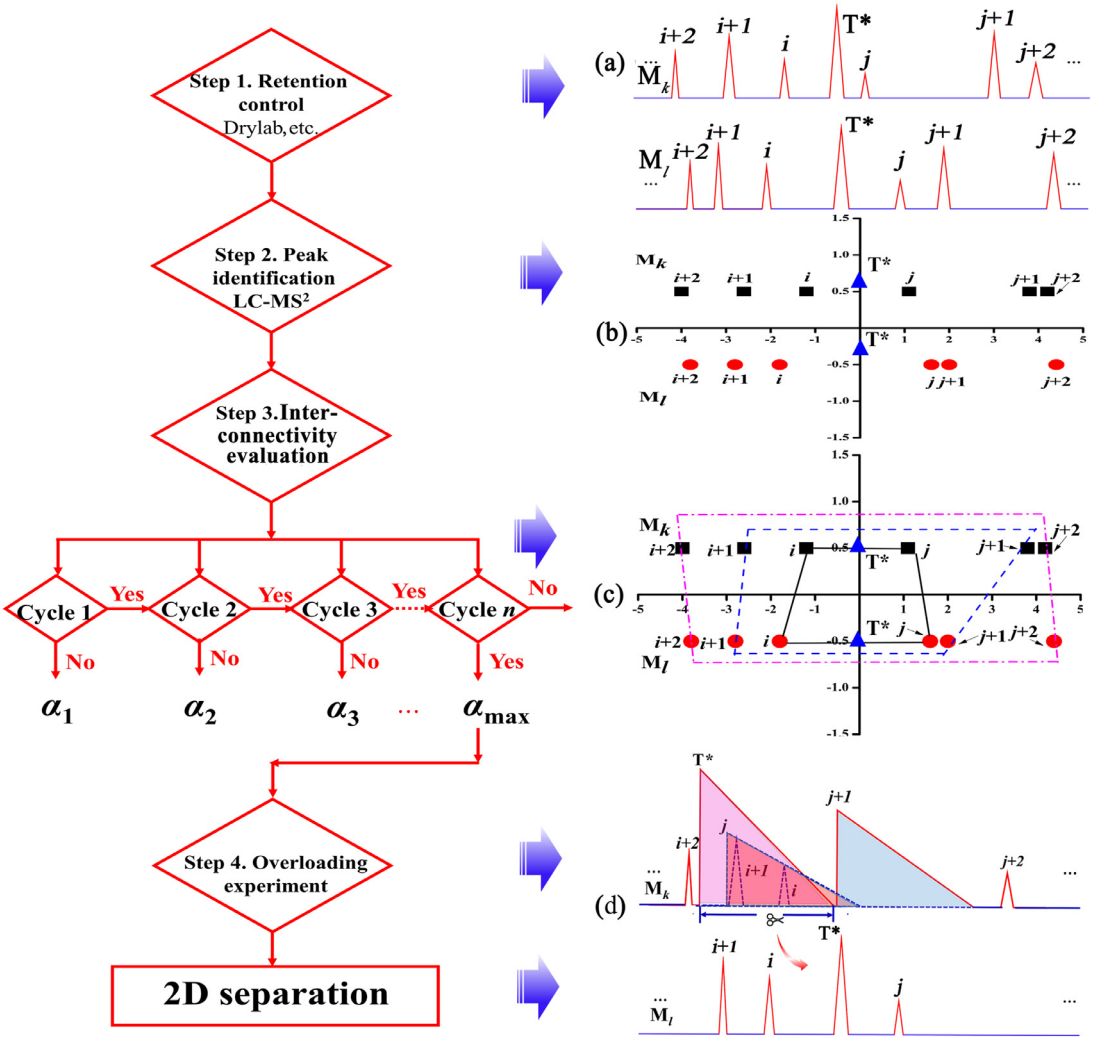
Table 4
The influence of column length on the peak area ratio of corilagin in the fractions obtained from purification on LH-20 columns.

Fractions	7.0 cm		13.5 cm		20.0 cm	
	Content* (%)	Volume (mL)	Content (%)	Volume (mL)	Content (%)	Volume (mL)
1	56.4	2.4	61.3	2.7	61.3	0.8
2	17.3	0.7	31.1	2.7	10.7	1.1
3	69.9	0.7	14.2	1.6	1.2	1.1
4	87.6	0.7	4.6	1.4	0.9	1.1
5	95.8	0.5	27.0	1.4	6.5	1.0
6	97.8	0.8	98.1	9.0	97.7	10.9
7	99.4	0.7	98.5	8.2	98.6	11.3
8	98.5	6.8	98.5	6.4	97.6	12.0
9	97.7	10.3	99.6	8.6	95.3	8.6

* The content of corilagin in fractions was determined by HPLC with area normalization method. The HPLC conditions used throughout these analyses were: column, ACCORM C18 (250 mm × 4.6 mm, 10 μm) mobile phase, ACN-0.1% HCOOH/H2O (10:90, v/v); flow rate, 1.0 mL/min; temperature, 30°C.

Table 5
Comparison of corilagin purification by COC and previously reported methods.

Methods	Purity (%)	Recovery (%)	Corilagin obtained (mg)	Time (h)	Corresponding efficiency (mg/h)
COC and LH-20	98.4	80.0	228.0	2.0	114.0
A1	60.1	82.2	-	0.6	-
B1	61.6	88.0	-	1.1	-
Adsorption Chromatography [26]	92.4	88.0	-	12.5	-
Revere phase/normal phase-HSCCC [27] HSCCC [28]	91.0/ 92.2 >95.0	82.9/ 94.6	70	2.0/ 7.0 3.7	18.9



Scheme 1. Construction of COC. Left: Four steps of COC. Right: (a) Distribution of peaks on the simulated methods of M_k and M_l. (b) Distribution of α-points between impurities and the target in (a) on the simulated methods of M_k and M_l. (c) The construction of closed-cycles based on α-points. (d) Simulated overloading experiments using 2D separation based on M_k and M_l.

impurity is well studied. Finally, owing to its ability to be accurately scaled-up between analytical and preparatory separations, we envision that COC can find wide application within the industrial sector.

4. Conclusions

In this work, we describe capacity orthogonal chromatography, a new technique for purifying large quantities of biologically active compounds from complex natural products. The central merits of this method are focused on obtaining the optimum production rate in sequential purification using different purification methods and extending the application scale of preparative 2D separations. We also describe efficient guidelines for simultaneously optimizing both the selectivity and loading capacity of the purification method. As an example of the utility of our process, 228 mg of >98% corilagin was successfully prepared from pomegranate flower extracts by COC, supporting its application for the purification of biologically active compounds from complex natural products. We envision that COC can be extended to other separation technologies, such as membrane separation and HSCCC, and large-scale purifications, such as those currently used in industry.

Author contributions

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Munire Abuduaini: Writing: Review & Editing.
Yongxin Zhao: Writing: Review & Editing.
Rahima Abdulla: MS detection.
Haji Akber Aisa: Idea, Review & Editing.

Declaration of Competing Interest

The authors have declared no conflict of interest.
We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461129.

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